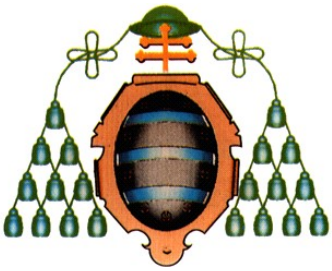

Universidad de Oviedo
Departamento de Química Física y Analítica

"The application of mercury-containing labels in protein analysis using complementary mass spectrometric techniques"

Tesis doctoral

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Contents

1 Introduction

Proteins are biopolymers built of amino acids, which are bound together by a very stable peptide bond, formed between the amino group of one amino acid with the carboxyl group of a second. The sequence of the chain for a given protein is coded in the DNA of an organism. In most organisms, proteins are constructed from 20 individually encoded amino acids, but also the substitution of selenium for sulphur in selenocysteine is regarded as an additional amino acid, which is also included in some proteins. Whereas the genome (entity of all genes of an organism) of many living beings is known [1], the proteome (entity of all proteins expressed by a genome, cell, tissue or organism) is a more complex systems to describe, as the expression and residence time (and therefore the actual concentration) of all proteins is not constant, but underlies changes in its composition [2].
Proteins are synthesized in the mitochondrial system of an organism. After their synthesis, the proteins may undergo different types of post-translational modifications, as for example phosphorylation (enzymatic addition of PO_4^{3-}), glycosylation (enzymatic addition of saccharides to proteins) or glycation (non-enzymatic addition of normally glucose or fructose to proteins) that may change their function or activity in a drastic manner.
As proteins have different tasks and functions in an organism, their structure and their properties are highly diverse: There are huge membrane and structure proteins (e.g. keratin in hair or silk proteins), giving shape to tissue and cells, there are various transport proteins (huge size static ion channels as well as highly mobile transport proteins for different elements such as haemoglobin for oxygen and transferrin for iron), and there are regulating proteins of different size as enzymes and hormones, expressed to control different processes in an organism. Some proteins (e.g. the proteins of the cytochrome P family) are involved in synthesis or decomposition of certain substances, as for example synthesis of second messengers (compounds involved in the intracellular signal transduction). Smaller proteins work as hormones, as for example insulin, that regulate the uptake and storage of glucose. Disturbance in its expression leads to diabetes mellitus, but it was also reported to be abused as a performance enhancing substance in sports [3]. Another remarkable protein is the so-called green fluorescent protein (GFP), that was discovered in 1961 and is known for its outstanding property of emitting green fluorescence light after excitation due to an amino acid sequence Ser_{65}-Tyr_{66}-Gly_{67}, which represents

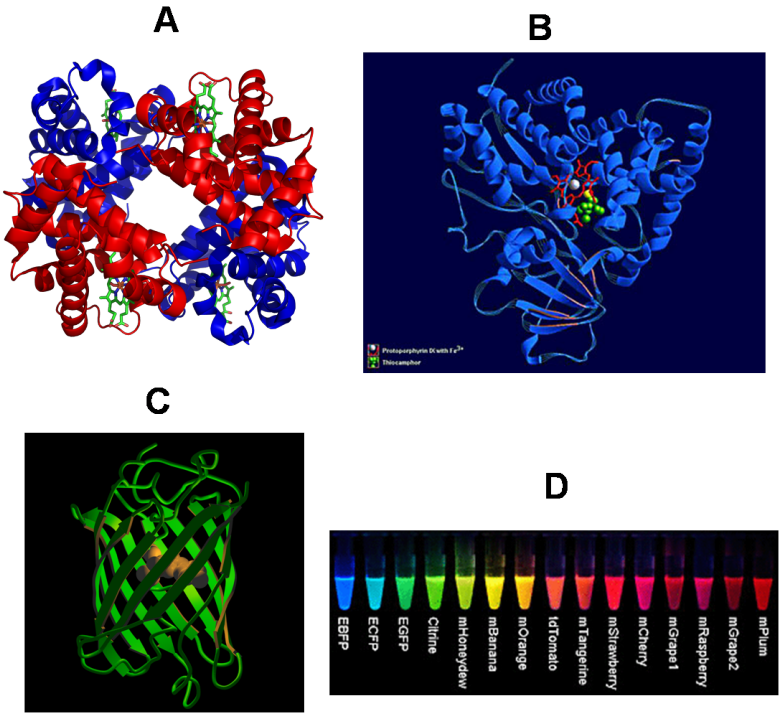


Figure 1.1: Structures of some proteins: haemoglobin (A), cytochrome P 450 (B), green fluorescent protein (C), colours of some isoforms of GFP (D), from [6]

an internal chromophore. Nowadays, it is used to visualize the expression of other proteins after embedding it into the sequence of the gene encoding the target protein. Its discovery and its exploitation for scientific purposes was honoured with the Nobel Prize in Chemistry in 2008 ([4], [5]). Figure 1.1 shows the structures of some above mentioned proteins. Due to the diversity in structure and function, there is no standard recipe for the analysis of proteins, making detailed investigation of sample preparation and analytical methods necessary for every target protein.

Mass spectrometry has contributed significantly to the knowledge of structure and functions available up to now. In contrast to classical methodologies for protein sequencing such as Edman degradation, mass spectrometry based protein sequencing is not only faster (in terms of seconds instead of hours), but also less sample consuming (several fmol instead of μ- or mmol). Characterisation of a given protein can either be accomplished by determination of its molecular mass or its sequence by MS/MS analysis after fragmentation. The possibilities offered by modern instrumentation are highlighted in several reviews, such as [7]-[9]. There are various fields in modern life science, that require accurate analysis of proteins in almost all concentration ranges, but with special interest to the trace level, as for example biochemical research (the expression and procession of proteins controls all biological functions of an organism, so that minor changes in protein concentrations can have drastic effects on a biological system), and clinical diagnostics (discovery of biomarkers, for example Troponin T is known to be present at elevated levels after heart attacks, [2]). A cluster of authors from different research groups have com-

piled a non-redundant list of total 289 proteins documented in human plasma at different concentration rates [10] using data proportionated by different analytical techniques. For clinical purposes, a list of relevant proteins was also compiled in 2010, including 205 individual proteins that are approven by the US Food and Drug Administration and are assayed on a regular basis in plasma and serum samples, excluding immunoglobulin proteins [11].

Normally, the concentration of proteins in living organism varies over several orders of magnitude. For the human serum, the most abundant proteins like albumin and transferrin can be found in the range of mg mL^{-1}, whereas some proteins only occur in the low pg mL^{-1} range, among them biomarkers for certain diseases or proteins that can be used for diagnostic purposes [2]. Figure 1.2 shows the abundance of different proteins in human plasma.

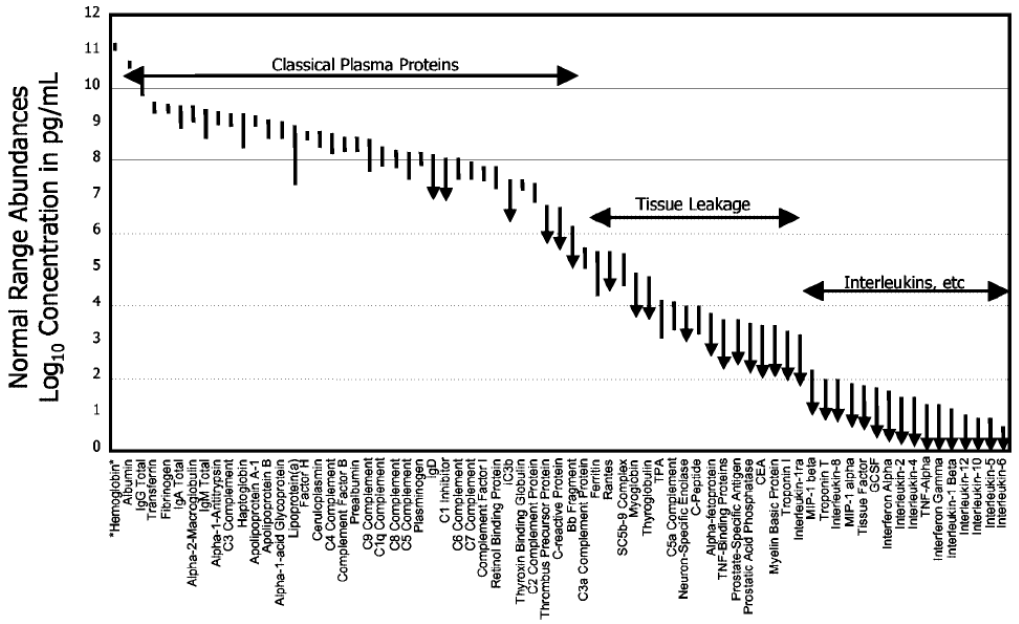


Figure 1.2: Abundance of different proteins in human plasma, from [2]

For analytical chemists, the analysis of proteins in complex samples is a tremendous challenge, as the target protein may be a low abundant protein in a complex matrix of other proteins in a small absolute amount of sample [12]. In contrast to DNA analysis, no technique like PCR (polymerase chain reaction) exists up to now to amplify the total amount of protein in the sample, so that analytical methods have to be highly sensitive and specific. Methods based on mass spectrometry (MS) have shown to be able to cope with this task, so that MS techniques have become versatile and indispensable tools as well for the qualitative investigation of protein structures as for their quantitative analysis. The description of the entity of all proteins in a biological system (both qualitative and quantitative) has lead to the formation of a new scientific branch, the so-called proteomics [13]. Besides advances in the understanding of biological systems, the contribution to instrumental development

in mass spectrometry (especially using soft ionisation techniques like electrospray) is also considerable. Not only the development of new kinds of instrumentation can be directly related to proteomics, but also the close connection between the generated data (mass spectra) and powerful tools for their software-based evaluation. Today, identification of a huge number of proteins in a sample is state of the art, and also methods for their quantitative determination have been developed.

For protein quantification using mass spectrometry, techniques have been developed which include the introduction of stable isotopes directly into the proteins of an organism, or the artificial introduction of a label which contains the isotopic information. After sample treatment, the expression of equal proteins in different samples is determinable by comparison of the respective signal intensity of the different isotopically labelled forms in the mass spectrum. At the same time, information about the nature of the quantified protein is available via database supported identification. A detailed overview of so far developed methods for protein quantification using labelling techniques will be given in chapter 3.3, and can be also found in some excellent reviews (references [14]-[16]) published in recent years.

However, all of these methods have in common that they were designed for the application in combination with soft ionisation techniques such as electrospray (ESI) or matrix assisted laser desorption/ionisation (MALDI). Due to the limited potential for quantification of these methods, mainly relative quantification is attainable, or in other words, comparison of a protein amount present in one sample (biological system after the alteration of external conditions or treatment with other stimuli, e. g. pharmaceuticals) relative to a second sample (e. g. control sample). This methodology is feasible, if a comparable sample is available, as for example in cell cultures, but this is not always the case. Then, absolute quantification of proteins is neccessary, accomplishable by the direct correlation of a measurand to the amount of analyte.

The combination of such quantification strategies involving the use of labels or other techniques such as stable isotope dilution with modern mass spectrometry instrumentation as for example multiple reaction monitoring (MRM) on triple quadrupole instruments is certainly a way to further facilitate the discovery and clinical determination of biomarkers in human derived samples such as blood or urine [17]. This methodology meanwhile permits the highly multiplexed and specific analysis of various biomarkers in one run with high detection sensitivity (a more detailed description of the experimental approach will be given in chapter 3.2.5).

In contrast to other established techniques that can be used for quantitative protein analysis such as immunoassays, the use of mass spectrometry seems to be highly advantageous. Despite all progress made in this field, the actual fraction of accurately quantified proteins in a given sample is still very limited. Also limited by the lack of knowledge about potential proteins encoded in the genome of target organisms,

there is certainly a gap between the number of proteins present in a sample and number of the proteins identified in a sample. Due to the high differences in the physicochemical properties and the abundance of certain proteins, the number of quantified proteins is even lower. This was illustrated by *Bantscheff et al.* [16]:

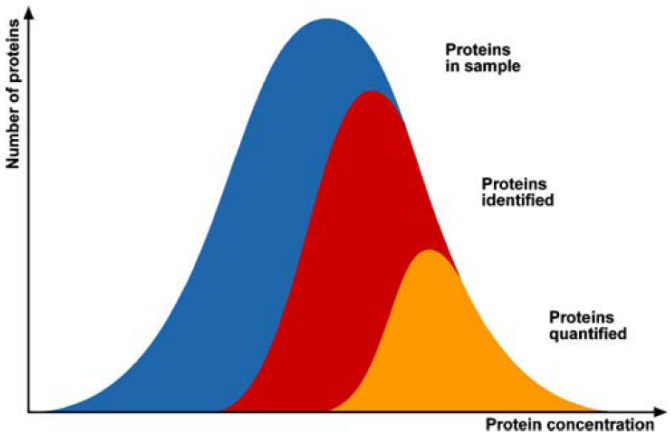


Figure 1.3: Fraction of the number of proteins potentially present in a sample, identified and quantified proteins, from reference [16]

However, from the analytical point of view, questions may arise concerning the accuracy of methods that aim on simultaneous quantification of a huge number of proteins, as there are only a limited number of certified reference materials available for their proper validation [18]. This must lead not as far as to mistrust results obtained in the last years in the field of quantitative protein analysis, but certainly more information regarding the accuracy and precision of the proposed methodologies is desirable. Supporting this statement, a study about mass spectrometric protein identification (not taking into account possible results for quantification) between different laboratories was conducted [19]. Therefore, an equimolar sample consisting of 20 purified recombinant proteins was provided to 27 independent laboratories together with the task to identify these proteins via mass spectrometry. The results indicate, that accurate protein identification of an unknown sample is already a more sophisticated task setting high demands to laboratories with known expertise in protein analysis and thus far from being conductable on a routine base. Further examples for the growing interest on the actual quality of the results generated in typical proteomics experiments are studies on the accuracy and precision attainble with one of the most popular labelling reagents nowadays, the iTRAQ reagent, (isobaric tag for relative and absolute quantification, [20]), and the examination of a complete uncertainty budget for the analysis of a growth hormone by isotope dilution mass spectrometry [21].

In the last years, the interest in inductively coupled plasma-mass spectrometry (ICP-MS) as element selective detector for heteroatoms in proteins was steadily growing due to its outstanding properties for quantification [12]. In contrast to other mass spectrometric techniques, the signal intensity obtained in ICP-MS can be directly

related to the concentration of a given element in a sample. The subsequent use of complementary mass spectrometric techniques (both molecular and elemental) can help to improve the quality of the data generated for the quantitative determination of proteins. Unfortunately, the detection of the most abundantly occuring ICP-MS detectable elements in proteins, sulphur present in the amino acids cysteine and methionine and phosphorous being part of an often found post-translational modification, is hampered by low ionisation yields and numerous spectral interferences. Therefore, also here techniques were developed for the artificial introduction of elements easily detectable in ICP-MS [22]. As normally elements such as lanthanides are used, which are not naturally occurring in proteins, limits of detection can be further improved, and absolute quantification is in principle possible due to the direct proportionality of the obtained signal to the analyte concentration in the sample. Additionally, the isotopic information provided by ICP-MS can help to subsequently introduce isotope dilution analysis as a quantification strategy in protein analysis in order to correctly address possible error sources occuring during the sample treatment and improve accuracy and precision of the obtained results [18]. For these reasons, the use of ICP-MS in combination with elemental labelling can be highly beneficial for the application in absolute quantification of proteins.

2 Objectives

2.1 Objectives

Accurate quantitative analysis of proteins is one major task for analytical chemists nowadays. There is an urgent need for reliable absolute quantification methods for proteins in various concentration levels, but of special interest in the lowest concentration level in real samples as for example blood derived samples or urine. Labelling in combination with mass spectrometry has already taken an important role in proteomics, but still absolute determinations of proteins at trace level are not possible. Therefore, the combination of labelling together with elemental mass spectrometry, especially inductively coupled plasma-mass spectrometry and the concept of isotope dilution analysis, might help to accomplish this objective.
In this project, the development of a methodology based on labelling of proteins with p-hydroxymercuribenzoic acid (pHMB) and its application to the quantitative analysis of proteins is the main objective. pHMB seems to be a very promising labelling agent, taking into account its chemical properties (stable binding of mercury to sulphur, moderate size for fast and complete derivatisation) as well as practical reasons (easy handling as it is soluble in diluted sodium hydroxide, not volatile). Furthermore, consequent use of ICP-MS can help to achieve complete traceability over the whole analytical process by following the signal of mercury. As there are many possible pitfalls in the use of labelling for quantitative purposes, as for example the necessity of the exact knowledge of the stoichiometry of the labelled protein, special attention should be paid to avoid possible sources of error in the method. Also, special attention should be drawn to the compatibility of the mercury containing label with typical workflows for peptide and protein identification using molecular mass spectrometry (e. g. influence on efficiency of enzymatic digestion and software based identification). Finally, the experience acquired during the first part of the project should be applied to the investigation of possible mercury binding proteins in living beings due to exposure to environmental contaminants such as methylmercury ($MeHg^+$) or other alkylated mercury compounds.
This general objective is divided into 4 subobjectives:

1. Elucidation and optimisation of the proposed derivatisation process using complementary mass spectrometric techniques. The experimental procedure should be optimized in order to selectively derivatise the targeted binding

sites. The stoichiometry of the adducts should be confirmed by the use of molecular mass spectrometry. Finally, the potential of the method should be demonstrated in terms of analytical figures of merit and applicability in protein analysis.

2. Development of analytical methods for the determination of selected standard proteins using typical workflows in protein analysis and application of isotope dilution as means for relative and absolute protein quantification. Due to the potential complexity of samples and the related need for multi-dimensional experimental procedures, the advantages of label-specific isotope dilution analysis for protein quantification should be demonstrated in combination with multidimensional chromatography or gel-based separation followed by tryptic digestion and peptide analysis.

3. Elucidation of possibilities for the application of molecular mass spectrometry for identification of peptides and proteins containing mercury labels and development of methodologies for absolute quantification using these techniques. As an alternative to the detection with ICP-MS, potential advantages and drawbacks of commonly applied techniques for protein and peptide quantification such as ESI-MS and MALDI-MS should be elucidated.

4. Investigation on potential binding partners for $MeHg^+$ in tuna fish muscle tissue. In a first series of experiments, the binding of MeHg to a model protein should be investigated in order to confirm cysteine residues as binding sites. Second, tuna fish muscle tissue material should be screened for the molecular weight range, in which $MeHg^+$ is found, and, if associated to proteins, more detailed investigations on the identity of these proteins should be conducted.

2.2 Objectivos

La cuantificación exacta de proteínas es una de las tareas más importantes para los químicos analíticos hoy en día. Existe una necesidad urgente de desarrollar métodos fiables para la cuantificación absoluta de proteínas en todos los rangos de concentración, pero sobre todo, en las concentraciones más bajas en muestras reales, como por ejemplo, de sangre u orina. El marcaje en combinación con espectrometría de masas ocupa una posición elevada en proteómica, pero todavía no es posible la determinación absoluta de proteínas a nivel de trazas. Por tanto, la combinación del marcaje y la espectrometría de masas elemental, especialmente el empleo de plasmas de acoplamiento inductivo y el concepto de análisis por dilución isotópica, pueden ayudar a cumplir dicho objetivo.

El objetivo principal de esta tesis es el desarrollo de una metodología analítica basada en el marcaje de proteínas con ácido hidroximercuribenzoico (HBM) y su aplicación para el análisis cuantitativo de proteínas. El compuesto HBM parece ser un agente de marcaje muy prometedor teniendo en cuenta sus propiedades químicas (enlace altamente estable entre el mercurio y el azufre, tamaño moderado para una derivatización rápida y completa) así como por razones prácticas (fácil de manejar al ser soluble en hidróxido sódico diluido además de ser no volátil). Además, el uso del ICP-MS en cada etapa permite alcanzar una trazabilidad completa en todo el proceso analítico siguiendo la señal del mercurio. Como existen muchos problemas derivados del empleo del marcaje para fines cuantitativos, como la necesidad de conocer la estequiometría exacta de la proteína marcada, se debería prestar especial atención para evitar posibles fuentes de error en el método. Asimismo se hará un estudio comparativo entre la técnica desarrollada y otras técnicas tradicionales empleadas para la identificación de péptidos y proteínas usando espectrometría de masas molecular (como por ejemplo el efecto derivado de la eficacia de la digestión enzimática o la identificación empleando software informático).

Finalmente, los resultados obtenidos en la primera parte de esta tesis serán aplicados al estudio de posibles interacciones de mercurio con proteínas en seres vivos debido a la exposición a contaminantes medioambientales, como metilmercurio ($MeHg^+$) u otros compuestos alquilados de mercurio.

El objetivo general de esta tesis se divide en cuatro objetivos parciales:

1. Desarrollo y optimización de la metodología propuesta para el proceso de derivatización utilizando técnicas complementarias basadas en espectrometría de masas. El proceso experimental será optimizado para derivatizar selectivamente los sitios de enlace sospechados. La estequiometría de los aductos formados será confirmada con el uso de espectrometría de masas molecular. Finalmente, el potencial del nuevo método será demostrado mediante sus características analíticas y su aplicabilidad en el análisis de proteínas.

2. Desarrollo de métodos analíticos para la determinación de proteínas estándar mediante el empleo de protocolos tradicionales para el análisis de proteínas y la aplicación de la dilución isotópica como herramienta para la cuantificación relativa y absoluta de dichas proteínas. Debido a la complejidad de las muestras y la consecuente necesidad de protocolos de separación multidimensionales, las ventajas de la dilución isotópica específica al compuesto de marcaje serán demostradas en combinación con cromatografía multidimensional o métodos basados en la separación en geles seguida por la digestión enzimática y el análisis de péptidos.

3. Elucidar las posibilidades de la aplicación de la espectrometría de masas molecular en la identificación de péptidos y proteínas marcadas con el compuesto de mercurio y desarrollar metodologías para su cuantificación absoluta utilizando dichas técnicas. Como alternativa a la detección via ICP-MS, serán investigadas las ventajas e inconvenientes de las técnicas generalmente aplicadas para la cuantificación de proteínas y péptidos.

4. Investigación de moléculas que potencialmente pueden enlazarse con el $MeHg^+$ en tejido muscular de atún. En los primeros experimentos, será investigado el enlace de $MeHg^+$ con una proteína modelo para confirmar el aminoácido cisteína como punto de enlace. Después, se examinará tejido muscular de atún para determinar el rango de peso molecular en el que se encuentra el metilmercurio y si se encuentra asociado a proteínas se llevarán a cabo estudios más detallados para identificarlas.

3 Theoretical background

In the following chapter, different methods known and applied frequently today for the analysis and quantification of proteins will be presented. Their advantages and drawbacks are highlighted in order to point out, why the use of ICP-MS in combination with labelling as a tool for absolute protein quantification is of high interest and what benefits can be taken from it.

3.1 Analytical tools for protein analysis

3.1.1 Commonly applied methodologies for protein analysis

Photometric methods

The use of colouring dyes has been established long ago, and still they are used frequently due to their simplicity. The most known colouring dyes are the so-called Lowry assay (1951, [23]), the Bradford assay (1976, [24]) and staining with silver [25]. The Lowry assay consists of two reactions steps. The first reaction step is the binding of Cu^{2+} to the peptide bonds, and its subsequent reduction to Cu^{+}, giving a violet colour. This is the so-called Biuret-reaction, which is already a specific reaction for proteins. To further improve sensitivity, heteropolyacids of tungsten and molybdenum are added (Folin-Ciocalteu‘s reagent) in a second step, giving an intensive blue colour, that can be measured by absorption between 540 and 750 nm. The Lowry assay is very sensitive, protein quantities of 0.1 to 1 μg mL^{-1} can be determined, but it is very laborious and prone to interference. With time, several enhancements have been published to tackle these drawbacks [26]. The Bradford assay uses the dye coomassie blue (also known as Brilliant Blue 250). The dye is binding to cationic and as well neutral and hydrophobic amino acid side chains. After binding, the absorption maximum of the dye shifts from 470 nm to 595 nm that can be easily determined by absorption measurements. After external calibration (for example with bovine serum albumin), the amount of protein can be calculated. The Bradford assay responds to proteins in general, so that the parameter determined is always the total protein concentration, if not pure samples were used.
In modern protein analysis, different types of dyes are used mainly for gel staining and thus visualization of the separated protein spots in the gel, although some dyes are also capable of quantification. An overview on the most commonly used dyes

is given in reference [27]. Besides Coomassie Blue staining, which was one of the tools in the early stage of proteomics ([28], [29]), many other dyes are used today. The limits of detection that can be achieved vary in the order of low ng amounts (between 1 ng for example silver staining and 10 to 100 ng for Coomassie staining). However, their response is restricted only to the presence of proteins in general, and especially the aforementioned dyes show only a limited dynamic range and reproducibility (1 to 1.3 orders of magnitude and a variation coefficient between 10 to 15 % for the majority of all protein spots investigated, [27]). One alternative dye, which shows a higher dynamic range of three or more orders of magnitude is the so-called SYPRO stain [30]. The compound is a ruthenium metal chelate that binds to the basic amino acids in proteins via electrostatic interactions. Besides the higher dynamic range, it shows less interference with mass spectrometry for protein identification and can be combined with other staining techniques (multiplexed staining) sensitive to post translational modifications, such as phosphorylated proteins or glycoproteins [27].

Difference gel electrophoresis

In the late 1980ies, the concept of difference gel electrophoresis (DIGE) was introduced for the observation of potential differences in protein expression in individual samples [31]. Although this technique is rather considered as a tool for proteomics, it is discussed here, as it involves the use of dyes and detection due to their photometric characteristics. For DIGE, two (or more) protein samples are incubated separately with special dyes that can bind to primary amine groups (as the α-terminal amino group and the side chain of lysine) [32]. After incubation, the samples are mixed and subjected to two-dimensional gel electrophoresis. Using their characteristics as fluorescence markers, images for the distributions of proteins from both samples can be generated using one single gel, so that significant improvement of reproducibility and a reduced number of analysis can be achieved. The most important characteristics of the dyes commonly used (Cy2, Cy3 and Cy5 [1]) are, that they are designed to be charge- and size matched to each other, so that identical proteins in two samples show identical migration behaviour in two-dimensional gel electrophoresis. However, usually only a small fraction of the total amount present of each protein is labelled in order to avoid multiple labelling in different binding sites of the same protein. Therefore, especially for small proteins, a difference in migration distance (in the order of 0.5 kDa) can be observed between the labelled form of the protein and the unlabelled bulk [27]. Using software based data evaluation, even (relative) quantification of protein expression in different samples is possible. The main advantage of this approach is, that in studies aiming at multiplexed analysis of independent sam-

[1] Nomenclature of the dyes follows the nomenclature of the manufacturer. As the dyes are based on cyanine dyes, they are called CyDyes with the number for further specification.

ples fewer gels have to be run for statistical evaluation. To increase the attainable accuracy, an internal reference sample should be added to all gels, which can be a pooled sample in order ensure representation of all proteins on all the gels analysed. The main advantage of DIGE in comparison to MS-based methodologies is, that the dynamic range accessible is larger. However, the use of MS-based identification is still the consecutive means for the identification of differentially expressed proteins. During the last decade, DIGE has been subsequently extended with further dyes and other target amino acids to offer capacity for multiplexed analysis.

Immunometric methods

Radioimmunoassays (RIA)

The use of radioimunoassays was first published in 1959 by *Yalow* and *Berson* with human insulin [33]. The quantitative aspect of this technique is based on the competitive behaviour of antigens for antibodies after being labelled with an element that can also be introduced as one of its radioactive isotopes. The antigen (target protein) in its radioactive labelled (hot) form (normally with ^{3}H, $^{125,131}I$) is bound to a specific antibody in solution. Incubation with the same antigen (standard solution of the target protein) labelled in its normal (cold) form will lead to increasing competition of both for the binding sites of the antibody with increasing amount of the cold antigen added. Measuring the radioactivity left from the hot antigen bound to the antibody after separation of the free antigen will give a calibration curve for the concentration of cold antigen added, so that an unknown amount of antigen can be determined. *Yalow* was honoured with the Nobel Prize for medicine in 1977 for the development of the radioimmunoassay approach [34]. Due to the extreme sensitivity of radiometric detection methods, this technique is characterized by outstanding limits of detection. Contrary, the necessity to work with high amounts of radioactivity, and therefore the means of protection necessary for the scientific personal and the environment, limit its widespread application.

Enzyme-linked immunosorbent assay (ELISA)

The so-called ELISA (enzyme-linked immunosorbent assay) is also based on the highly specific antigen-antibody reaction [35]. ELISA represents a separation or purification method for complex samples as well as a detection method. Nowadays, the most common form is the so-called sandwich-ELISA, where not only one antibody is used, but two, binding to two different binding sites (epitopes) of the target protein. Figure 3.1 shows a scheme with the fundamental steps involved in an ELISA essay. The first antibody (specific to one epitope) is bound onto a solid phase support (see step 1 in figure 3.1, mainly a 96-well plate for high-throughput analysis), and is incubated with the sample (blood serum, urine etc.). As the antibody reacts specifically with only one target (protein, toxin etc.), other components of the sample can

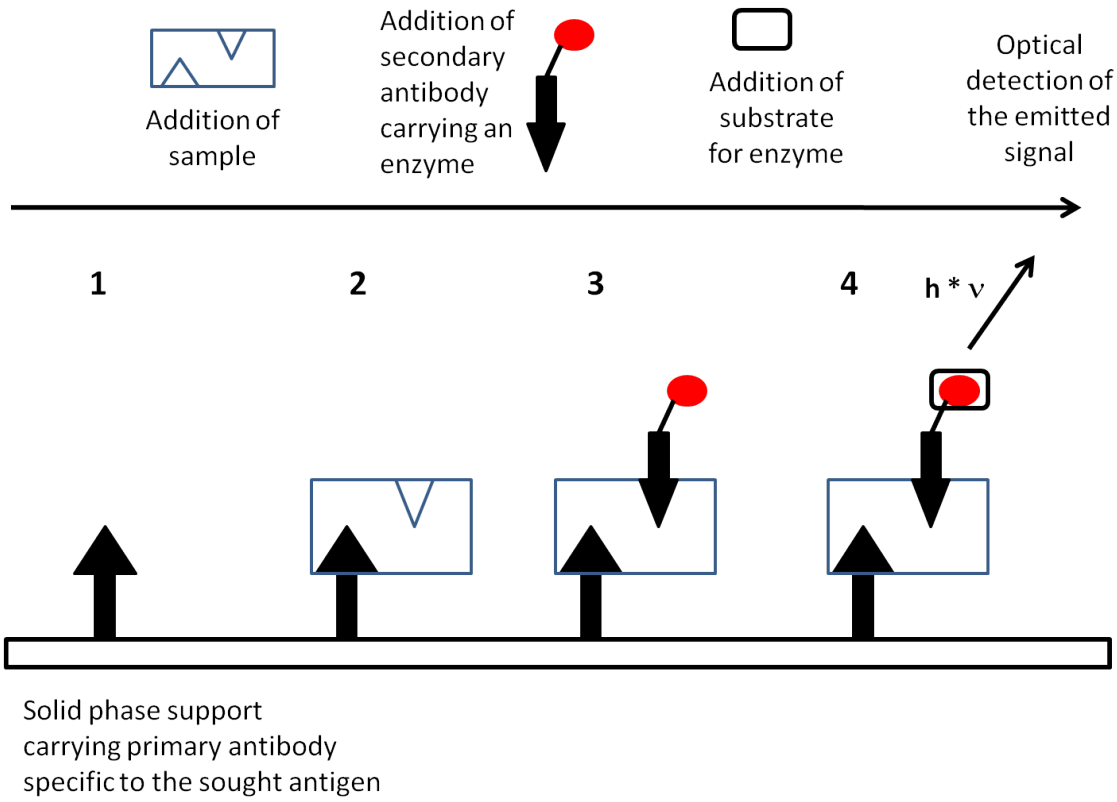


Figure 3.1: Scheme of a so-called sandwich ELISA

be washed off the well. After that, a second antibody is added, binding to a second epitope of the same antigen (step 2). The excess of the second antibody can also be washed off. This second antibody is modified with a fluorescence marker or an enzyme (e.g. horse radish peroxidase), that can give a signal in a detection system (fluorescence, or light emission after chemical reaction). The detection based on chemical luminescence is shown in steps 3 and 4 in the figure. Also other detection methods, as for example using absorption measurements are commonly used in ELISA essays. Signal intensity is directly related to the concentration of the secondary antibody carrying the detection marker and therefore to the concentration of the target (after calibration). Some of these detection markers have been adapted to the specific means of ICP-MS as a detector for ELISA based assays, as for example europium containing fluorophores ([36], [37]) or gold nanoparticles [38]. Although ELISA is known to be a relatively cheap technique for high-throughput analysis, this is only true if the applied antibodies are known and available. If not, the antibodies have to be developed in a very expensive and time-consuming way [17], relativising the above mentioned advantage of low cost analysis. Another important restriction for ELISA is, that the sequence of the protein has to be known or a sufficient amount of it is available to produce the related antibody.

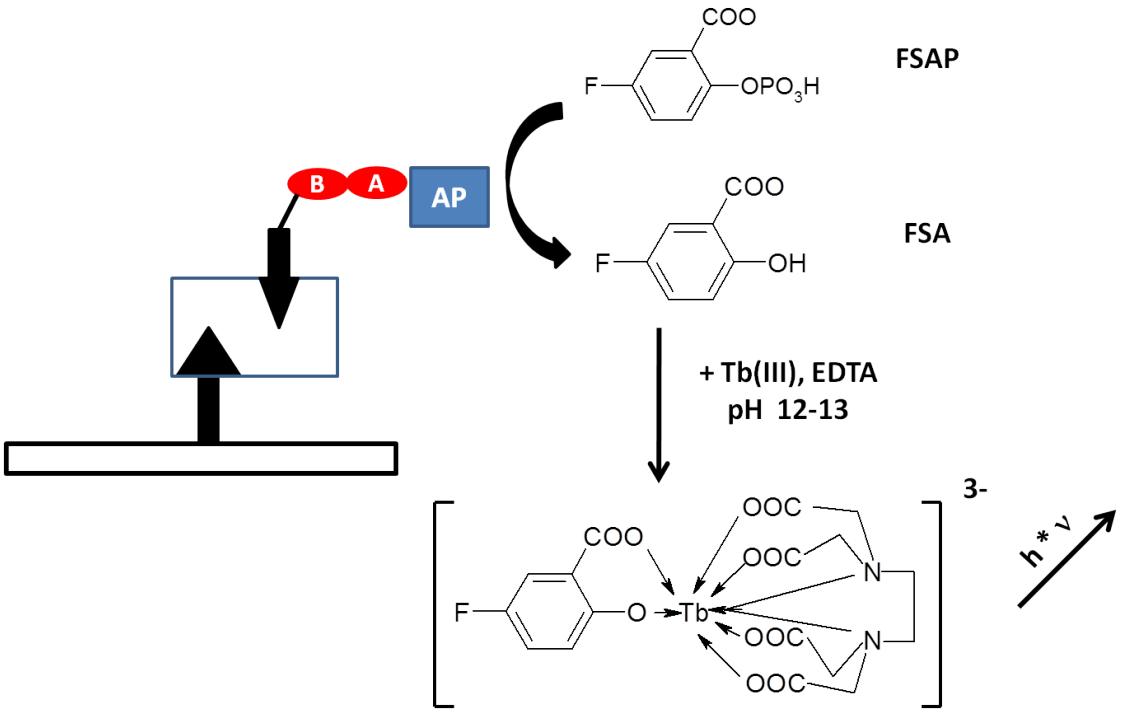


Figure 3.2: Working principle for the detection in ELISA essays using enzyme-amplified lanthanide luminescence, EALL. Adapted from [40]

Techniques based on fluorescence detection

Fluorescence detection is a very likely tool, as the intensity of the emitted light depends on the intensity of the light source, so that high intensities can lead to high fluorescence signals. Here, the use of laser systems for excitation is very common, as they are very intense sources offering monochromatic light with narrow line width. Detection takes place under an angle of 90°, or in the confocal plane to keep background irradiation on a minimum level (confocal laser scanning microscopy or surface plasmon resonance imaging, [39]). The highly specific and selective properties of ELISA have been improved in the last years in terms of detection limits by the use of time-resolved lanthanide luminescence detection [40], either by involving lanthanide ions into an enzyme catalyzed reaction (enzyme-amplified lanthanide luminescence, EALL) or the use of nanoparticles that contain a huge amount of luminescent lanthanide chelates. The principle of this detection method is shown in figure 3.2. In the pioneering work from *Evangelista et al.* [41], a method for the determination of alkaline phosphatase (AP) with the use of 5-fluorosalicylic phosphate (FSAP) and Tb(III) has been applied as a means of detection for an ELISA essay for rat IgG. AP is bound via a biotin-avidin (B and A in figure 3.2) linker to the secondary antibody. After enzymatic dephosphorylation of FSAP to FSA, this compound can participate in a chelate complex of Tb (III) and EDTA, resulting in strong luminescence.

Also in recent years, a class of signal enhancing substances for the fluorescence

detection of biopolymers was developed, that can take part in a photophysical phenomenon called Förster resonance energy transfer (FRET) [42]. Here, the energy absorbed by one fluorophore (donor) is transmitted to a second (acceptor) via a short distance. The acceptor can now emit the absorbed energy as fluorescence light on its characteristic emission wavelength. The most interesting information obtainable by this method is the dynamic behaviour of protein-protein interaction, as FRET is only possible on short distances, so that for example substrate-enzyme reactions can be observed. Additionally, excitation of the target analyte can be done in a more selective way, if conventional excitation leads to bad signal-to-noise ratio due to other emitting compounds being present in the sample. One of the possible excitation fluorophores is the already in the introduction mentioned green fluorescent protein, but also self-luminescent proteins as for example luciferase is commonly used, so that background irradiation can be kept to a minimum as no external light source has to be used. In modern life science, techniques enabling imaging of protein distribution or protein interaction have gained importance to follow not only static but also dynamic processes on living cells (live cell imaging). Care has to be taken by the choice of the dye and its concentration, in order to obtain a high and stable fluorescence signal but to maintain integrity of the biological system observed.
As a matter of fact, the above presented techniques have been developed rather for qualitative analysis of basic interactions than for quantitative analysis, nevertheless they can also be used for quantification for example in ELISA [43], if calibration with standards is done.
Although it is not directly used as a method for protein quantification, also fluorescence activated cell sorting (FACS) should be mentioned here. Based on flow cytometry it is basically used to count cells which were previously dyed with substances, which respond to different properties or states of the cells. The dyed cells are aspired through a tube and directed into a light beam (normally laser beam) to activate the fluorescent dye. The emitted light is detected in a photomultiplier. The number of signals acquired corresponds to the number of cells in a certain state. As fluorescent markers, also dyed antibodies can be used, which bind to a certain epitope on the cell surface, so that the number of cells carrying this epitope can be determined. This procedure is applied, for example, in cancer diagnostics [44].
The use of quantum dots conjugated to biomolecules was first described by *Chan* [45]. In comparison to fluorescent dyes, they exhibit a higher brightness, are more stable against photobleaching and their spectral line width is relatively narrow. For quantitative measurements, quantum dots conjugated to antibodies can be used as detection unit in ELISA assays [46]. One drawback in the use of quantum dots is their reduced biocompatibility.

Analysis of mRNA level

The sequence information of the proteins is saved in the genome of an organism, encoded in the DNA. For protein expression, the sequence is transmitted to the mitochondrials by the so-called messenger ribonucleic acid (mRNA) and read by certain enzymes to start protein synthesis. Short after decryption of the genomic code, it was proposed, that the so-called transcriptome (entity of all mRNA molecules) could describe a biological system in terms of its expressed proteins, as analysis of the mRNA expression is easier to handle as analysis of proteins. Even before, the basic correlation between both, mRNA expression and protein abundance was realised [47]. In a first comparative study using quantitative two dimensional gel electrophoresis and transcript image methodology [48], a correlation coefficient of 0.48 was obtained between the mRNA and protein abundances determined for 19 proteins expressed in human liver. The authors suggested, that post-transcriptional regulation of gene expression is a frequent phenomenon in higher organisms which might lead to this result.
Gygi et al. also investigated this idea, comparing the expression of proteins from *Saccharomyces cerevisiae* after 2D gel electrophoresis, tryptic digestion and metabolic labelling of the peptides to the expected value given by serial analysis of gene expression (SAGE) [49]. They concluded after the analysis of 156 identified proteins, that correlation between the mRNA level for a given protein and its actual concentration value is not straightforward, as they observed partly correct prediction of the protein concentration, but as well differences of about the 20-fold value.
Although all of the above mentioned methods have proven their potential and their applicability, and especially ELISA is nowadays an established routine method frequently applied for protein quantification, mass spectrometry has become the method of choice in protein identification and also quantification. To stress its importance, the technique and its application in the field will be presented in a more comprehensive way in the next chapter.

3.2 Mass Spectrometry

Besides the aforementioned techniques, especially mass spectrometry plays an important role in modern protein analysis. However, instrumental development and new applications in protein analysis have always been closely related. Starting from the development of ionisation sources that enable the generation of ionised biopolymers without extensive fragmentation, mass spectrometry is nowadays far away from being a simple tool for the measurement of molecular weights [7]. For example, using tandem mass spectrometry, determination of the amino acid sequence of a given protein is one of the major duties carried out in analytical laboratories working in the field of protein analysis and proteomics. Compared to classical chemical methods

for peptide sequencing, as for example Edman degradation [50], mass spectrometry based methods offer breathtaking advantages. For instance, the quantity needed for the identification of a certain peptide is much smaller (several fmol instead of μmol), and the time necessary is also considerably smaller (in terms of seconds instead of hours). Also, modifications do not hinder the sequencing process, but can be identified in the same run and therefore add valuable information about the protein/peptide of interest [8]. In the same way, the structural information provided by mass spectrometry can be used for the identification of proteins in a sample after enzymatic digestion and database search.

The analytical technique mass spectrometry is based on the generation of charged atomic or molecular species, which are afterwards separated in a mass analyzer according to their mass to charge ratio (m/z). Finally, an appropriate detection system counts the number of ions of a particular mass to charge ratio in order to generate a mass spectrum (ion intensity versus m/z) or to obtain an intensity profile for one or various m/z ratios during a chromatographic run (single ion monitoring mode, SIM). Depending on the ion source and further features of the mass analyzer used, different kinds of information can be obtained, such as the molecular mass of the intact compound, different kinds of fragments (which can give information about the chemical structure), or information about the elemental composition. The important parts of a mass spectrometer are shown in figure 3.3. Two important pa-

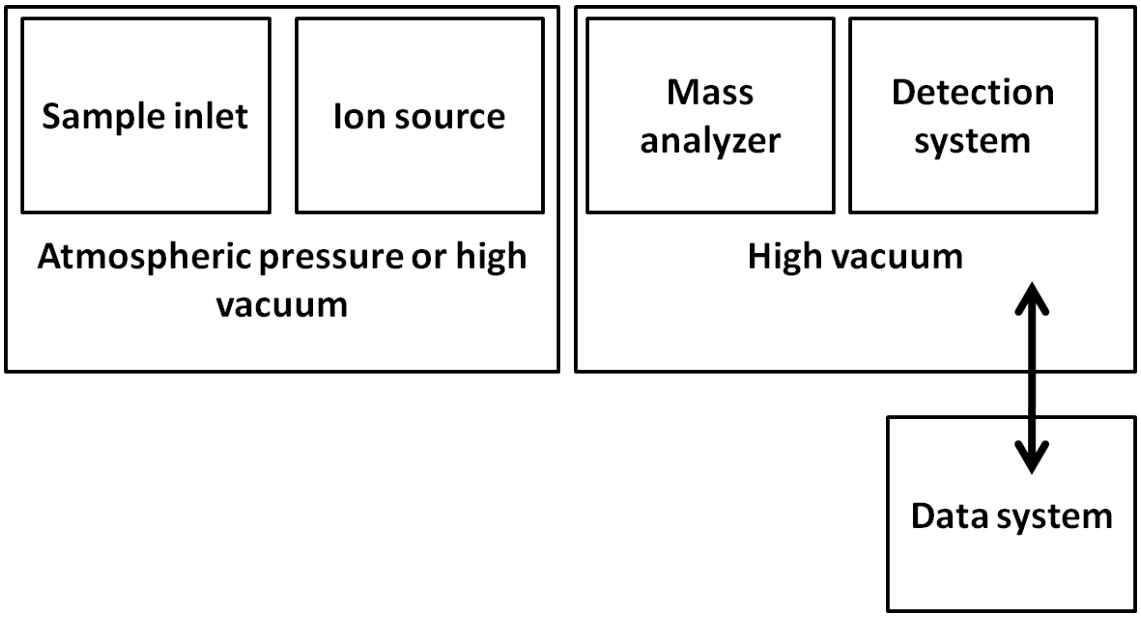


Figure 3.3: Scheme of a mass spectrometer, adapted from [51]

rameters that characterise a mass spectrometer are the resolution (R) and the mass accuracy attainable. Both parameters will be explained briefly in the following. The resolution is given by the following equation, in which m denominates the mass of interest and Δm the width of the peak at usually 10% of the peak height.

$$R = \frac{m}{\Delta m} \tag{3.1}$$

Sometimes, this definition also refers to the full width at half maximum (FWHM), which makes a considerable difference. Due to its definition, the resolution is dimensionless. The second parameter, mass accuracy is defined in the following way.

$$\text{mass accuracy} = \frac{m_{measured} - m_{calculated}}{m_{calculated}} \tag{3.2}$$

For the calculation of the mass accuracy attainable, the difference between the *measured mass* and the *calculated accurate mass* of a given compound is divided by the *calculated accurate mass*. Therefore, this parameter can indicate the quality of the obtained data and is important for calibration of the instruments. In general, it is usual to express mass accuracy in a relative manner as *parts per million* (ppm).
Due to the manifold variations of ion sources, mass analyzers and detection systems, only an overview can be given in this work. More detailed information can be found in the literature, as for example [51]. Therefore, only the relevant ion sources for protein analysis, matrix assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI), as well as inductively coupled plasmas (ICP) for elemental analysis will be briefly discussed in the following chapters together with the typically used mass analysers for the respective technique.

3.2.1 Instrumentation typically used for protein analysis

Due to the demands set by biopolymers such as proteins, the ionisation process must be extremely soft in order to conserve the structure of the analyte during its transition into the gas phase. In modern mass spectrometry for proteins and peptides, two ion sources predominate, namely matrix assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI). Their development has been awarded with the Nobel Prize in chemistry in 2002. The principle of both ionisation techniques will be discussed together with its possibilities and restrictions for the analysis of biopolymers such as proteins and peptides.

3.2.2 Matrix-Assisted Laser Desorption/Ionisation (MALDI)

The continuous evolution of laser based methods for desorption and ionization of analyte molecules for mass spectrometry was deeply affected by the idea of embedding the analyte into a matrix of light-absorbing compounds. The use of organic compounds as a matrix was proposed by *Hillenkamp and Karas* [52] and is nowadays state of the art for the determination of molecular weights of all kinds of compounds. Shortly after its discovery, its potential for the analysis of proteins was recognized

[53]. An alternative approach developed by *Tanaka*, which made use of mixing ultrafine Co-powder with the sample in glycerol was not further investigated [54]. Figure 3.4 shows a scheme of the process occurring during ionisation in a MALDI source.

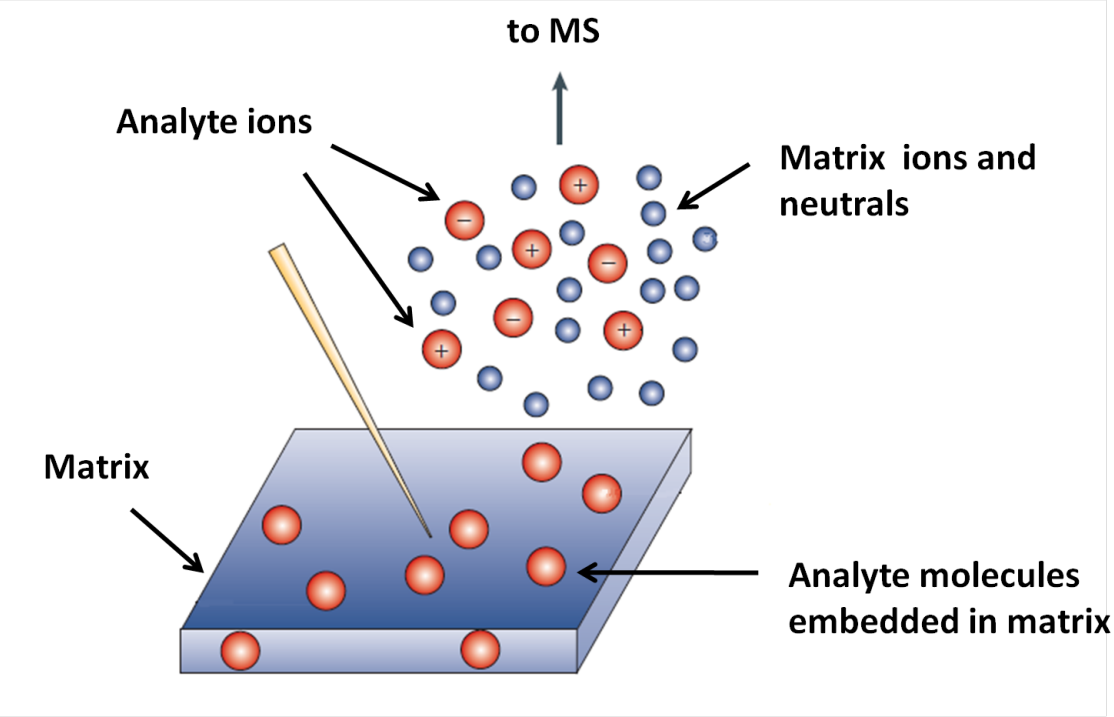


Figure 3.4: Processes occurring during ionisation in a MALDI source, adapted from [8]

For MALDI analysis, the analyte is mixed with the matrix solution and dried so that co-crystallisation between both occurs. The matrix molecules are present in great excess (1,000-10,000 : 1) to effectively protect the analyte from direct laser radiation. In the majority of instruments, UV-lasers are used, although also IR has been used for special applications. As the matrix should effectively absorb the laser radiation, mostly compounds with aromatic structures are used for UV-MALDI. Table 3.1 gives an overview of the most commonly applied matrix substances. In some cases, also combinations of different matrix substances or addition of other components can be useful for better results. For sample preparation, small aliquots (typically 0.5 μL) of sample and matrix solution are directly mixed on the surface of a target and then crystallized by air drying (dried droplet technique). The addition of a volatile organic solvent leads to the formation of a thin layer of crystals, which is advantageous for repeatability and mass accuracy.
However, detailed knowledge about the ionisation process in MALDI is still scarce. In earlier days, a two-step mechanism was suggested, were the primary ionised species (matrix) help to desorb the analyte-matrix cluster, and finally secondary reactions as for example proton transfer lead to the formation of $[M+H]^+$ ions of the analyte [59]. Later, a concerted mechanism was proposed, in which the protonated analyte is liberated after the decay of a cluster ion of the analyte with various

Table 3.1: Commonly used matrix substances for MALDI

Abbreviation	Name	Application	Reference
NA	Nicotinic acid	proteins and peptides	[53]
CHCA	α-cyano-hydroxy-cinnaminic acid	peptides	[55]
DHB	2,5-dihydroxy-benzoic acid	oligosaccharides	[56]
SA	Sinapinic acid	proteins	[57]
THAP	2,4,6-trihydroxyacetophenone	phosphorylated peptides	[58]

matrix molecules [60]. For more detailed information about the ion formation process in MALDI the reviews [61] and [62] are recommended.
As MALDI predominately generates single-charged ions ($[M+H]^+$, but also other ions such as $[M+2H]^{2+}$ and $[2M+H]^+$ are observed for entire proteins), it is more useful for the analysis of peptides than for entire proteins. As the mass resolution in time-of-flight mass analyzers widely used in MALDI mass spectrometers depends from the square root of the m/z value observed, the determination of $[M+H]^+$ for a protein is not very accurate. For smaller molecules like peptides, mass resolution is more than sufficient, so that the almost unique presence of $[M+H]^+$ leads to relatively simple mass spectra. In contrast, electrospray leads to the formation of multiple charged ions, so that even huge proteins can be detected at relatively small m/z values, enabling for example detection with quadrupole mass analyzers, or high resolution when using time-of-flight analyzers. Another important difference of MALDI-MS in contrast to ESI-MS is its inherent incompatibility for online detection in chromatographic separations. In the case of μ- or nanoLC separations, this can be overcome by fraction collection and subsequent spotting on a MALDI target followed by addition of the matrix. However, the combination of fast sample preparation, relatively high tolerance to salts, limits of detection in the fmol range and easily interpretable spectra has made MALDI an effective tool in protein analysis, as the entity of peptides resulting from an enzymatic protein digest can be analyzed in one mass spectrum, which can afterwards be matched to a database for protein identification. This procedure is called peptide mass fingerprint or peptide mass mapping [9], [63].

Mass analyzers for MALDI-MS

As MALDI is a pulsed ion source, the use of a pulsed mass analyzer is quite appealing [64]. Therefore, most instruments are equipped with time-of-flight (TOF)

analyzers. In this type of analyzer, the ions are accelerated with a certain potential (usually in the range of several kV), and then let to drift in a field-free region, the so-called drift region. After passing the accelerating potential all ions have received the same kinetic energy, but due to their different masses, their actual velocity during the trajectory towards the detector is different. Smaller ions will reach the detector earlier than heavier ones, separating them according to their mass. In order to achieve a higher mass resolution, a so-called reflectron can be included in the analyzer. This device consists of a set of ion lenses generating a repelling potential towards the charged ions (ion mirror). The ions are first decelerated and afterwards rejected into the contrary direction into a second field free region. This leads first to extended flight path, but can also reduce resolution limiting effects caused by different initial velocities of ions with the same mass. As faster ions will penetrate deeper into the ion mirror, their flight path will be prolonged in comparison to an ion with same mass, but lower initial kinetic energy. One important characteristic of time of flight mass analyzers is, that the mass resolution attainable decreases with the square root of m/z. For this reason, ions with a lower m/z ratio are resolved more efficiently than ions with a higher m/z ratio. Meanwhile, also TOF/TOF systems have been developed, which are capable of MS/MS analysis to elucidate more structural information of the analyte.

3.2.3 Electrospray Ionisation (ESI)

In contrast to MALDI, electrospray permits the generation of single- and multiple charged ions from biomolecules in solution, thus enabling on-line coupling of chromatography to the mass spectrometer as detection unit. Due to the extremely soft ionisation process and the generation of multiply charged ions, this technique takes a special place among all ion sources used for the analysis of biopolymers, such as proteins [65]. Especially the development of miniaturized ion sources for electrospray has had a deep impact on the development of proteomics as a scientific branch [66].

The working principle of ESI is based on the generation of charged droplets from the liquid sample. Therefore, the sample is introduced to the ESI interface via a syringe pump (or HPLC system) through a small capillary (diameter 50-100 μm) to a needle, which is kept at high potential (3-6 kV) towards the inlet of the mass spectrometer. Additionally, a flow of heated gas (typically nitrogen) is applied in order to desolvate the generated droplets. Figure 3.5 shows a scheme of an ESI ion source. Organic modifiers such as acetonitrile or methanol are useful for ESI, as they can reduce the surface tension of the sample and help to generate smaller aerosol particles. The addition of acids such as formic or acetic acid is also mandatory, as protonation of the analyte is the basic form of charge transfer to the analyte.

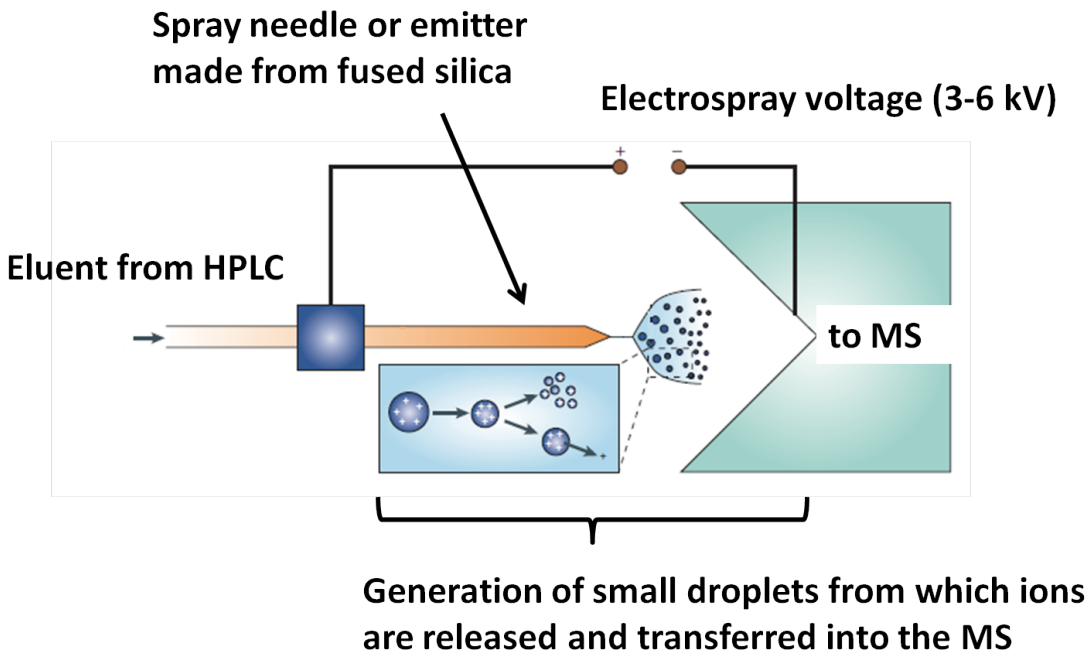


Figure 3.5: Scheme of an ESI ion source, adapted from [8]

During desolvation of the droplets, positive (or negative) charges accumulate on the surface of the droplet, which finally leads to the generation of smaller droplets after repelling electrostatic forces exceed the opposing surface tension of the droplet. After further evaporation of solvent, analyte ions are generated by either complete desolvation until only the charged analyte remains (charged-residue model, CRM), or by subsequent liberation of desolvated ions directly from the droplet surface (ion evaporation model, IEM). However, similar to MALDI, further discussion of both models is still necessary [67]. Reduction of the diameter of the spray capillary to a few μm leads to an ESI source with extremely low flow rates and sample consumption [66]. Also, these so-called nanoESI sources show an improved sensitivity and matrix tolerance in comparison to conventional ESI sources. As ionisation in an ESI source is predominately an electrochemically controlled process, the reduction of the flow rates leads to a better provision of charges to the analytes. Limits of detection for peptides are in the fmol range, but when operated in single ion monitoring mode (SIM), limits of detection can be similar or even better than for ICP-MS [68].
For ESI-MS instruments, various combinations of mass analyzers are commercially available. Due to the manifold applications where structure elucidation is one of the major goals of mass spectrometric analysis, almost all instruments commercially available are so called tandem mass spectrometers capable of MS/MS analysis [2]. Especially instruments equipped with three quadrupole analyzers are often the method of choice for routine analysis, as these instruments are highly sensitive when

[2] A more detailed explanation of some terms used in this paragraph, namely tandem mass spectrometry, MS/MS analysis and SRM will be presented in one of the following chapter. They are necessarily mentioned here for fit-to-context reasons.

operated in the selected reaction monitoring mode (SRM). Their limited mass resolution and accuracy excludes them from challenging applications like *de novo* peptide sequencing. For these applications, combinations of quadrupoles and TOF analyzers are suitable, as for example realised in the instrument used during this work (Q-TOF-MS, see chapter 4.2.6 for details). In these instruments, a full scan for the generation of a mass spectrum can be done using the quadrupole as ion guides only, whereas the TOF mass analyser can generate a highly resolved spectrum (resolution typically in the range of 10,000 to 15,000). For tandem mass spectrometry, a precursor ion is selected in the first quadrupole, whereas the second quadrupole serves as a collision cell. The mass spectrum of the product ions is once again generated in the TOF mass analyzer.
In recent years, the combination of a linear triple quadrupole system and a new type of mass analyzer, the so-called Orbitrap, was commercialised mainly with ESI ion sources which since then has overrun the market for high resolution and high mass accuracy mass analyzers. Theoretically, resolutions up to 200,000 and mass accuracies up to 1-2 ppm can be achieved [69].

3.2.4 Top-down and bottom-up approach for protein identification

For the mass spectrometric identification of proteins, there are in principle three strategies that one can pursuit. These strategies are called after the fundamental target for mass spectrometry, either the mass of the entire amino acid chain including possible modifications (top-down), or the masses of its corresponding peptides after proteolytic digestion using different enzymes (bottom-up). Figure 3.6 shows the experimental approach in both cases. Similar to the bottom-up approach, the so-called shotgun approach is based on the enzymatic digestion followed by mass spectrometric peptide identification. In contrast to the former, the shotgun approach is not restricted to a given target protein, but refers to the treatment of all proteins being present in a sample at the same time. The main advantage of this approach is, that even proteins that are hardly detectable due to their characteristics such as extreme pI values or molecular weights can be successfully identified, although they are easily lost when using a prior separation on the protein level, as e. g. two dimensional gel electrophoresis [70]. This however requires the use of powerful separation techniques based on multidimensional chromatography to previously separate individual peptides in order to reduce signal suppression in the ion source caused by other peptides present.
Whereas the top-down approach can deliver information about post-translational modifications which might change the activity or function of a given protein, this kind of information may be lost during the necessary sample treatment in the

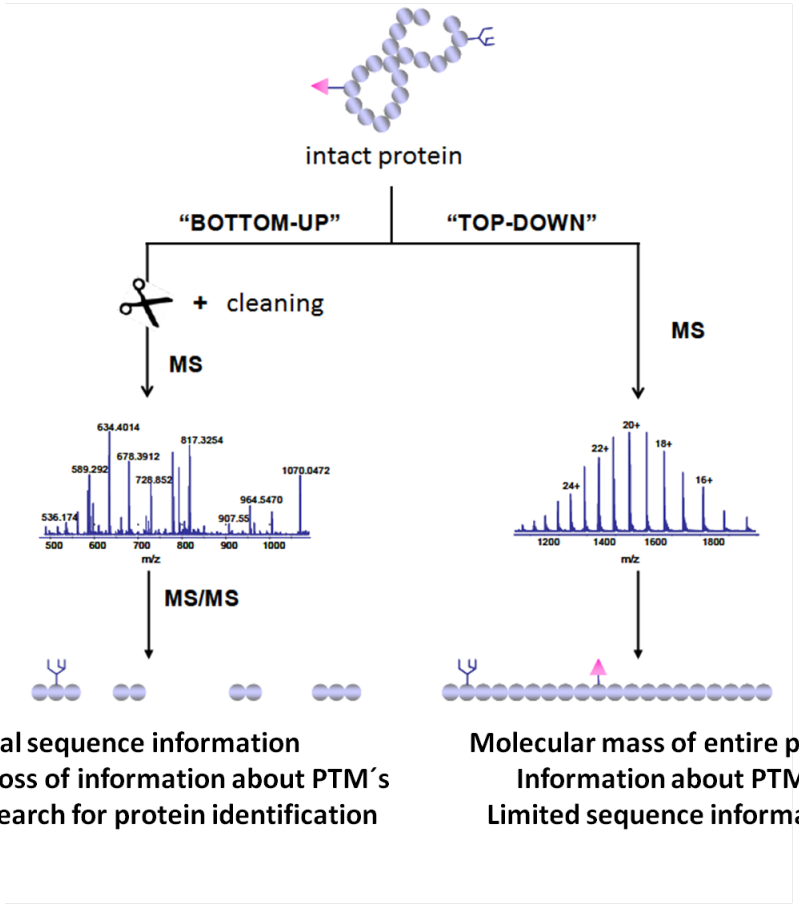


Figure 3.6: Experimental approach for protein identification using mass spectrometry following the "bottom up" and the "top-down" approach

bottom-up approach. Nevertheless, the latter one can be more specific, as a set of peptides may help to unambiguously identify a protein (peptide mass fingerprint), whereas a molecular mass may not be sufficient, especially when it has to measured with relatively high uncertainty. However, also ions from intact proteins can be subjected to MS/MS analysis for gaining information about their amino acid sequence, but straightforward sequencing is difficult, when its size superates 50 kDa (or 500 amino acids). Especially non covalent interactions like hydrogen bonds that arise due to protein folding during desolvation in an electrospray source can reduce the number of fragment ions that are formed after dissociation of an ionised protein [71].

For bottom-up approaches, the protein is digested and afterwards cleaned up before mass spectrometric identification. This approach can also help to identify unknown peptides and proteins (or parts of it) with help of the so-called *de novo* peptide sequencing [72]. Another important point for the analysis of peptides instead of proteins is, that efficient chromatographic separations are accomplished more easily, as the analytes are smaller [14].

3.2.5 Mass spectrometric peptide sequencing

As pointed out in the previous paragraph, mass spectrometric peptide and protein identification is nowadays one of the main tasks in systems biology and proteomics. Since the development of miniaturized ion sources such as nanoESI, tremendous progress has been made in the field, leading to the discovery of several hundreds of proteins in a sample [63], [73]. Especially the development of experimental sample treatment procedures, such as in-gel tryptic digestion [74] has enabled the role that is played by mass spectrometry today. Still, powerful separation techniques such as reversed-phase chromatography are mandatory for the effective separation of all peptides present in a sample, in order to assure conditions, under which ion suppression is avoided as far as possible [16].
For the elucidation of the structure of a peptide, the ion of the entire peptide (precursor ion) is selected in the mass analyzer and subjected to fragmentation using different mechanisms for energy transfer to the ion. The resulting new ions (product ions) can be analysed in a second mass analyzer. This methodology is called tandem mass spectrometry or MS/MS analysis. Depending on the type of instrument used, even the product ions can be subjected to further fragmentation if necessary. Different methods for energy transfer to a selected molecule have been developed and will be explained briefly in the following. Using different fragmentation mechanisms, different kinds of product ions are generated and hence different structural information can be obtained with regard to the precursor ion. The basic terms used nowadays for the product ions resulting from the fragmentation of peptides have been established in the early 1980ies. Following the nomenclature developed by *Roepstorff* and colleagues [75], the fragments of a peptide are denominated as shown in figure 3.7.

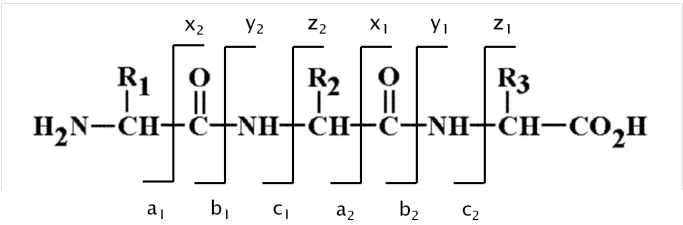


Figure 3.7: Nomenclature of peptide fragments following *Roepstorff et al.* [75]

Depending on the fragmentation mechanism used, different types of ions can be generated. The most valuable information about the sequence of a given peptide can be supplied by b- and y-ions. Here, bond breaking follows the lowest energy pathway along the peptide backbone, inducing cleavage of the amide bonds. The ions are called b ions if the charge is retained on the amino-terminal end, and y ions if retained on the carboxyl-terminal fragment. The formation of b- and y-ions is typically observed when using collision induced dissociation (CID) with gas molecules

like N_2 or He. For tryptic peptides, y-ions normally predominate due to their higher potential for charge retention of the basic lysine and arginine amino acids [9]. The number in the index of a, b, c ions corresponds to the number of amino acid residues present in the ion starting from the original amino terminus. Vice versa, the number in the index of x, y, z ions corresponds to the number of residues present starting from the original carboxyl terminus. Other fragmentation mechanisms such as electron capture dissociation (ECD) lead to the transfer of higher amounts of energy to the precursor ion, so that other fragmentation mechanisms can occur. In ECD, c and z ions typically predominate. This technique is more useful for the fragmentation of bigger biomolecules, as it can generate more fragment ions [76]. Besides the above mentioned fragmentation methods, also infrared multiphoton dissociation (IRMPD) is frequently used for mass spectrometric peptide sequencing. Here, energy is transferred to the precursor ions via laser radiation in the IR range. Also here, fragmentation along the peptide backbone occurs and therefore y and b ions predominate. A complete overview on the different mechanisms and methods can be found in [77].

The fragmentation process described above can also be used for other purposes than peptide identification. If the observed signal intensity of a specifically selected m/z values corresponding to the precursor ion of a given peptide and one (or more) of its characteristic fragment ions are monitored over time, this type of mass spectrometric experiment is called selected reaction monitoring (SRM) [78]. The result of this experiment is similar to a chromatogram, resulting in a signal, if this particular fragmentation process occurs as the previously selected peptide elutes from the column. If several such transitions are monitored, e. g. various transitions for one peptide and several different peptides during one chromatographic run, one speaks about multiple reaction monitoring (MRM). As for these applications the generation of a complete mass spectrum is avoided, greater dwell times in the scanning mode of the mass analyzers enable reduction of detection limits between of one or two orders of magnitude. Furthermore, the contribution of other compounds to the signal can be reduced very effectively by choosing a mass window size for both, precursor and product ion, as small as possible.

In the last years, there is a growing interest in the use of SRM or MRM applications in targeted quantitative proteomics, the quantification of previously selected peptides and hence proteins [79]. One of the main advantages of this methodology in comparison to the data evaluation from mass spectra is the improved linear dynamic range of up to five orders of magnitude, which is an important prerequisite for the analysis of low-abundant proteins in a highly complex mixture. However, although this technique has an enormous potential for quantitative analysis, the basic drawback of electrospray and also MALDI in this context is still not overcome.

Unfortunately, these ion sources commonly used for the studies on protein expres-

sion are known to have ionisation efficiencies depending on the particular compound ionised and furthermore these can be easily affected by other compounds being ionised in the same moment. Therefore, correlation of the observed signal intensity with the abundance of a particular compound in a sample is not possible. However, as pointed out in the introduction, quantitative information about protein concentrations is highly needed. Therefore, different techniques have been developed that allow relative quantification of proteins between two individual samples, mostly one sample in altered conditions and one control. Even if some of these methodologies claim to enable absolute quantification, it should be noticed that these methods still make use of relative quantification - relative to an internal standard [79]. One common feature of these approaches is, that additional information in form of a label is added on the protein- or peptide level. These labels normally contain isotopes which are not present under normal conditions or show low abundance, as e. g. ^{2}H, ^{13}C, ^{15}N, ^{18}O etc. The isotopic information is transported throughout the sample preparation and sample separation protocol and afterwards read out by mass spectrometry, resulting in the formation of signal duplets from identical compounds. Their signal intensity relative to each other should therefore not be affected by the ionisation process and can be compared to each other. The most outstanding examples for these methodologies will be presented in the following chapter.

3.3 Labelling in protein analysis

As pointed out in the introduction, proteins are involved in all functions in living beings. Their expression and possible modification are part of highly complex interacting processes, which can be influenced by a variety of external stimuli. As the exclusive consideration of gene expression is not sufficient for the description of the subsequent protein expression, methods have been developed to tackle the preferably complete description of a given proteome, both in qualitative (which proteins are expressed ?) as also in quantitative (how many copies of a given protein are produced ?) manner ([15], [16]). One of the fundamental objectives of this type of studies is to investigate the response of an organism to a change of its surrounding circumstances in order to gain insight to new biomarkers or potential target proteins. Within the last ten years, different types of labelling techniques have been developed to answer these questions. All of these methods have in common that they are fundamentally based in molecular mass spectrometry such as ESI-MS and MALDI-MS as a tool for protein identification. Due to their characteristics as ion sources, only relative quantitative information is accessible by comparison of isotopically labelled species or MS/MS-generated fragments of equal peptides, where the different isotopic composition encodes two (or more) individual states. Figure 3.8 gives an overview on the different strategies developed up to now.

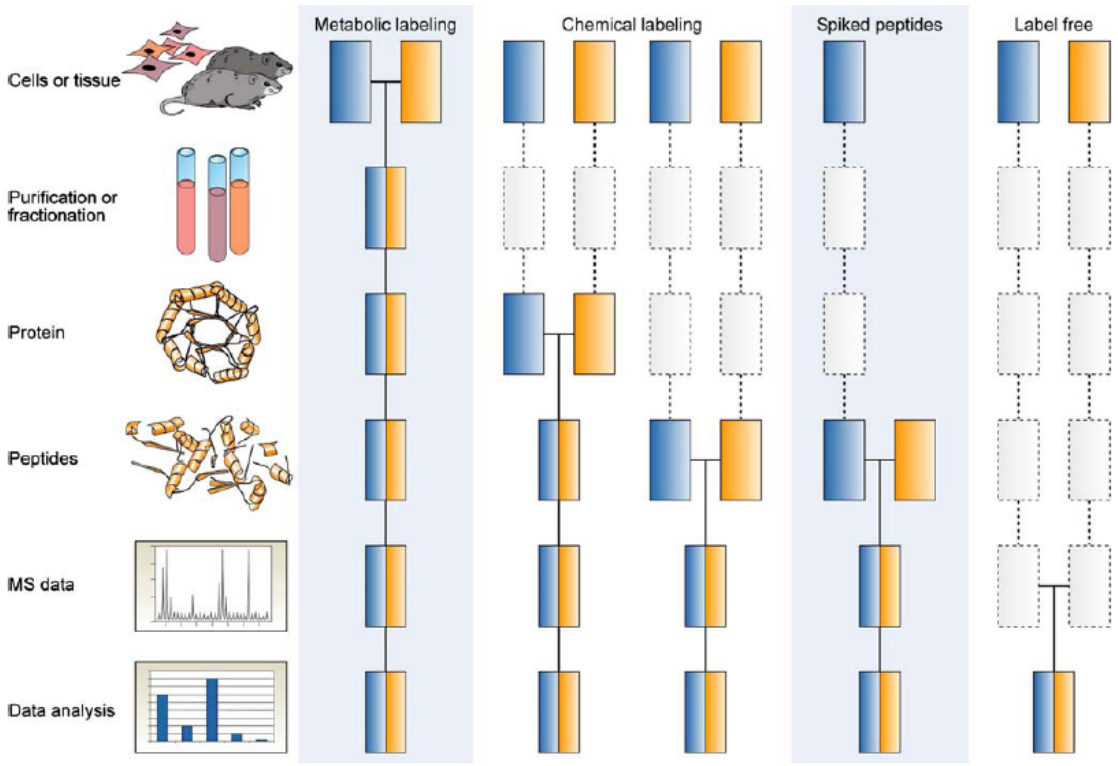


Figure 3.8: Overview on the labelling techniques developed up to now, adapted from [15]
Blue and yellow boxes represent individual states. The moment when both states are united is indicated by a straight line. Dashed lines indicate when both samples have to be treated separately and therefore quantification errors can occur.

Examples for all approaches will be given in the following chapter. As it can be seen in the figure, only metabolic labelling is able to compensate for quantification errors in all steps of sample preparation and -fractionation. Other methods, as for example chemical labelling, depend on the posterior introduction of the label by chemical reaction. Here, the moment of the introduction of the label is in all cases only possible after the first sample preparation steps, as for example protein extraction from tissue.

The same is true for the use of peptides, which were previously synthesised with enriched stable isotopes, as internal standards. The use of identical compounds with altered isotopic composition as internal standards for quantification has already been described for the analysis of smaller molecules such as cholesterol [80] and has been extended to peptides [81] and also proteins [82]. For the purpose of protein analysis, its great drawback is, that peptides of potential target proteins have to be known previously in order to synthesize the corresponding standards. Additionally, the unmanageable huge number of target peptides will impede this approach from a more general application.

3.3.1 Labelling techniques for molecular MS detection

Metabolic labelling

Metabolic labelling permits the incorporation of the label already on the protein level in the investigated organism *in vivo*. Therefore, most of the error sources present during sample treatment can be avoided. Likewise, the function of the respective proteins is not altered by the presence of the label, so that the expression of post-translational modifications can also be scrutinized in the same experiment. There are two fundamental ways in which metabolic labelling can be conducted. The growth of cell cultures on special media containing amino acids enriched in stable isotopes (as for example ^{15}N) was proposed by the group of *Mann* (SILAC = stable isotope labelling with amino acids in cell cultures, [83], [84]) and was frequently used for quantitative description of proteomes of a variety of organisms [85]. Alternatively, cell media which contain elements with altered isotopic composition can be applied. The isotopes are then subsequently included in the proteins during the growth of the organisms [86]. However, a certain degree of incorporation has to be accomplished in order to obtain significant results. Vice versa, the incorporation is not stoichiometric, so that the exact labelling degree has to be determined before quantification. Also here, studies on various organisms have been conducted, but the applicability of the approach is limited [87]. The experimental approach for the generation of quantitative data using this kind of labelling is similar to other labelling approaches and will be explained in the following paragraphs.

Enzymatic labelling

Another possibility for labelling of peptides is the introduction of isotopes during the enzymatic digestion of the protein [88]. As the binding between two amino acids is released by a protease, a hydroxyl group of the reaction medium (water) is introduced into the developing carboxyl group. If the hydrolysis of two samples is carried out in $H_2{}^{16}O$ and $H_2{}^{18}O$ respectively, chemically identical peptides with a mass difference of up to 4 Da are generated (due to the possible introduction of two oxygen atoms per amino acid). After combining both digestions, followed by chromatographic separation and MS analysis, relative quantification between both forms can be done via corresponding signals, as it is a common procedure for comparative proteomics [89]. However, the amount of single- and double substituted amino acids formed is depending on the reaction conditions and the type of enzyme used, so that in some cases a mixture between both is found which makes quantification difficult. However, this can also be used for discrimination between typical and non-typical peptides [90]. In principle, most of the commonly used serine proteases (as for example trypsin) can accomplish this type of labelling.

Chemical labelling

In contrast to metabolic labelling, the label is introduced into the protein via a chemical reaction, which leads to the selective derivatisation of certain binding sites within the protein. There are various amino acids which can be derivatised by different compounds. The most well-known examples will be presented in the following. One of the common features of most chemical labelling approaches is, that labelling is done on the peptide level instead of the protein level. Due to the smaller size of peptides, steric hindrance can be reduced and the chemical reaction leading to the attachment of the label is more efficient. This however, as shown in figure 3.8, implies that analyte loss occurring up to then are not compensated for by the label. Nevertheless, these approaches are commonly used in proteomic studies, as they are powerful tools for protein quantification and are not restricted to a special type of model organisms such as cell cultures.
The first published approach for a chemical labelling strategy was the ICAT reagent (isotope-coded affinity tags) presented by *Gygi et al.* in 1999 [91]. The structure of the reagent is shown in figure 3.9.

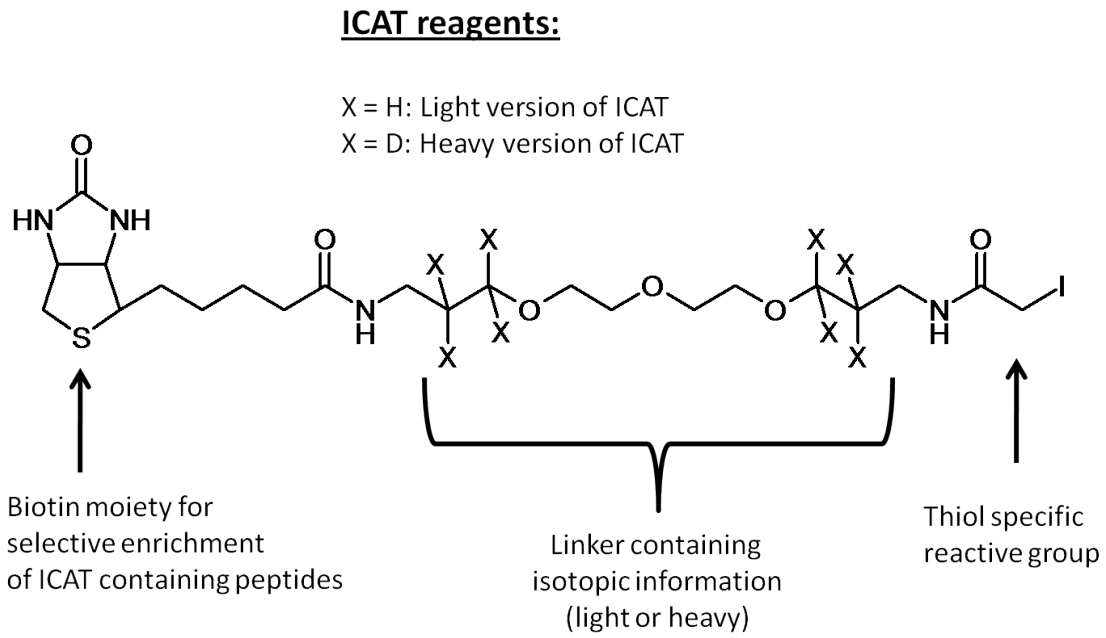


Figure 3.9: Structure of the ICAT reagent, adopted from ref. [91]

The reagent is constructed of a linker that permits binding to cysteine residues in peptides. The main part of the reagent contains a series of atoms x which can be H (light version of the reagent) or D (heavy version of the reagent). This part of the reagent is responsible for the characteristic mass shift between identical peptides coming from two samples. Their relative signal intensity can be used for relative quantification, as ionisation suppression effects will affect both forms to the same extent. The final part of the reagent is a biotin group, so that peptides that contain

the ICAT reagent can be selectively separated from all other peptides using biotin/streptavidin affinity chromatography. As there were indications, that the huge relative mass difference between H and D leads to isotopic fractionation for example in chromatographic separations [92] (complete separation of both forms, leading to possible quantification errors of possibly up to 500 %), an improved version of the reagent was produced, containing ^{12}C and ^{13}C, respectively, as the observed isotope effect is significantly reduced [93].
The experimental procedure for relative protein quantification using ICAT is similar to the SILAC approach, as both approaches are differential labelling approaches. Samples representing two different states of a biological system are labelled differentially with one form of the ICAT reagent. Afterwards, the samples are combined and fractionated. After separation of the labelled peptides to simplify the sample, relative quantification is achieved via comparison of the signal intensity of identical differentially labelled peptide. In the same analysis, identification of the peptides (and later via database search also their corresponding proteins) can be accomplished in an MS/MS run. Figure 3.10 shows the typically applied workflow.

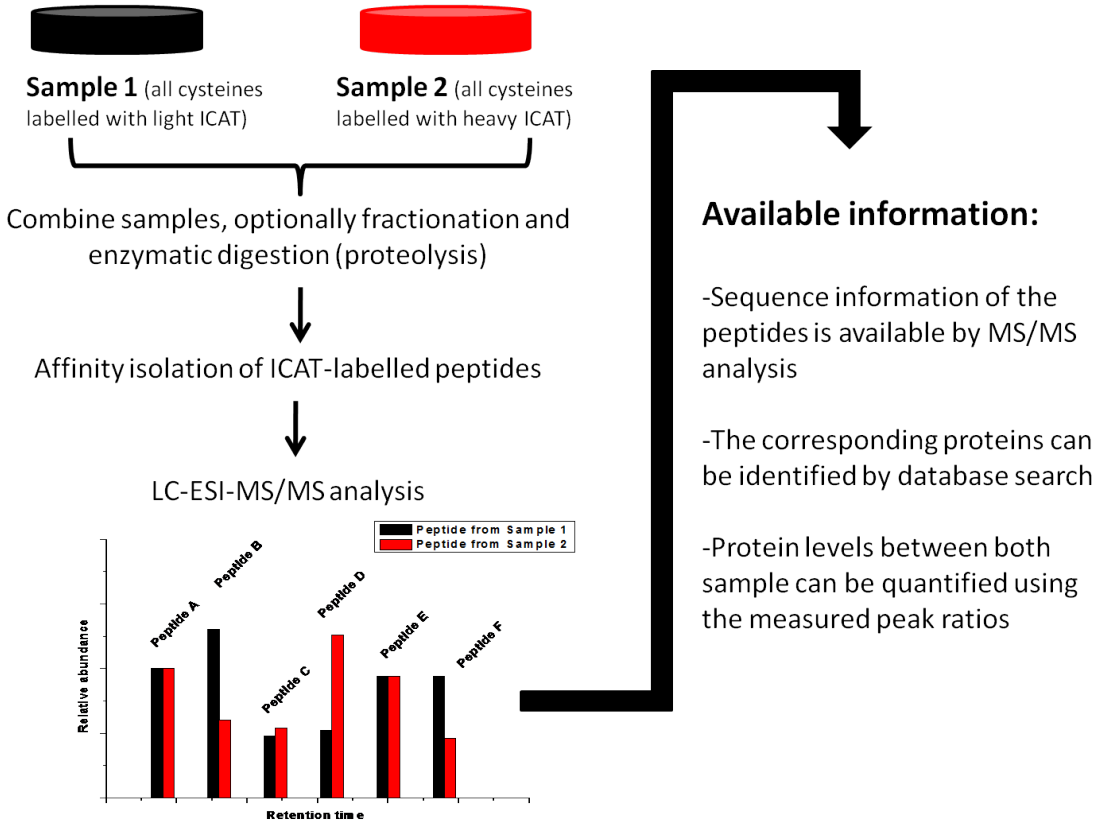


Figure 3.10: Workflow for relative protein quantification using the ICAT reagent, adopted from reference [91]

A similar approach to ICAT is the so-called iTRAQ (isobaric tag for relative and absolute quantitation, [94]) approach. Here, the label consists of different combinations of the enriched isotopes ($^{12,13}C$, $^{14,15}N$, $^{16,18}O$) in two different parts of the

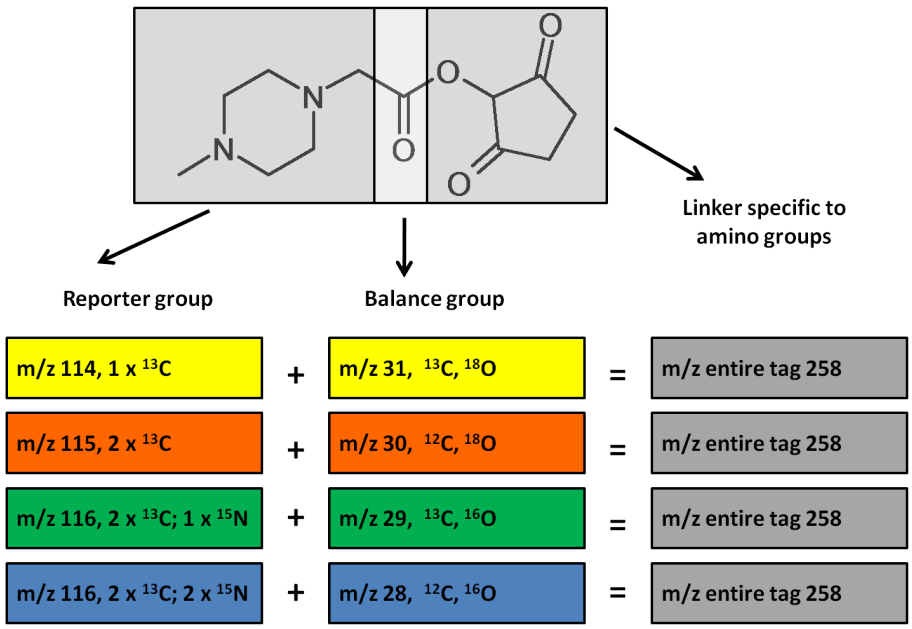


Figure 3.11: Structure of the iTRAQ reagent for the quantification of four samples

molecule (reporter-and balance group), giving always the same mass for the entire tag (isobaric), but different fragment (reporter) ions. Up to 8 samples can be compared to each other at a time using reporter groups of different masses, where each mass represents one of the samples investigated. After fragmentation, the ion intensities of the reporter groups reveal the relative amount of the peptides in the sample, so that after database matching the amount of protein can be deducted. Figure 3.11 shows the structure of this reagent for a simultaneous treatment of four individual samples.

One important difference of iTRAQ in comparison to ICAT is, that the reporter groups appear independently in the MS/MS spectrum in a zone, where no typical signals coming from peptide fragmentation appear. Therefore, they are easier to discriminate from other ion signals that do not correspond to the target peptides. If one of the treated samples contains an internal standard, which was previously added in a known amount, this can enable absolute protein quantification using the iTRAQ approach.

A further example for protein quantification after differential labelling of the corresponding peptides is the so-called GIST approach presented by *Regnier et al.* [95]. The derivatisation agent used is N-acetoxysuccinimide and its analogue containing three atoms of deuterium. In a first paper, overexpression of β-galactosidase in *Escherichia coli* was identified and quantified. However, this methodology is not commonly used, as similar to the first version of ICAT differences in retention times of identical peptides have been observed.

The use of spiked peptides is another possibility for the quantification of proteins. The first approach published was the so-called AQUA approach (absolute quantification (for proteins and peptides), [96]). Similar to species-specific isotope dilution

analysis (see chapter 3.6), isotopically enriched versions of the target peptides (or proteins) are added to the sample in a known amount. By comparing the signal intensities of the peptides of the sample (not enriched) to the intensity of the spiked peptide (mass shifted due to the enriched isotopes), absolute quantification is possible.

As the amount of peptide added for quantification has to be certified as well, the use of phosphorous and selenium detection and ICP-MS has been proposed as an accurate means ([97], [98]), although not used in the AQUA studies. The main disadvantage of this method is, that the structure of the target peptide must be known in advance to synthesize the enriched form of the peptide. Therefore, this approach is only suited for a very small percentage of proteins, and the analysis of unknown samples is not possible. However, in contrast to isotope dilution analysis, factors like enrichment or effects caused by isotopes of other elements being present in the species are not taken into account when quantitative information is generated. Due to the higher mass differences that separate ions from the same species, these superimposition effects are unlikely to be observed, and typical enrichment is set to be 100%.

However, isotope dilution analysis as a method for accurate quantification of substances using enriched stable isotopes and mass spectrometry has also been applied for protein and peptide quantification. *Arsene et al.* reported the exact and accurate quantification of human growth hormone in urine samples by IDA via two tryptic peptides (T6 and T12) [21]. The same peptides were used as internal standards after their synthesis with $^{13}C_6$, ^{15}N substituted leucine and $^{13}C_5$, ^{15}N-substituted proline. In this work, the complete uncertainty budget for the quantitative analysis of proteins in real samples was addressed. The authors calculated a recovery between 100 and 102 % (for quantification with T12 and T6 resp.) with standard deviations of 2.4 and 2.5 %. The combined uncertainty was calculated to be below 3.0 %, stressing the excellent results obtainable with isotope dilution analysis.

As it can be seen in figure 3.8, the use of labelling approaches on the protein level may be superior in comparison to labelling on the peptide level. In this way, one can already compensate for errors due to probable losses of peptides during the tryptic digestion step.

As a first method based in labelling on the protein level, the so-called IPCL (isotope-coded protein label) approach was presented [99]. This method aims on the derivatisation of primary amine groups and lysine side chains by N-nicotinoyloxysuccinimide containing ^{12}C and ^{13}C. Although it can additionally improve detection sensitivity due to the presence of the label also this approach is not as widespread as for example iTRAQ, probably because of his lacking multiplexing capacities.

In 2007, the PSAQ approach (protein standard absolute quantification) was published [100], using the target proteins synthesised with ^{13}C, ^{15}N enriched lysine and

arginine as spike compounds during analysis. The results obtained in a first comparative study to ELISA (determination of staphylococcal enterotoxins in food samples, [101]) showed the applicability of the concept and its higher accuracy in comparison to ELISA as a reference method. Recent advances and applications of this approach have been reviewed thoroughly [102]. As the certification of the concentration of the spiked protein is a crucial point in this approach, high effort has to be made to achieve the highest accuracy possible.

Another enhancement for the relative quantification of proteins with ESI-MS detection after labelling was proposed by *Whetstone et al* and *Liu et al.* in 2004 and 2006, resp. ([103], [104]). Whereas the first approach aimed at the derivatisation of cysteine residues with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), primary amine groups were the target for derivatisation with DTPA (diethylenetriaminepentaacetic acid) in the latter. After complexation of different lanthanides, a similar workflow as for the ICAT approach was applied. Here, the use of metals was introduced to the denomination of the tagging reagent: Element-Coded Affinity Tags (ECAT, [103]) and metal element chelated tags (MECT, [104]). The main advantage of this approach was the higher amount of available tags due to the 14 different lanthanide elements. It has to be mentioned in this context, that only five of the lanthanide elements are monoisotopic, whereas the others have a variety of isotopes, which would additionally complicate mass spectra. Additionally, one of the elements, promethium, is radioactive, and hence the number of reasonable accessible tags is surely lower. By using an affinity column that selectively binds lanthanide DOTA chelates, *Whetstone et al.* were able to purify and enrich the labelled peptides to simplify the sample before mass spectrometric analysis. Besides that, isobaric interference in ESI-MS detection of the peptides are also reduced, as smaller mass differences in multiple charged ions do not necessarily come from a label. For example, a mass difference of 6 Da in a triply charged ion can also be owed to a loss of water, so that two signals with a small mass offset may be mistakenly correlated although they are not related to each other by the label. Using lanthanide chelates as DOTA or DTPA, mass difference is typically in the range of 70-80 Da (for $[M+2H]^{2+}$ ions), making false identification of correlated peptides less probable.

Although the above presented approaches for protein quantification using different types of labelling have proven their applicability, they are mainly restricted to relative quantification. The main reason for this is non quantitative character of the ion sources typically used. Whereas for peptide and protein identification, mass spectrometric techniques such as ESI-MS and MALDI-MS are outstanding with respect to their possibilities, their potential for the generation of quantitative data is strongly compound-dependent. However, in many cases, absolute protein quantification is mandatory. Besides the techniques belonging to the field of molecular mass spec-

trometry, elemental mass spectrometry, especially inductively coupled plasma-mass spectrometry (ICP-MS) would be an appealing alternative for the absolute quantification of biopolymers and is presented as an alternative technique in the following chapter.

3.4 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

3.4.1 Inductively coupled plasma as an ion source

Since its introduction in the 1980s, inductively coupled plasma-mass spectrometry has become a benchmark technique for the analysis of the elemental composition of a sample [105]. In principle, all elements which have a first ionisation potential below 15.75 eV (first ionisation potential of Ar) can be ionised in an ICP. Therefore, all metals as well as most of the remaining semi- and non-metals (except C, H, N, O and F) can be determined. More details on the development and working principle of ICP-MS can be found in the literature ([106]-[108]). The extent of ion formation is depending of the plasma temperature and electron density and is described by the so-called Saha equation, here shown in a simplified form:

$$\frac{[M^+]}{[M]} \propto \exp^{\frac{-IP}{k_B T}} \tag{3.3}$$

with

[M$^+$], [M] Amount of ionised species and species in ground state, resp..

IP First ionisation potential of the respective element

k$_B$ Boltzmann constant

T Plasma temperature

Depending on the first ionisation potential, ion yield for metals is typically close to 100%, but significantly lower for semimetals like sulphur. The fundamental design of an ICP source has remained almost unchanged since its first introduction by *Fassel et al.* [109]. The plasma itself is a mixture of neutral atoms, ions and electron in the gas phase with a temperature between 5,000 and 10,000 K. Due to the presence of charges it is conductive (but neutral to its surrounding). After ignition with free electrons it maintains itself as long as sufficient power (typically 600 to 1,500 W) is applied. In the case of an ICP, this power is supplied by induction of an electromagnetic current and distributed by a coil surrounding the torch with typically three windings. Figure 3.12 shows a scheme of an ICP source. There are three independent gas flows, which are introduced via different channels of the

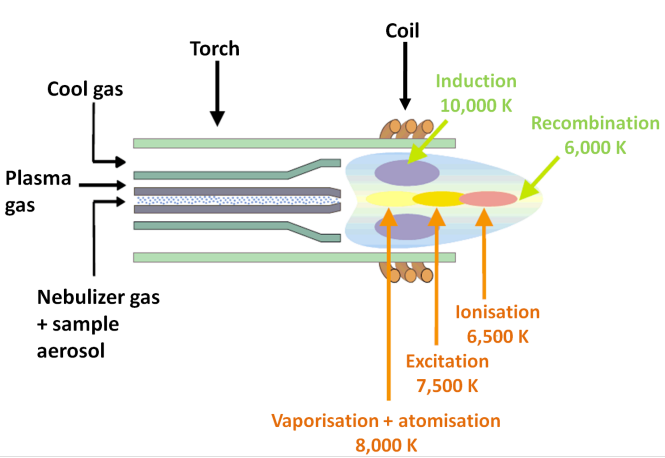


Figure 3.12: Design of an ICP torch by Fassel, [109]

torch. Through the inner channel the sample is introduced, either via pneumatic nebulisation using Ar for liquid analysis, or as a laser generated aerosol for solid samples. Gaseous samples can also be introduced to ICP-MS. The typically applied flow rates are between 0.8 and 1.5 L min^{-1}. In the second channel, an additional Ar flow between 0.7 and 1.0 L min^{-1} is introduced for the generation of the plasma, whereas in the outer channel Ar with a flow rate of approximately 15 L min^{-1} is introduced for cooling of the torch system. Due to the high thermal demands of the torch, it is manufactured of quartz. The plasma is generated in the induction zone (see figure 3.12 for details). Here, ionized Ar species are accelerated by the electromagnetic field and collide with neutral atoms (Ar or analyte atoms) to generate new charged species by either direct ionisation (electron impact or Penning ionisation) or secondary ionisation caused by charge transfer from ionised argon. Also other processes are possible, as for example photoionisation.
When the analyte molecules enter the plasma, they are first desolvated (in case of a liquid aerosol), then vaporised and atomised. This however leads to the loss of structural information about the analyte, so that only the total amount of the element is accessible, if the chemical composition of the sample is unknown. If information about different species is desired, the sample has to be previously separated by different analytical techniques. The high temperature permits that the corresponding atoms are then ionised, and the remaining species can be analysed by optical emission (ICP-OES), or by mass spectrometry. The latter technique is clearly superior in terms of detection limit and can also give information about the isotopic composition of the analyte, whereas the former has a higher matrix tolerance. To summarize, inductively coupled plasma is an ion source for mass spectrometry which has outstanding properties for elemental analysis, as for example:

- Limits of detection in the range of pg g^{-1} to ag g^{-1} for most of the elements
- High dynamic range up to nine orders of magnitude

- Species independent ionisation enabling quantitative and semiquantitative analysis
- Multielement analysis possible and isotopic information available

However, there are also some drawbacks, which have to be kept in mind when working with ICP-MS. As for all spectroscopic or spectrometric techniques, especially the presence of interferences can lead to errors. In the colder zone of the plasma, recombination between ions can occur, and this leads to the formation of polyatomic species (mainly caused by Ar, O, N, etc.), which can give an additional contribution to the signal on a certain mass to charge ratio corresponding to the analyte. Most of all, these so-called polyatomic interferences occur when measuring in aqueous solutions, as for example diluted acids or salt containing buffers and hamper the determination of almost all elements in the lower mass range (up to masses 80-90). Further interferences can occur as isobaric interferences caused by the presence of other elements having isotopes of the same nominal mass. Especially the most representative elements for the determination of proteins, sulphur and phosphorous are highly interfered. Table 3.2 shows some of the most frequently found polyatomic interferences for elements, which are of interest for the detection of proteins. The table also shows the resolution necessary for the complete separation of the ion of interest from the interference. Also, non-spectral interferences like the efficiency of nebulisation and vaporisation/atomisation can have an influence on the response of the detection system, if different matrix solutions are measured (for example due to the presence of organic modifiers such as alcohols or different combinations of acids).
The formation of spectral interferences such as oxides leads not only to the presence of potentially disturbing interferences, but it can also reduce the number of detectable ions of a certain isotope and should therefore be kept as low as possible by choosing appropriate operation conditions. As the formation of polyatomic interferences and their removal is of fundamental interest in ICP-MS, special techniques have been developed which will be discussed in the following chapters.

Table 3.2: Polyatomic interferences and necessary resolution for their complete removal

target isotope	**interference**	**resolution**
$^{31}P^{+}$	$^{14}N^{16}O^{1}H^{+}$	1,500
$^{32}S^{+}$	$^{16}O_2{}^{+}$	1,800
$^{34}S^{+}$	$^{16}O^{18}O^{+}$	1,300
$^{54}Fe^{+}$	$^{40}Ar^{14}N^{+}$	2,100
$^{56}Fe^{+}$	$^{40}Ar^{16}O^{+}$	2,500
$^{63}Cu^{+}$	$^{40}Ar^{23}Na^{+}$	5,000
$^{80}Se^{+}$	$^{40}Ar_2{}^{+}$	9,700

Quadrupole mass analysers are the most common in ICP-MS systems commercially available. Due to their simple working principle, they are robust, relatively small and can be operated easily. As a quadrupole works only by the application of electric voltages, it can be switched rapidly between different mass to charge ratios, allowing high scan speeds. A quadrupole consists of four rods made of stainless steel, arranged parallel to one another [110]. On one pair of opposing rods, a direct current is applied, which represents the ground potential of the quadrupole. Additionally, a second mouldable radiofrequency current is applied to the other pair. Under the influence of both currents, ions with a certain mass to charge ratio are forced on a circular trajectory towards the outlet of the quadrupole, where the detector is located. All other ions with different mass to charge ratios are forced to instable trajectories and collide with the rods before leaving the quadrupole. The ion trajectories can be described with the Mathieu equations. These equations introduce two parameters U and Q, which represent the two applied electric fields. For each mass to charge ratio, only a certain value for U/Q gives a stable trajectory through the quadrupole. Scanning different mass to charge ratios is achieved by increasing both parameters at a constant ratio, leading to temporarily stable trajectories for all ions at a time. The slope of the increasing of both voltages can affect the resolution attainable, but it also affects the number of ions which can pass the quadrupole under the applied conditions and therefore the sensitivity of the instrument.
However, mass resolution achievable with quadrupole mass analysers for ICP-MS is relatively poor (up to ≈ 300, as R is depending on the observed mass), so that only integer mass numbers can be separated from each other. Therefore, care has to be taken concerning spectral interferences, as these also contribute to the signal of a certain mass to charge ratio and will lead to wrong results.

Removal of spectral interferences with the use of collision/reaction cells

The introduction of collision/reaction cells has greatly improved the applicability of quadrupole based ICP-MS instruments for the analysis of interfered analytes. Here, a cell is installed in front of the quadrupole, which can be filled with a gas at low pressure. The cell can be a smaller quadrupole or a similar device (hexapole or octapole, depending on the manufacturer), in which the ions will collide with the gas present in the cell. Depending on the gas used and the applied voltage between the cell and the mass separating quadrupole, one can distinguish between operation as a collision- or a reaction cell. An exhaustive description of the technique can be found in [111].
Collision cells work with the so-called kinetic energy discrimination. Here, the cell is filled with a chemically inert gas (mostly He, but also Xe was described for the analysis of sulphur [112]). Additionally, an energy barrier (about 2 V) is set between

the collision cell and the quadrupole, so that only ions with sufficient kinetic energy can overcome this barrier to be finally detected. Analyte ions as well as polyatomics leave the interface and enter the cell with a certain kinetic energy and start colliding with the collision gas, leading to loss of kinetic energy. As the polyatomic interference consists of two or more atoms, it has a bigger cross section and therefore collides more often and thus suffers of a higher loss of kinetic energy, so that its remaining kinetic energy is not sufficient to enter the quadrupole. In contrast, the analyte has still sufficient energy to pass the barrier and is the only ionic species which is detected at a certain mass-to-charge ratio.
Reaction cells make use of reactions between the cell gas and either the polyatomic interference or the analyte. The use of different reaction gases for different analytes has been described, as for example H_2 ([113], [114]) for the detection of Se, O_2 for P and S [116], or CH_4 [115]. All of these approaches have in common, that either the previously charged interference is converted into a neutral or not-affecting charged species, or the analyte mass is shifted to a not interfered region. Therefore, the charged species are to be extracted out of the cell by a potential difference towards the separating quadrupole.

Removal of spectral interferences with the use of sector field instrumentation

A second possibility for the removal of spectral interferences is the use of so-called sector field instruments [117]. Here, the behaviour of charged particles in magnetic fields is used for ion separation with different mass to charge ratios. As the ions generated in an inductively coupled plasma show a broad discrimination of kinetic energy, an electrostatic sector is situated after the magnetic sector to focus ions with the same mass to charge ratio, but different kinetic energies in order to achieve maximal resolution. Both sectors are located in a manner that their focussing points overlap in a common point, which leads to the term double focussing mass analyser. The mass resolution is adjustable by varying the diameter of the entrance- and exit slits of the mass analysers, so that resolutions between 300 and 10,000 are attainable. This enables the mass spectrometric separation of the observed element and the undesired spectral interference. However, diminishing of the slit diameter also correlates with exclusion of ions, so that sensitivity has to be sacrificed for resolution. In any case, it has to be mentioned that although in ICP-MS working with instrumentation that permits resolutions of 3,000 or 10,000 (what is often referred to as "medium" or "high" resolution in ICP-MS), the achievable resolution is poor in comparison to organic mass spectrometry, due to the smaller masses of the analysed ions. One of the main reasons for this is the broad kinetic energy distribution of the ions generated by an inductively coupled plasma. Sector field mass spectrometer are more expensive and require skilled operators. However, they have been established as mass analysers for ICP due to their higher sensitivity and

the ability for complete removal of most spectral interferences from the analyte signal.

3.4.2 Hyphenation of ICP-MS with separation techniques

Although ICP-MS is a powerful tool for the determination of most of the elements in the periodic system, it can only give information about the presence of a given element in a sample, but not on its chemical form. The chemical form however can have dramatic effects on different properties, as for example toxicity [118]. In order to address the distribution of a given element in its different species, the combination of ICP-MS with a highly efficient separation technique is necessary. These combinations are called hyphenated techniques. For the analysis of different species of an element, the term speciation was coined [119]. In many cases, the hyphenation of separation techniques to ICP-MS is relatively straightforward. In the case of gas chromatography (GC), the outlet of the column is directly connected to the ICP-MS via a heated transfer line to avoid condensation of analytes due to the temperature difference. Although possible applications for GC for the analysis of biomolecules are limited, it has been frequently applied in environmental analysis, for example for the determination of alkylated species of metals and semimetals [120]. The combination of liquid chromatography (LC) to ICP-MS was first described by *Thompson et al.* [121]. Since then, many applications using all kinds of chromatographic separation mechanisms have been published [122], [123]. Also here, hyphenation to ICP-MS is accomplished easily by direct connection of the column outlet to a pneumatic nebulizer, since typical flow rates for LC (0.5-1.0 mL min^{-1}) match the uptake rate of the usually applied nebulizers. However, due to the ongoing miniaturization of analytical systems, column dimensions and therefore also flow rates were reduced, so that the demands set by pneumatic nebulizers could no longer be fulfilled without significant dilution of the eluting analytes. Similar needs came up for the hyphenation of capillary electrophoresis (CE), a technique offering outstanding separation capacities, but very low flow rates [124]. Furthermore, especially reversed-phase chromatography requires high amounts of organic solvents for gradient elution of proteins and peptides, which causes severe problems with plasma instability. Driven by this, new types of miniaturized nebulizers were designed, in order to successfully couple this kind of separation techniques to ICP-MS ([125]-[127]). Due to the low flow rates (between 0.5 and 15 μL min^{-1}), these nebulizers can achieve a complete nebulisation of all incoming liquid. Therefore, it was possible to work with spray chambers without a drain connection, so-called total consumption spray chambers. The development of the hyphenation of μ- or nanoLC to ICP-MS was the basis for its application in proteomic studies and its role is of steadily growing importance [128]. This also opened up new fields for ICP-MS, as for example the determination of trace metals in oil samples [129]. Using low flow rates, it is possible to intro-

duce pure organic solvents to ICP-MS, which previously lead to huge problems with plasma instability. However, the introduction of varying amounts of organic modifiers as for example acetonitrile causes changes in detection sensitivity caused by the so-called carbon enhancement effect [130]. Whereas for some elements like P, As or Se, improved detection sensitivity is observed, other elements as for example metals are detected less sensitive. In recent years, strategies have been developed to compensate for changing detection sensitivity during gradient elution. Especially for the quantification of phosphorylated peptides, where isotope dilution analysis is not possible, different strategies were pursuit, as for example correction of the sensitivity by using a mathematically adopted response curve [131], or the continuous post-column addition of acetonitrile to minimize the effect [132].
Another important separation technique for protein samples is gel electrophoresis (GE). In combination with ICP-MS, there are in principle two ways of interfacing gel electrophoresis. The use of laser ablation (LA) for the offline analysis of gels is a major part of this work and will be discussed in a separate chapter (see chapter 5.2.3). Some years ago, *Brüchert et al.* established an online coupling of gel electrophoresis to ICP-MS. To achieve this, the gel is stuck in a glass tube (similar to a chromatographic column). On the end of the glass tube, a membrane is placed, where eluting compounds are washed away by an elution buffer proportionated by an external peristaltic pump [133]. Due to the membrane, conductivity between both electrodes on either side of the gel is established. This set-up has been successfully applied to the separation and determination of *cis*-platin incubated oligonucleotides [134], but as well for size determination of nanoparticles [135] and speciation analysis of iodine in aerosols [136]. Only recently, *López Fernández et al.* were able to quantify DNA damage caused by oxidative substances [137].

3.4.3 Application of hyphenated techniques for absolute protein quantification

Due to the versatility of ICP-MS in combination with different separation techniques, many different studies have been published on the separation and determination of proteins during the last years. As a first subject of studies, metallothioneins (MT) were investigated due to their function in the detoxification mechanism of toxic metals, as e. g. Cd [138]. Therefore, the signal trace of the metal could be easily used for detection of the targeted protein species in complex samples, such as liver or kidney extracts from various organisms. For the effective separation of MT isoforms, the use of multidimensional separation techniques (such as size-exclusion chromatography combined with reversed phase chromatography) was pursuit, and the combination of ICP-MS detection of metal-containing species followed by their identification using complementary molecular mass spectrometry was proposed.

As pointed out before, detection of abundantly occurring heteroelements (sulphur and phosphorous) in biopolymers such as proteins and peptides using ICP-MS is hampered by their low ion yield and the numerous spectral interference. However, several studies have aimed at absolute quantification using both Q- and SF-MS instrumentation. In a pioneering work from *Lehmann et al.* [139], phosphorylated β-casein was determined from polyacrylamide gels by laser ablation after electrophoretic separation. In a first study involving the use of reaction cell technology for the detection of sulphur in proteins, metallotheonein proteins extracted from liver samples were analysed using capillary electrophoresis coupled to ICP-MS [112]. *Zinn et al.* have introduced the combination of μLC coupled to SF-ICP-MS for the detection and absolute quantification of sulphur containing proteins [140].
Besides that, many metals naturally present in proteins have been used for identification and quantification of proteins using classical means of speciation analysis. Among these, iron containing proteins such as haemoglobin and transferrin ([141], [142]) have been studied thoroughly. In the case of transferrin, the combination of anion exchange chromatography and ICP-MS could help to separate and accurately quantify different isoforms of the protein, which are related to chronic alcohol abuse. Using the same experimental approach, a study on the health status of harbour seals was published, monitoring the transferrin quantity and its isoform distribution [143]. Also the iron containing enzyme catalase, which plays a role in the defence mechanisms towards oxidative stress has been studied [144]. Here, a two-dimensional separation protocol was applied in order to completely separate the target protein from other Fe-containing proteins, such as impurities of ferritin. Also proteins containing other metals, such as the Cu and Zn containing enzyme superoxide dismutase ([145], [146]) was successfully analysed with ICP-MS.
Another element which can be present in proteins and is likely to be detected by ICP-MS is selenium. Whereas Se in its inorganic forms is toxic, it is an important trace element in its other species. In serum, it can be present in the amino acid selenocysteine, and thus for example being incorporated in e. g. extracellular glutathione peroxidase (eGPx) or selenoprotein P [147].
Besides that, also incorporation of metals ions with exogenous origin into proteins has been subject of study, as for example Ti [148], which can be liberated by different kinds of protheses and seems to be transported by transferrin in the blood cycle. For a more detailed summary, a review has been published recently [12].
However, although the potential of ICP-MS in protein analysis is proven, its broader application in trace analysis of proteins is limited by the facts, that the elements ubiquitously present in proteins suffer from spectral interferences and high limit of detections, whereas metals do only occur in a limited number of proteins. Therefore, the artificial introduction of an ICP-MS detectable element into proteins might be the key to new analytical possibilities, namely exploiting the superb limits of detec-

tion and the outstanding potential for absolute quantification offered by ICP-MS.

3.5 Labelling in combination with ICP-MS detection

As pointed out, the use of ICP-MS in combination with elemental labelling could be an appealing alternative for absolute quantification of proteins and peptides. Besides its outstanding quantitative properties, ICP-MS can also help to reduce the complexity of mass spectra [12], as only the signal intensity of a few isotopes are measured, either as a mass spectrum or as a function of retention time in a chromatographic run. On the other hand, all structural information is lost, so that the species that are to be quantified have to reach the plasma purified from other species containing atoms of the same element, and their structure has to be known and characterised with respect to the number of analyte atoms they contain. However, as coupling of a wide range of powerful separation techniques (HPLC, GE, CE) used for protein analysis is easily possible, many approaches have been published in recent years, that try to explore the capabilities of ICP-MS for the detection of proteins and peptides after the introduction of an ICP-MS-detectable element [149]. Also here different strategies have been developed, which differ in the target for labelling. Many of them have in common, that already well-established reagents or procedures were adapted to meet the demands of ICP-MS in terms of sample conditions and to later on explore its potential for absolute quantification. Figure 3.13 gives a basic overview of the yet proposed strategies.
One example for this proceeding is the use of antibodies labelled with lanthanides or gold nanoparticles, as these are frequently used for fluorescence detection purposes in ELISA assays (A in figure 3.13). Using the high stability of chelate complexes of metals, the complexing agents can be attached to proteins to incorporate the label (see B in figure 3.13). Finally, some elements easily detectable in ICP-MS can be attached directly to proteins, or in form of smaller molecules (C in figure 3.13). Examples for all these approaches will be presented in this chapter together with their potential advantages and drawbacks.

3.5.1 Protein quantification using labelled antibodies

The concept of using labelled antibodies for absolute protein quantification was first described independently by the groups of *Tanner* (lanthanide containing chelating agent, [150]) and *Zhang* (gold containing nanoparticles, [151]). The advantage of this methodology is, that the unmatched specificity of antibodies towards their corresponding antigens (e. g. proteins) can be used for the separation of a target protein from the sample as used in ELISA assays. As all structural information is lost in an ICP source, the analyte has to be completely separated from other species

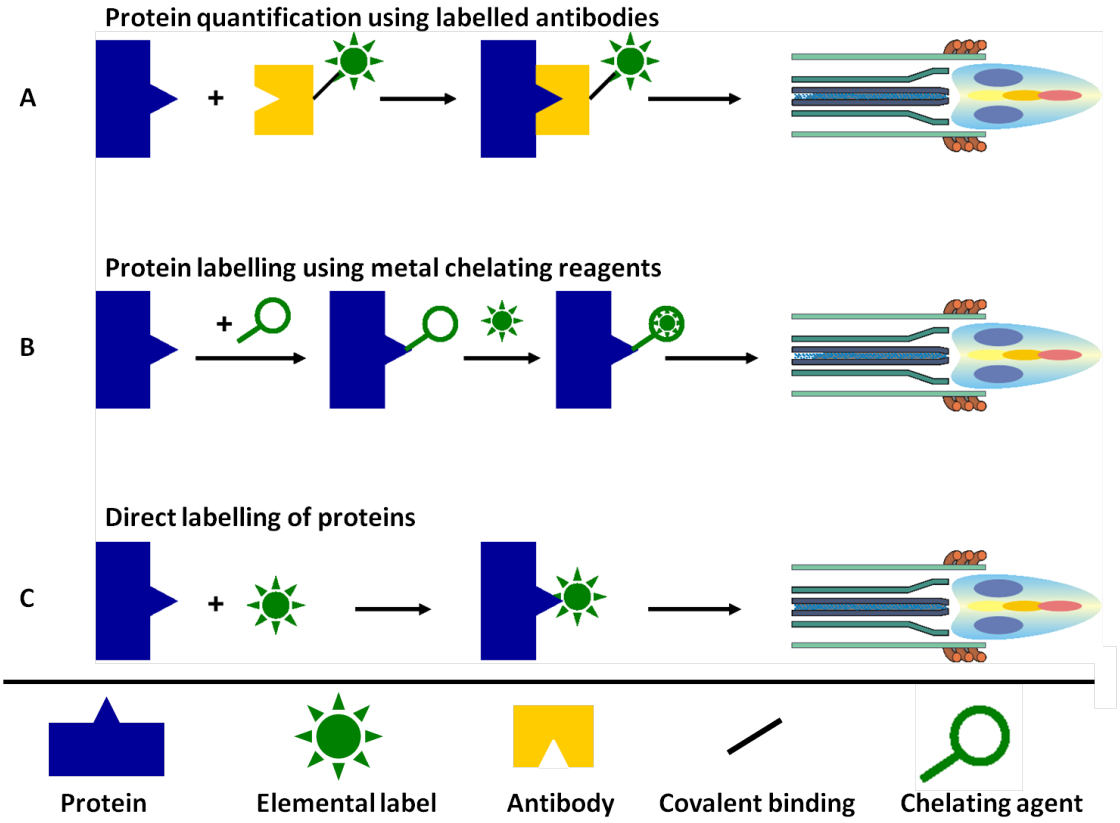


Figure 3.13: Labelling strategies published for the use in combination with ICP-MS

containing the same element. Here, first of all a primary antibody is immobilized onto a surface. In a second step, the sample is applied, and the target antigen binds to the antibody and gets also immobilized. After several washing steps, a secondary antibody, which binds to a different epitope of the same target molecule and carries the elemental label, is applied. After solubilisation of the entire complex using acids and addition of an internal standard the total concentration of the element is measured and from the results, the concentration of the sought antigen can be calculated. This procedure is frequently applied in biochemistry and similar fields, as for example food analysis and environmental toxicology [152]. Meanwhile, even direct comparison of ICP-MS and fluorescence based ELISA essays was accomplished, showing that low limits of detection is not an exclusive trademark of ICP-MS [153]. However, one of the main advantages of ICP-MS in comparison to other techniques is its multielement capacity, making multiplexed analysis of various target analytes possible if they are specifically encoded with different elements [154]. The subsequent development of the proposed methodology by *Tanner* and co-workers has lead to the development to a new type of ICP-TOF-MS instrumentation for the description of cell states after lanthanide-antibody labelling [155]. Whereas the conventional method (flow cytometry) works with dyes, which are then activated with lasers to detect their fluorescence, this new method instead encodes the expression of different target biomarkers with isotopes, which are then detected in ICP-MS.

It could be shown, that this methodology enables multiplexed analysis of up to 20 individual antigens, which greatly exceeds the number of antigens accessible with conventional instrumentation due to the spectral overlap of the signals [156]. In a recently published study, the authors were able to determine a total of 34 individual parameters in one single run, of which 31 were proteins or surface markers previously derivatised with metal-containing antibodies [157]. The potential of using labelled antibodies together with ICP-MS was also recognized by the group of *Jakubowski*. In their approach, antibodies against proteins from the cytochrome P-450 family (CYP1A1 and CYP2E1) were applied for the detection of their antigens. The antibodies were labelled with p-SCN-Bn-DOTA to derivatise the ϵ-amino group of lysine residues, which was afterwards incubated with Eu. After electrophoretic separation of the samples (rat liver microsomes), the proteins were blotted onto nitrocellulose membranes and the membranes were analyzed by LA-ICP-MS [158]. As ICP-MS offers multielement detection, they were able to quantify five proteins from the cytochrome P 450 family in one run, using a specific antibody labelled with different lanthanides for each of them [159].
The potential of the use of labelled antibodies for protein quantification was also demonstrated by *Terenghi et al.* using classical methodology of speciation analysis, SEC coupled to ICP-MS [160]. After antibody labelling using DOTA and incubation with different lanthanide elements, the authors were able to determine five cancer biomarker proteins in one run. Although limits of detection were similar to ELISA, the proposed methodology enabled multiplexed analysis due to the multielemental detection capabilities of ICP-MS.

3.5.2 Protein labelling using metal chelating reagents

The first direct labelling approach of proteins with lanthanide chelating agents and ICP-MS detection was published by *Linscheid´s* group [161]. These complexes show very high stability constants for the binding of lanthanides and should thus enable complete and enduring derivatisation. The so-called MeCAT (Metal-coded Affinity Tag) approach uses DOTA complexes (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) of different lanthanides together with a specific binding site (maleinimido group) for binding to cysteine residues in proteins. Figure 3.14 shows the structure of the reagent.
Besides the metal chelating part of the molecule, the DOTA macrocycle (part A in the figure), a spacer (B) was included, as well the maleinimido group (C), which is finally responsible for binding to cysteine residues. Additionally, a biotin group can be introduced into the structure of the reagent in order to enrich MeCAT labelled peptides via affinity purification (D in part B in figure 3.14). Further systematic studies on this approach showed, that the use of the label is compatible with typical workflows in biochemistry and proteomics [162]. The stability of the binding was

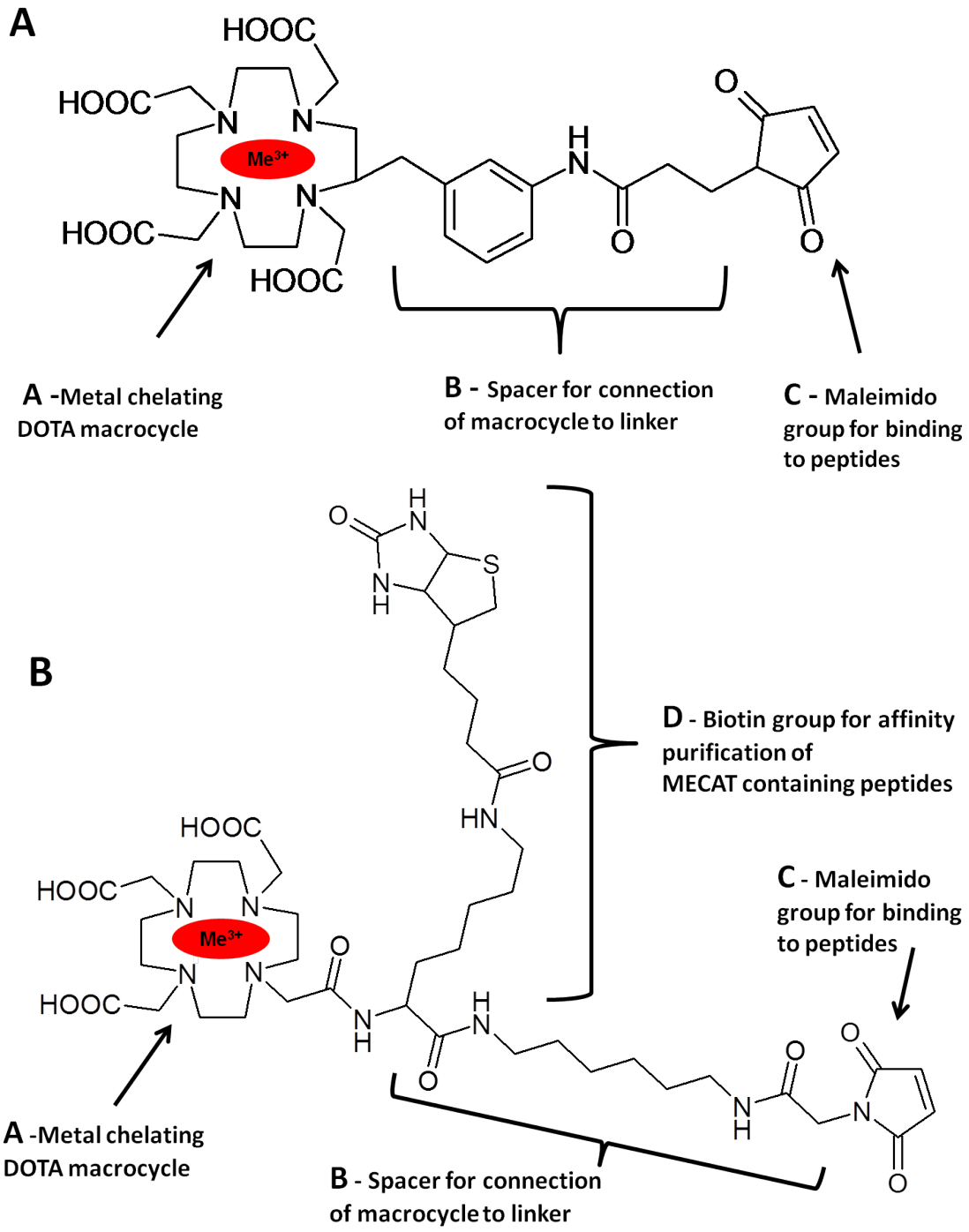


Figure 3.14: Structure of the MeCAT reagent proposed by *Ahrends et al.*, [161]. A shows the reagent, whereas a biotin group was included in B for affinity purification.

tested under typical conditions, as for example separation with nanoLC coupled to ESI. It could also be shown, that the label does not hinder the mass spectrometric elucidation of peptide sequences due to altered fragmentation behaviour [163].
The use of DOTA as a chelating agent was also proposed with In as ICP-MS detectable element for labelling and subsequent quantification of Bβ_{15-42} peptide, a cleavage product from fibrin [164]. Although sensitivity and limit of detection was similar for LC-ICP-MS and LC-ESI-TOFMS, the higher selectivity and robustness of ICP-MS as a detection method was highlighted by the authors. Similar to the MECT approach, *Patel et al.* [165] proposed a labelling approach using cyclic diethylenetriaminepentaacetic anhydride (cDTPA or DTPAA). They also used a two-step derivatisation, where first the cDTPA was bound to target peptides (Bradykinin and substance P), and afterwards chelated in a second step with Eu. This reagent is binding specifically to the N-terminus of a protein or peptide chain, and as well as to the side chain of lysine. Applying also isotopically enriched Eu, a differential labelling approach could be realized, where the quantity of a peptide could be determined by measuring the isotope ratio of ^{151}Eu/^{153}Eu after mixing the naturally labelled sample with a standard of the same peptide labelled with enriched europium and subsequent LC separation and ICP-MS detection.
The direct labelling of proteins using 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, a DOTA derivative, followed by incubation with lanthanides was described by *Jakubowski et al.* [166]. After electrophoretic separation, the proteins were blotted onto nitrocellulose membranes (NC membranes) and detected by laser ablation coupled to ICP-MS. Another direct labelling approach was published by *Rappel et al.* [167], also using a two step derivatisation with DTPAA, followed by complexation of Lu in the second step. As quantification method post-column isotope dilution analysis on ^{176}Lu was used to determine possible side reactions or loss of efficiency of the derivatisation reaction caused by other components in the reaction mixture, as for example buffers or reducing agents. It was shown in this study, that labelling becomes difficult when more and more sample preparation steps have to be applied, as the use of reduction agents like DTT or THCP reduced labelling efficiency in a drastic manner. Recently, the same reagent was tested for its applicability in mass spectrometry based proteomic studies, with regard to possible effects in detection sensitivity in MALDI-MS, fragmentation behaviour and identification of single protein and protein mixtures after tryptic digestion [168].
As an alternative to chelating mainly lanthanides, also organometallic compounds, such as metallocarbonyl complexes or metallocene based complexes [169]. Whereas the former have not been analyzed in combination with atomic spectroscopy devices, there are several publications on the latter. The reagent introduced by *Seiwert et al.*, ferrocenecarboxylic acid(2-maleimidoyl)ethylamide (FMEA) was originally de-

veloped for the simultaneous determination of oxidized and reduced thiols [170]. Due to the presence of iron in the complex, it can also be used as an ICP-MS detectable tag. Although the most abundant isotope, ^{56}Fe is highly interfered by $^{40}Ar^{16}O$, the use of collision cell technology is able to reduce the observed background significantly. Its potential as a derivatisation agent was recently demonstrated for the determination of phytochelatins from green alga, *Chlamydomonas reinhardtii*, [171].

3.5.3 Direct labelling of proteins

Among the approaches that aim on direct labelling of discrete binding sites in a protein or peptide, only two elements were investigated for their possibilities. Originally developed for the introduction of ^{125}I [172], *Jakubowski et al.* adapted a protocol using a solid-phase derivatisation with iodine for labelling of different proteins with the stable isotope ^{127}I [173]. After electrophoretic separation end blotting onto NC-membranes, laser ablation was used for the interrogation of the membrane by ICP-MS. Complementary studies using ESI-FTICR-MS on intact lysozyme and BSA showed, that the supposed binding sites (tyrosine and histidine) are derivatised by the label, but complete derivatisation was not achieved.
Navaza et al. used the compound bis(pyridine)iodonium tetrafluoroborate (IPy_2BF_4) for selective iodine labelling of tyrosine residues in peptides [174] followed by quantification using 2-iodobenzoic acid as external standard. For detection and quantification they used capHPLC coupled to elemental mass spectrometric techniques (ICP-MS), and molecular mass spectrometry (ESI-MS) for compound identification. Besides that, mercury containing substances have been investigated thoroughly due to the highly covalent binding between Hg and sulfhydryl groups present in free cysteine residues. On the one hand, this leads to their extreme toxicity, but on the other hand it also exhibits promising properties for their use as elemental labels. Typically, mercury compounds partly substituted with organic molecules (R-Hg-X) are applied in order to prevent bridged binding of one mercury ion to various sulfhydryl groups or insertation in existing disulfide bonds, as it may occur with the more reactive Hg^{2+}. As substituted mercury compounds have only one side left for binding, it cannot bind to more than one sulfhydryl group per molecule, if other effects do not come into play during reaction. Doubly substituted compounds (R-Hg-R) should not react any more as they are completely saturated [175]. In a first publication, the binding of p-hydroxymercuribenzoic acid to proteins was described back in 1954 [176]. Herein, the reactivity of the compound to various sulfhydryls in different proteins has been described by means of spectrophotometric detection. In 1992, this concept was rediscovered by *Takatera et al.* and applied to the analysis of metallothionein-like proteins [177]. By modification of the cysteine residues (which play an important role in the function of metallothioneins), their number could be estimated. Here, ICP-MS was introduced as a detector for the first

time. In a second paper published by the same group in 1993 [178], the concept of derivatisation of cysteine residues (of ovalbumin) was examined more in detail concerning reaction rates of five different mercury compounds (ethylmercuric chloride, mersalyl acid, p-chlorobenzoate, p-(chloromercuri)benzenesulfonic acid, fluorescein mercuric acetate), showing that the size of the molecule is a major influence as steric hindrance can play a role in the derivatisation process. They also determined the analytical figures of merit of the method, e.g. detection limit (less than 1 pmol for ovalbumin) and reproducibility (10% for 5 successive injections).

In 1994, a study involving the derivatisation of peptides and proteins using different organomercurials and detection with MALDI-TOF-MS was published [175]. Here, the structure of the final reaction product was determined for the first time, indicating that the binding of mercury is site-specific, as it is a necessary requirement for labelling. The main intention of the study was to develop an easy tool for differentiating between cysteine and cystine residues (disulfide bridges) in peptides and proteins.
In 2005, the use of pHMB for the determination of low molecular weight thiols in human whole blood was described by *Bramanti et al.* [179]. These compounds such as glutathione in reduced and oxidized form can be used as biomarkers for certain diseases or expression to potential hazards like oxidative stress. After derivatisation, the thiols were separated with reversed-phase chromatography, and detected with an atomic fluorescence detector after on-line chemical vapour generation. In 2008, this methodology was extended to other low molecular weight S-nitrosothiols [180]. In 2010, the same group applied their derivatisation methodology to the determination of low-molecular weight thiols in yeast, which act in the sulphur metabolism. In contrast to earlier studies, they used a LC-ESI-MS method for detection using an Orbitrap mass analyzer which was compared to ICP-MS as a detection method [181]. Contradicting to common expectations, they could show that the Orbitrap instrument was superior for quantification with respect to LOD and RSD.
In 2008, another comparative study about different mercury-containing agents (monomethylmercury, monoethylmercury and p-hydroxymercuribenzoic acid) was published [182]. Here, first experiments with ESI-MS were conducted to explain the structure of the derivatised proteins. Once again, as a potential application, counting of disulfide bridges by subsequent derivatisation before and after reduction of the protein was mentioned. A similar study on the availability of cysteine residues in human and rat hemoglobin by mass spectrometric analysis after reaction with pHMB was published by *Lu et al.* in 2010 [183]. This methodology was also applied to the absolute quantification of different proteins (bovine pancreatic ribonuclease A, lysozyme and insulin) using external standardisation with $MeHg^+$ [184]. The same group published an advanced labelling procedure using methylmercurithiosal-

Table 3.3: Organomercury compounds mainly used for protein derivatisation

Abbreviation	Compound	Remarks	Reference
$MeHg^+$	Methylmercury	Fundamental study	[184]
$MeHg^+$	Methylmercury	MeHg was liberated in situ from methylmercurithiosalicylate IDA using ^{204}Hg	[185]
pHMB	p-(Hydroxymercuri)-benzoic acid	Application to Metallothionein proteins Human whole blood Hemoglobin (human and rat)	 [177] [179] [183]
EtHgCl	Ethylmercury chloride	Fundamental study	[178]
PCMBS	p-(Chloromercuri)-benzenesulfonic acid	Fundamental study	[178]
MA	Mersalyl acid	Fundamental study	[178]
FMA	Fluorescein mercuric acetate	Fundamental study	[178]

icylate for the in-situ generation of the actual labelling agent methylmercury [185]. Using this reagent, they could avoid the need for handling the extreme toxic and volatile methylmercury. In this work, they also describe absolute protein quantification of glutathione, β-lactoglobulin and ovalbumin via label-specific isotope dilution analysis using their labelling agent previously synthesised with ^{204}Hg.
Besides its outstanding detection capabilities for metals and its feature of multielement analysis enabling multiplexed protein determinations, ICP-MS also offers the possibility for conducting multi-isotope analysis. Therefore, the concept of isotope dilution analysis (IDA) can be applied as a means for absolute protein quantification. Due to its fundamental characteristic that isotope ratios are measured instead of absolute intensities, the quantification result becomes independent from outer circumstances of the analysis, as for example instrumental drift. The basic features of this quantification technique will be presented in the next chapter.

3.6 Isotope dilution analysis (IDA) for absolute protein quantification

Isotope dilution analysis (IDA) has proven to be a very accurate method for quantitative determination of the concentration of an element of interest. As only isotope ratios are necessary for quantification, no external calibration or internal standardisation is needed, as the target element itself is used as an ideal internal standard correcting for various types of disturbances during analysis, as for example analyte

loss or instrumental drifts. Isotope dilution analysis is also known to be a very versatile technique as there are many examples for its use with various analytes and as well different instrumental techniques ([186], [187]). Recently, a comprehensive review was published on the application of isotope dilution analysis for protein quantification [18].

3.6.1 Introduction to IDA

For isotope dilution analysis, the isotopic composition of the element of interest in the sample is altered by the addition of a spike with a different isotopic composition. Therefore, at least two stable (and interference free) isotopes of an element are necessary to perform isotope dilution analysis. For some monoisotopic elements, IDA is also possible, when radioactive isotopes are available, which have sufficient long enough half lifes, so that the isotope can be considered stable on the time scale of the measurement. One example is iodine, whose isotope ^{129}I has a half life of about 15.7 million years. Normally, the isotope ratio monitored (A^a/A^b [3]) refers to the relation between the most abundant isotope in the sample (a), and the enriched isotope in the spike (normally a minor isotope in the sample, b). A graphic illustration of the principle of IDA with Hg as a role model can be found in figure 3.15. Here, the most abundant isotope in the sample is ^{202}Hg with an abundance of 29.86 %, whereas ^{199}Hg is the enriched isotope in the spike.
The relationship between the amount of both components in the mixture and the isotope ratio can be expressed in the isotope dilution equation:

$$c_S = c_{Sp} \cdot \frac{m_{Sp}}{m_S} \cdot \frac{M_S}{M_{Sp}} \cdot \frac{A^b_{Sp}}{A^a_S} \cdot \frac{(R_m - R_{Sp})}{(1 - R_m R_S)} \tag{3.4}$$

with:

m_{Sp}, m_S: mass taken of the sample and the spike, resp.

M_S, M_{Sp}: atomic weight of the element in the sample and the spike, resp.

A^b_{Sp}: abundance of the enriched isotope b in the spike

A^a_S: abundance of the most abundant isotope a in the sample

R_m: isotope ratio (a/b) in the mixture

R_{Sp}: isotope ratio (a/b) in the spike

R_S: isotope ratio (b/a) in the sample

[3] A = Abundance of the respective isotope

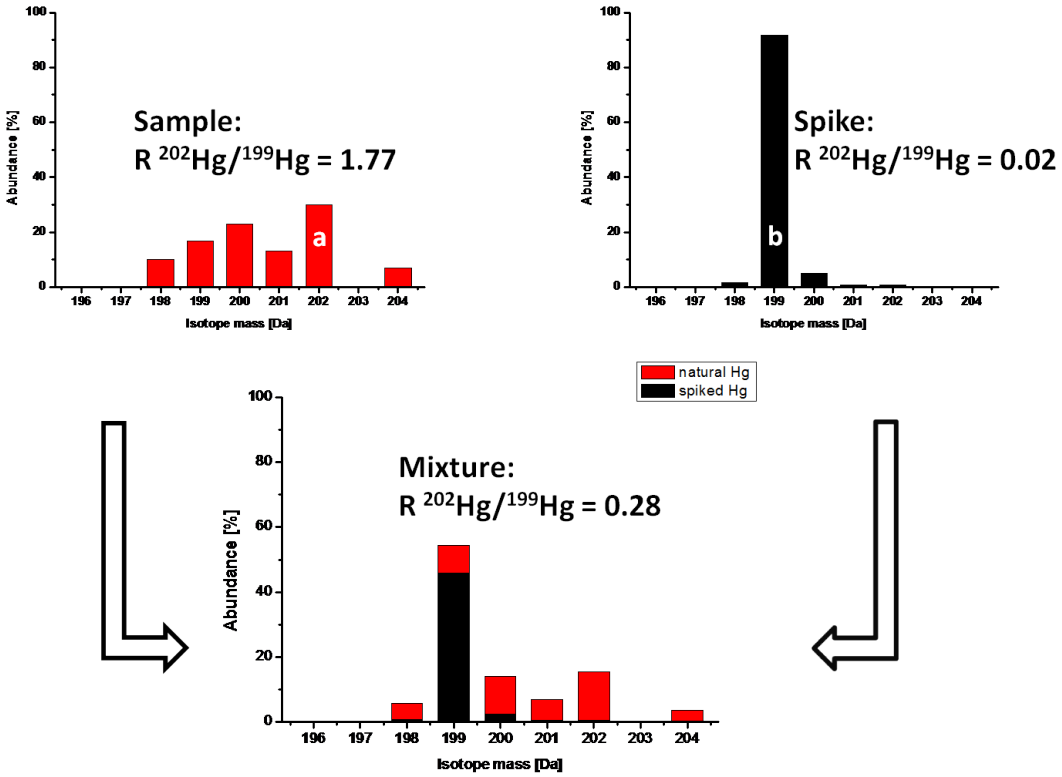


Figure 3.15: Principle of isotope dilution analysis

However, this equation is only valid for determinations of the total amount of the element of interest in a given sample. It can also be used for a species of interest, if the spike is added in the same chemical form, and only the isotope ratio for this species is determined experimentally. This so-called species-specific isotope dilution analysis requires the synthesis of the compound using enriched stable isotopes and its handling without possible contaminations with the element with natural isotopic abundance, which might not be always possible ([186], [187]).

Based on the fundamental work of *Heumann et al.* [188], an alternative method for isotope dilution analysis in chromatographic separations was developed, which allows the quantification of eluting species containing the element of interest. The principle of species-unspecific (or post-column) isotope dilution analysis implies the continuous addition of the spike to the eluting analyte after chromatographic separation. With the knowledge of the amount of spike added per time unit (mass flow of the spike, Mf_{Sp}), all eluting species containing the element of interest can be quantified for their content of this element. Knowledge of the exact stoichiometry is absolutely mandatory to calculate the amount of compound derived from the quantity of element. For LC separations using higher flow rates, the spike is added with a peristaltic pump and mixed in a T-piece with the LC eluent. The resulting mass flow of the spike can be determined exactly with the knowledge of the concentration and the amount of liquid delivered by the pump. In the case of

μLC separation, the determination seems to be more difficult due to the low flow rates. An alternative method for the determination of the mass flow is the injection of a standard solution of the target element. After acquisition of the chromatogram with ICP-MS, the measured intensities are converted into the relevant isotope ratio for every data point. Using the following equation, the resulting isotope ratio chromatogram is converted into the so-called mass flow chromatogram, which enables direct determination of the eluted amount of the analyte after peak integration.

$$Mf_S = c_{Sp} d_{Sp} f_{Sp} \frac{M_S}{M_{Sp}} \cdot \frac{A^b_{Sp}}{A^a_S} \cdot \frac{(R_m - R_{Sp})}{(1 - R_m \cdot R_S)} \tag{3.5}$$

with:

Mf_S**:** Mass flow of the sample

c_{Sp}**:** concentration of the element in the spike

d_{Sp}**:** density of the spike solution

f_{Sp}**:** flow rate of the spike

The values for c_{Sp}, d_{Sp} and f_{Sp} can be unified to give the mass flow of the spike (Mf_{Sp}). The amount of element of interest (and therefore the amount of analyte of interest) eluting from the chromatographic column in species-unspecific IDA can be calculated as shown in the following equation:

$$m_S = Mf_{Sp} \cdot \int_{t1}^{t2} \frac{Aw_S}{Aw_{Sp}} \cdot \left(\frac{A^b_{Sp}}{A^a_S} \cdot \frac{R_m - R_{Sp}}{1 - R_m \cdot R_S}\right) \tag{3.6}$$

with:

m_S**:** mass of the element eluted

t_1**,** t_2**:** Integration limit of the chromatographic peak

In general, isotope dilution analysis is considered to be an extremely accurate method for quantification, as only the isotope ratio has to be determined experimentally. This disconnects the analytical result from absolute intensities, which might be biased by changes in the actual sensitivity of the instrument, or potential losses of the analyte during sample treatment. However, care has to be taken, as there are certain pitfalls which can lead to serious errors in the analytical result [190]. In general, gravimetric determination of the amounts of spike and sample has to be accomplished as exact as possible. This can be achieved by using a highly precise balance and weighing of sufficient quantities to keep relative errors as small as possible. One of the crucial points is the accuracy of the determination of the isotope ratio. Here, different factors have to be kept in mind:

- The isotope ratio measured should be in the range, where the relative error for its determination is in the minimum range (not extremely biased towards the isotope ratio of the spike or the sample, respectively).
- In all cases, it is mandatory to correct mass bias effects caused by the mass spectrometer. This can be done by mathematical correction as shown in reference [187].
- The detector dead time (minimum time span between two individual ions, in which no further signals are recorded) has to be corrected as well, as the actual count rate might be higher than the measured one.

Especially fractionation effects between different isotopes of the same element are observed and must be corrected for very carefully when measuring isotope ratios. After transition of the ion beam into the vacuum stage, the lighter isotope(s) of a given element is deflected more easily due to the high amount of positive charges in the ion beam and additional effects of diffusion (space charge effect, [189]). This phenomenon is called mass discrimination and can reach values between 0.5-10 % per mass unit depending on the observed mass range. However, the extent of the mass bias can be addressed by measuring the isotope ratios of a standard solution with known isotopic composition followed by mathematical correction, as shown in references [187] and [189].
In general, contamination with the element to be determined have to be avoided, as all contributions not coming from the analyte species will affect the measured isotope ratio and therefore bias the analytical result. Especially when conducting species-specific isotope dilution after chromatographic separation, it has to be assured, that the peak of the desired species is completely separated from other species containing the element of interest. For species-unspecific isotope dilution analysis, it has to be kept in mind that this technique is only able to accurately quantify the amount of element which elutes from the chromatographic column, so that any losses up to then remain uncompensated. This means, that at least the column recovery of the analyte has to be known and kept in mind for the analytical result.

3.6.2 Application to protein quantification

As pointed out in the introduction, there is no standard recipe for the extraction, purification and separation of complex protein samples, so that often complex sample protocols have to be applied, which contain many points where errors or at least considerable uncertainties due to non-quantitative steps or uncompensated analyte losses can occur, as for example due to incomplete digestion of the protein or desalting procedures as for example the use of ZipTips. Independent of the quantification strategy, this leads to differences in the result obtained for the amount of a given pro-

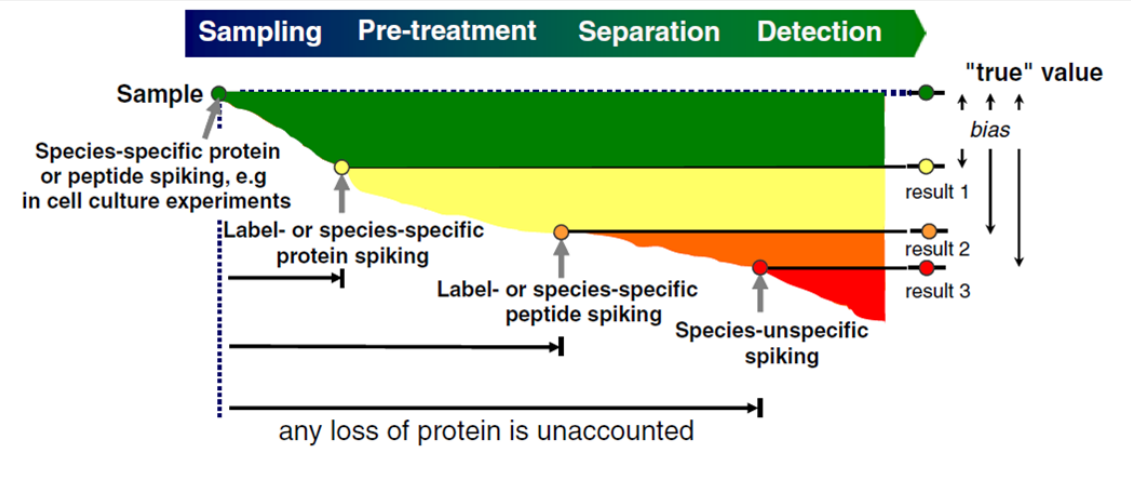


Figure 3.16: Accuracy obtainable with different IDA techniques, from ref. [18]

tein in a sample, and the real amount which was actually present. This is illustrated in figure 3.16. As pointed out before, only when complete mixture between spike and sample is accomplished, isotope dilution analysis can compensate for possible errors during sample treatment. For species-unspecific isotope dilution analysis, this is only the case short before detection, so that a considerable bias towards the real amount of analyte can occur. In contrast, when the spike is added in an earlier stage of the sample procession, as it is the case in species-specific IDA, this bias can be significantly reduced.

During the last years, many examples for the use of isotope dilution analysis for quantitative protein analysis have been published. A detailed review on the topic has been published which is recommended for a complete overview [18], so that only the most striking examples are presented here. The use of species-unspecific IDA was proposed in the majority of the yet published studies, due to its easier experimental handling. Using this mode, proteins containing various elements have been quantified, such as metallothioneins via Cu, Zn and Cd [191] or constituting elements like sulphur ([116], [140]) or selenium [192]. There are also examples for the quantitative determination of proteins and peptides after the chemical introduction of a label containing an ICP-MS detectable element. For example, *Rappel et al.* [167] made use of post-column IDA for the determination of the attainable labelling degree using DTPAA as a chelating reagent and Lu as elemental tag. On the protein level, a study on Cu, Zn superoxide dismutase indicated, that the results obtained for the quantification of this protein in red blood cells using the proposed ICP-MS based methodology is in good agreement with other methods, e. g. measurement of the enzyme activity [193].

A simplified method for the use of species-unspecific isotope dilution analysis in combination with μ- or nanoHPLC system was proposed by *Schaumlöffel et al.* [194]. By the addition of the species-unspecific spike directly to the mobile phase, they were able to spare a second HPLC-pump otherwise needed for reliable spike deliv-

ery in the low flow rates needed for this kind of instrumentation.
The first example for the use of species-specific isotope dilution for metalloproteins was proposed by *Harrington et al.* for the determination of the Cu-containing peptide rusticyanin [195]. Another examples for the successful quantification of Cu containing proteins was published for plastocyanin (synthesized with ^{63}Cu, [196]). The iron containing protein transferrin and its isoforms were successfully quantified using both spiking methodologies, species unspecific spiking using ^{57}Fe, and species specific spiking after saturation of the iron-free protein with the same isotope [142]. Both methodologies were proven to be suitable for these kind of analysis. Using a methodology based on species specific spiking using ^{57}Fe-transferrin and post column addition of ^{54}Fe, the same authors were able to determine simultaneously important parameters concerning the clinical iron status in human serum [197]. The same protein was also analyzed in a study on the binding behaviour of Ti using both methodologies [148]. Another iron containing protein, Ferritin was also synthesized as a spike for species specific IDA using ^{57}Fe [198]. In this case however, due to the nature of the protein, no definite iron to protein stoichiometry is amenable, so that iron analysis using ICP-MS can only give information about the iron content in a sample (iron is preferentially stored in Ferritin), and not the amount of protein molecules [199].
Another example for the introduction of enriched stable isotopes to a protein for absolute quantification was the synthesis of Cu, Zn-superoxide dismutase with ^{65}Cu and ^{68}Zn [145]. This protein spike was used for a comparison between internal standardisation and species specific IDA for the determination of SOD in red blood cells, showing the superiority of IDA [200].

4 Experimental

4.1 General remarks

All reagents used were of highest purity available if not otherwise stated. For all solutions, high purity MilliQ water was used (Millipore, Bedford, USA). For preparation, the desired amount of solid was weighed using an analytical balance and afterwards dissolved in the respective solvent. Standard solutions for calibration of the instruments were also made by weight. Mobile phases for RP-μLC and solutions for ESI/MALDI-MS were prepared as v/v solutions. Protein solutions were prepared as stock solutions of the respective protein in either water (ovalbumin) or other solvents (further specified in the following chapter). The solutions were kept at -18°C if storage for longer than 1h was necessary, but were never stored longer than 1 month. A complete list of all reagents used throughout this study can be found in table A.1 in the appendix. All gases for ICP-MS and ESI-MS were delivered by Air Liquide (Madrid, Spain) and had a purity of at least 99.999%.
As mercury compounds are very toxic, appropriate means of protection (laboratory coat, gloves and protection goggles) had to be applied when working with mercury containing solutions. When handling protein and peptide containing solutions for mass spectrometric identification, similar means of protection were taken in order to avoid possible contamination of the samples with keratin proteins. In order to reduce the potentially occurring memory effects when analyzing Hg, chromatographic columns were frequently cleaned by injection of 5 % of 2-mercaptoethanol. When total amounts of Hg were determined using ICP-MS, the complete sample introduction system (tubings, nebulizer and spray chamber) were cleaned with diluted hydrochloric acid (2 %) in order to remove remaining traces of mercury after formation of soluble chloro-complexes like $[HgCl_3]^-$ and $[HgCl_4]^{2-}$. These proceedings significantly reduced the extent of the otherwise critically disturbing memory effects, especially in combination with measurements of isotope ratios.

4.1.1 Proteins

As model proteins, ovalbumin and insulin were used in this study. Ovalbumin (SwissProt No. P01012) consists of 386 amino acids, resulting in a molecular mass of 42,221 Da without further modifications [201]. It has overall 6 cysteine residues, from which 4 are available for derivatisation, whereas the other two are forming an

intramolecular disulfide bridge (see figure 4.1). Bovine and human insulin (SwissProt No. P01317 and P01308, resp.) are smaller protein consisting of three independent amino acid chains. In the commercially available insulin, only a and b chain are present (see also figure 4.1). The molecular weight is 5,733 Da for bovine and 5,803 Da for human insulin. Both proteins are very similar in their structure. There are overall 6 cysteine residues present, 4 of them in the a-chain, two in the b-chain. All of them are bound in two intermolecular disulfide bridges, and on intramolecular bridge within the a-chain, so that insulin has to be reduced before derivatisation [202], [203].

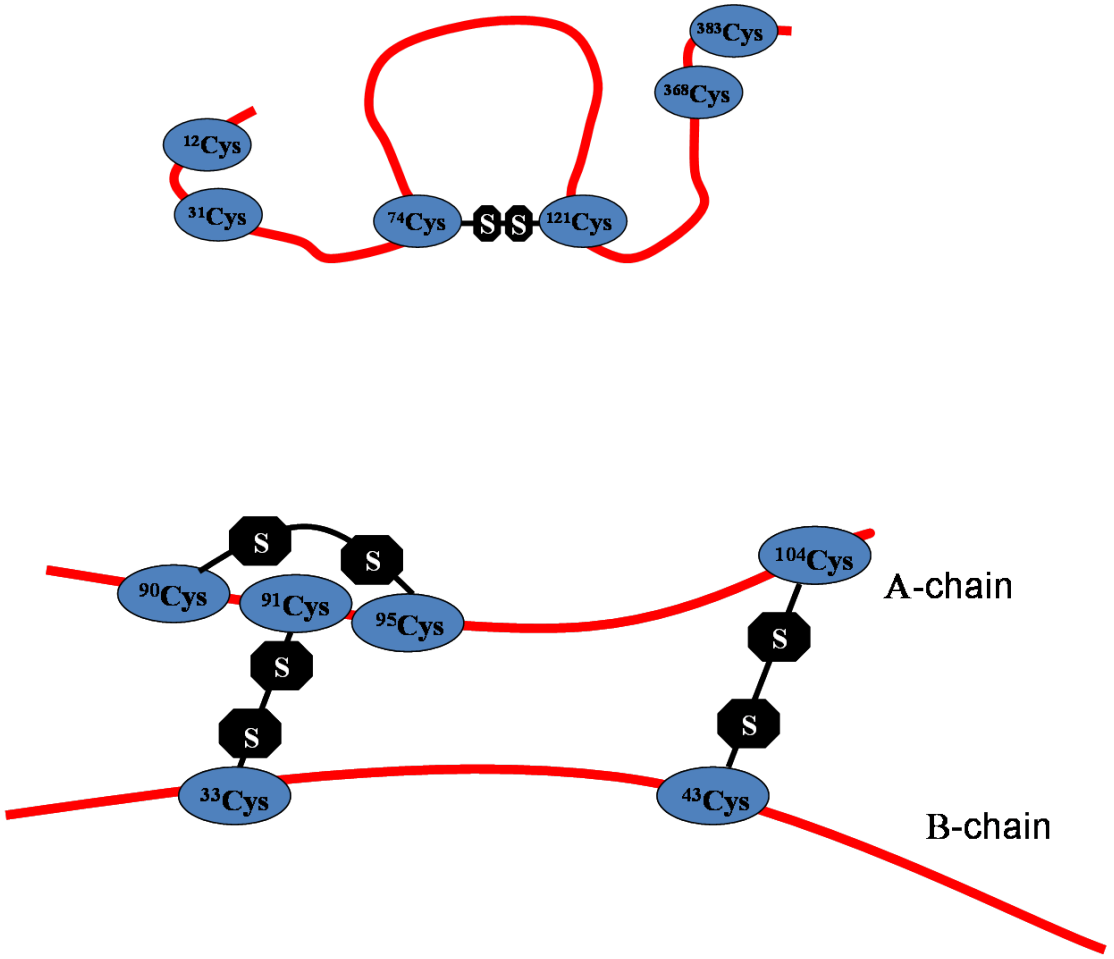


Figure 4.1: Structure of ovalbumin (up) and insulin (down)

4.1.2 Enriched Stable Isotopes

During this study, two different mercury spikes with enriched stable isotopes were used. The spike containing ^{199}Hg was commercially available as HgO, whereas the spike enriched in the isotope ^{200}Hg was obtained as Hg^{2+} in aqueous solution. In the following, denomination of the respective isotopic composition is referred to by the most abundant isotope. The abundance of all isotopes for both spikes is shown in table 4.1. The abundances for natural Hg were taken from the periodic

Table 4.1: Abundances of all isotopes of Hg with natural composition and in both spikes used throughout this study, ^{199}Hg and ^{200}Hg

Isotope	**Abundance** ^{nat}**Hg**	**Abundance*** ^{199}**Hg**	**Abundance*** ^{200}**Hg**
196	0.15	0.006 ± 0.001	0.001 ± 0.0009
198	9.97	1.55 ± 0.02	0.13 ± 0.03
199	16.87	91.70 ± 0.03	0.99 ± 0.01
200	23.10	4.91 ± 0.01	96.46 ± 0.05
201	13.18	0.72 0.01	1.45 ± 0.02
202	29.86	0.09 ± 0.02	0.90 ± 0.01
204	6.87	0.21 ± 0.05	0.09 ± 0.02

* Correction of mass bias effects is included in the values and was accomplished as shown in chapter 4.2.7

table, whereas the abundances for the spikes were determined using ICP-MS with pneumatic nebulisation.

4.1.3 Bradford Assay

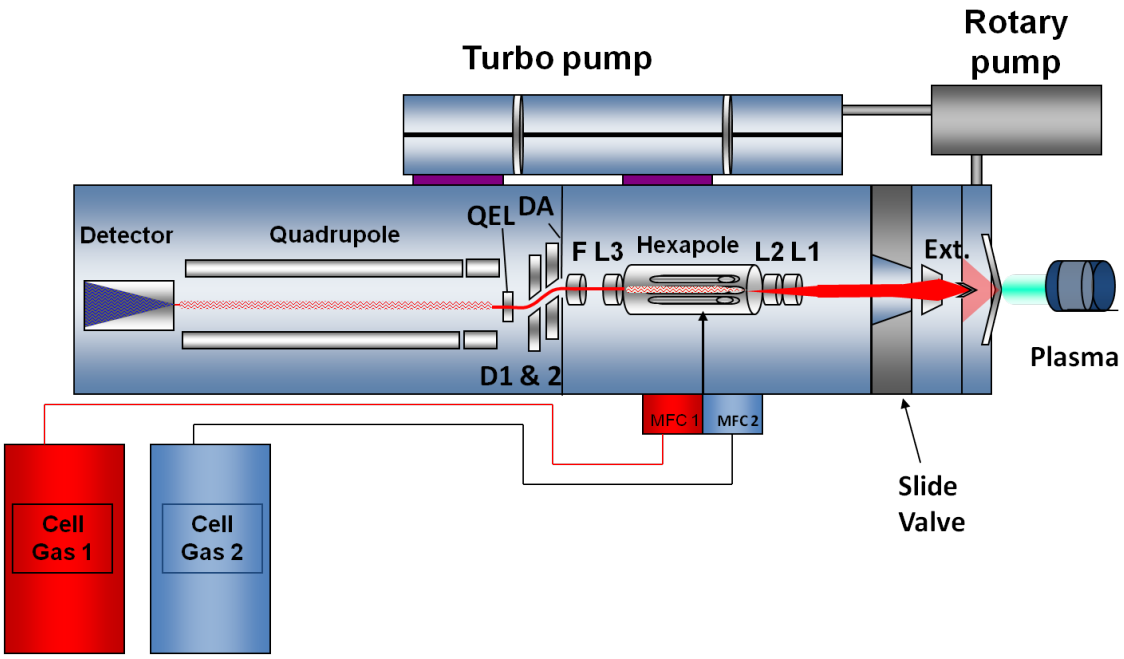
Determination of protein concentration via the Bradford assay was carried out using a Genesys Spectrophotometer (Thermo Fisher). The Bradford reagent commercially available was freshly diluted in MilliQ water before every measurement. Calibration was carried out using bovine serum albumin. All solutions contained 50 μL of 0.15 mol L^{-1} NaCl for adjustment of ionic strength and 50 μL of 1 mol L^{-1} NaOH for protein denaturation. After addition of the Bradford reagent, solutions were vortexed thoroughly and directly subjected to analysis. Spectra were acquired in the range between 350 and 700 nm wavelength with a resolution of 1 nm. Representation of the relative absorption [595 nm]/[465 nm] against the concentration was used for quantitative evaluation.

4.2 Instrumentation

4.2.1 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Liquid sample introduction

The ICP-MS used was a Thermo Fisher Scientific X-Series^{II} ICP-MS (Thermo Fisher, Bremen, Germany), equipped with a quadrupole mass analyser and a hexapole collision cell for the reduction of polyatomic interferences. A scheme of the instrument can be found in figure 4.2.



Abbreviations:
Ext: Extraction lens
L1, L2, L3: Ion lenses 1 to 3
F: Focussing lens
DA: Differential aperture
D1, D2: Deflection lens 1 and 2
QEL: Quadrupole entry lens

Figure 4.2: Scheme of the Thermo X-Series^{II} ICP-MS instrument used in this study

For the analysis of mercury, the use of collision/reaction cell technology is not necessary due to the absence of any significant interferences in the investigated samples, so that the cell was not activated to obtain higher sensitivity. The instrument was tuned daily with a multielement tuning solution containing 10 ng g^{-1} of various elements, and a solution containing 10 ng g^{-1} of Rh for μLC-ICP-MS. For measurements with conventional nebulisation a pneumatic nebulizer with a nominal flow rate of about 1 mL min^{-1} and a glass spray chamber with an impact bead delivered with the instrument was used. Typical parameters are displayed in table 4.2. For analysis with μLC-ICP-MS a miniaturized nebulizer and a self-constructed total consumption spray chamber were used to match the low flow rates coming from the LC-system. Figure 4.3 shows the experimental set-up. A detailed description can be found in reference [140]. A foto of the experimental set-up is shown in figure 4.4.

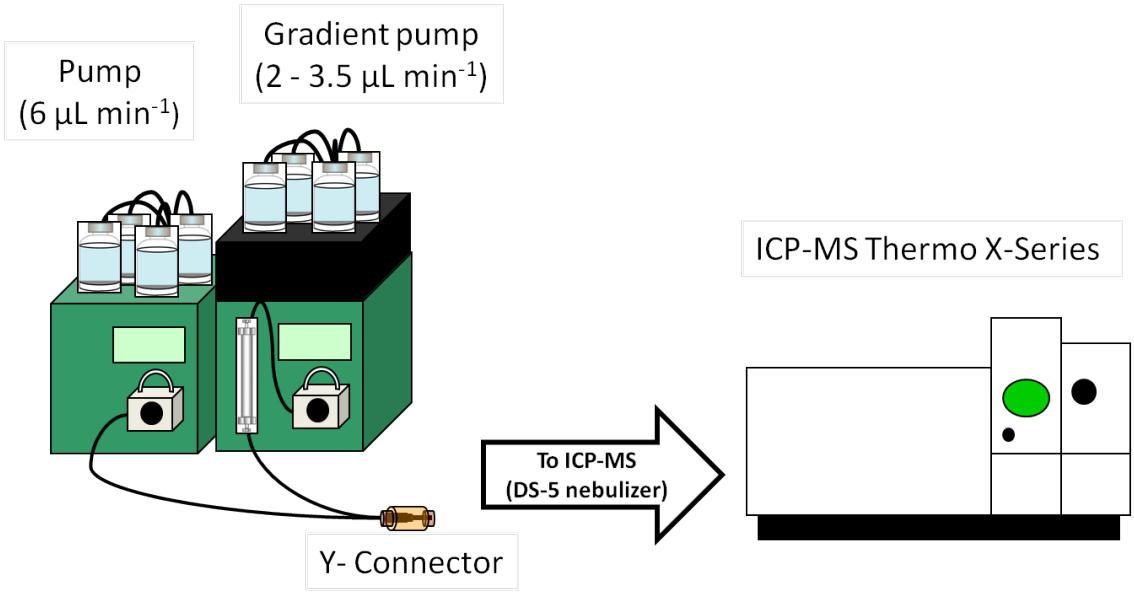


Figure 4.3: Experimental set-up for the analysis with μLC-ICP-MS

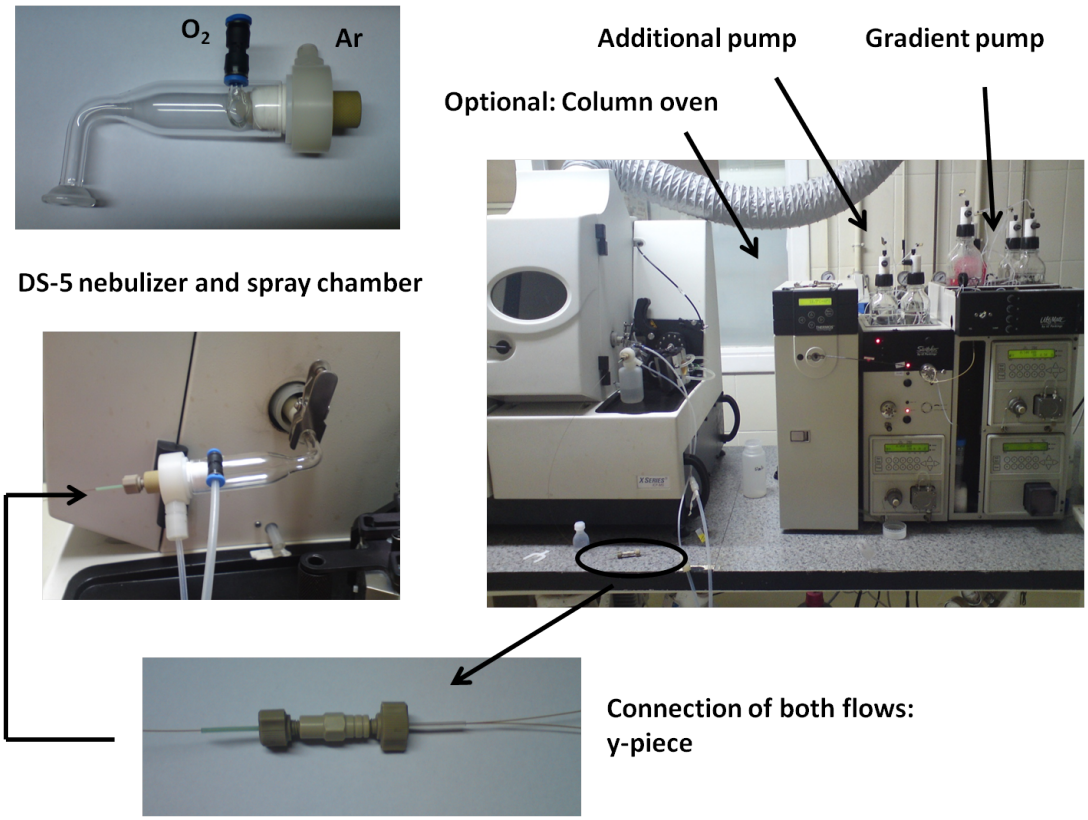


Figure 4.4: Foto of the experimental set-up

To the column flow between 2 and 3.5 μL min^{-1} an additional flow of 6 μL min^{-1} was added to match the optimum flow rate of the DS-5 capillary nebulizer (CETAC, Omaha, USA). This make-up solution contained 20 ng g^{-1} of Rh in 50 % acetonitrile (0.05 % TFA) as an internal standard. Mixing of both was achieved in a nano Y-connector with a void volume of 19 nL (Upchurch Scientific, Oak Harbor, USA) to prevent extensive peak broadening after separation. Due to the high back pressure

caused by the nebulizer (5-6 bar), Ar supply could not be accomplished by the instrument, but had to be realized by an external mass flow controller. During gradient elution of proteins high amounts of acetonitrile had to be used (up to 95 %), so that oxygen (40 mL min^{-1}) was added to the plasma to avoid carbon deposition on the cones and therefore reduced sensitivity with time. The addition of oxygen was also realized by an external mass flow controller. Platinum cones were used due to the oxidizing plasma conditions. Table 4.2 shows the normally applied plasma conditions. A more detailed description of the set-up can be found elsewhere [140].

Table 4.2: Optimised operation parameters for ICP-MS measurements carried out with different sample introduction devices during this study

ICP-MS parameter using conventional nebulisation	
Instrument:	Thermo X-Series^{II}
parameter	**typical value**
Forward power	1400 W
Nebulizer gas	0.96 L min^{-1}
Plasma gas	0.7 L min^{-1}
Cooling gas	13 L min^{-1}
Dwell time per isotope	100 ms
Spray chamber temperature	2°C
Data acquisition	3 x 100 replicates
ICP-MS parameter using μLC-ICP-MS	
Instrument:	Thermo X-Series^{II}
parameter	**typical value**
Forward power	1300 W
Nebulizer gas	0.8 L min^{-1}
Plasma gas	0.7 L min^{-1}
Cooling gas	13 L min^{-1}
Oxygen	0.04 L min^{-1}
Dwell time per isotope	100 ms
Spray chamber temperature	ambient (21°C)

With the objective of reducing the complexity of the instrumental set-up, a second miniaturized nebulizer was tested (ENYA Mist, Burgener Research, Mississauga, Ontario, Canada). This nebulizer was constructed for optimum performance with flow rates between 0.5 and 50 μL min^{-1} with a reduced back pressure of 3.5 bar. Therefore, the additional Argon supply and one mass flow controller could be spared. In comparison to the other nebulizer, similar analytical figures of merit could be observed [204]. However, in order to maintain compatibility between all experiments,

the set-up remained unchanged during the study, although the new configuration was easier to handle and seems to be a promising alternative for the future.

4.2.2 High Performance Liquid Chromatography (HPLC)

The μLC system used was a Ultimate gradient pump (LC packings, Amsterdam, Netherlands) equipped with an internal split to deliver flow rates in the low μL range. Mobile phases were 98 % MilliQ water, 2 % acetonitrile with 0.05 % TFA (A), or 98 % acetonitrile, 2 % MilliQ water with 0.05 % trifluoroacetic acid (TFA) (B) resp.. A low amount of acetonitrile had to be delivered constantly to maintain optimum nebulisation of the capillary nebulizer. All mobile phases were constantly flushed with He for degassing. A typical gradient for protein separation started at 2 % B and increased to 80 % B within 25 minutes. For peptide separation of tryptic digests, typical gradient conditions started at 2 % B and increasing to 40 % B in 40 minutes. All gradients included a 10 minute cleaning step at 95 % B as well as 15 minutes column equilibration at the initial solvent composition. All columns used were supplied by Grace Division (Columbia, USA). If not otherwise stated, a reversed phase column (diphenyl, 5 μm particle size, 300 $\mathring{A}$ pore size, 200 mm x 150 μm i. d.) was used for protein separation. For peptide separations, a C-18 column was used (5 μm particle size, 300 $\mathring{A}$ pore size, 200 mm x 150 μm i. d.). All connections were made of fused silica capillaries with 50 μm inner diameter and 365 μm outer diameter (Upchurch Scientific). Coupling of the chromatographic system to the ICP-MS was realised as shown in figure 4.3. For μLC-ESI-MS analysis an Agilent 1100 System was used (Agilent, Waldbronn, Germany), consisting of a quarternary pump with online degassing, and an autosampler. Mobile phases, columns and gradient conditions were the same as for both systems. For size-exclusion chromatography, the LC set-up consisted of a single pump (Shimadzu LC A10, Shimadzu, Duisburg, Germany), because separations were conducted in isocratic mode. Sample injection was achieved with a Rheodyne 9125 injection valve (Rheodyne, Oak Harbor, USA) and an injection loop of the desired volume made from PEEK. Predominately, a Superdex 75 column was used (GE Healthcare, 5 x 150 mm, optimum separation range 3 - 70 kDa). The flow rate was 0.3 mL min^{-1} of 50 mmol L^{-1} NH_4HCO_3 and the injection volume 10 μL. For the separation of peptide mixtures a Superdex peptide column was used (GE Healthcare, 10 x 300 optimum separation range 0.7 - 10 kDa). Here, the optimum separation conditions were 0.75 mL min^{-1} of 50 mmol L^{-1} NH_4HCO_3 with an injection volume of 50 μL. The columns were calibrated on a regular basis with protein standards corresponding to the separation range of the respective column. The following proteins were used: Superdex 75: Transferrin (80 kDa), bovine albumin (66 kDa), ovalbumin (44 kDa), lysozym (chicken, 14 kDa); Superdex 200: Thyroglobin (300 kDa), IgG (160 kDa), bovine albumin (66 kDa),

ovalbumin (44 kDa); Superdex Peptide: α-lactalbumin (14.2 kDa), cytochrome C (12.3 kDa), vitamin B12 (1.4 kDa), tryptophane (0.2 kDa). All connections were made from PEEK. Coupling to the ICP-MS was realized by direct connection of the column outlet to the pneumatic nebulizer. SEC with UV/Vis detection was carried out using an Agilent 1100 System equipped with a diode array detector. Also here, conditions for chromatographic separation were identical as in the case of ICP-MS detection. For fraction collection, the outlet of the detector was disconnected and the column eluent was collected after observing the signal of the desired species.

4.2.3 Gel Electrophoresis (GE)

Slab gel electrophoresis was carried out using the MiniProtean Tetra cell system (BioRad, Munich, Germany). Precast gels delivered by the same manufacturer were used with acrylamide concentrations of 7.5 %, if not otherwise stated. The running buffer was prepared freshly and consisted of 25 mmol L^{-1} tris(hydroxymethyl)aminomethane (Tris), 192 mmol L^{-1} glycine and 0.1 % sodium dodecylsulphate (SDS). Samples were incubated in 62.5 mmol L^{-1} Tris, 2 % SDS, 25 % glycerine, and 0.1 % bromophenol blue at pH of 6.8 before 20 μL were applied into each well. Addition of DTT and reduction of cystine was avoided due to possible side reactions with the derivatised protein. Electrophoresis was carried out with 200 V at ambient temperature until bromophenol blue had migrated to the end of the separation gel. After separation, the gels were stained using coomassie blue (1 g L^{-1} in 10 % methanol, 10 % acetic acid) for at least 1 h. Afterwards the gels were destained using 40 % methanol and 10 % acetic acid until the protein bands were clearly visible. The destaining solution was exchanged up to three times if necessary. Protein containing spots were cut out with a razor blade and either digested in concentrated nitric acid for analysis with ICP-MS or enzymatic digestion.

Gels for laser ablation were prepared using the Phastgel system (GE Healthcare). Also here precast gels were used with acrylamide concentration of 12.5 % if not otherwise stated. Electrophoresis was carried out at 250 V at 15°C. After electrophoresis, all gels were kept in 30 % methanol and 10 % acetic acid to precipitate the proteins. Gels for laser ablation were shaken in glycerine to remove remaining water before drying at 70°C for 2 h. This was necessary, as not to do so would lead to deformation and cracking of the gel during the drying process [205]. Finally, the gels were cut in the middle and attached to a glass slide using double-sided tape and mounted into the ablation cell. Alternatively, gels were also stained with coomassie blue for visual comparison of the migration distance of the protein sample.

4.2.4 Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS)

An ArF excimer laser operated at a wavelength of 193 nm (GeoLasC, Lambda Physik, Göttingen, Germany), with a computer-controlled xyz-stage was used during this study. Observation of the sample surface was possible by using an Olympus BX 51 microscope. Helium was used as ablation gas at a flow rate of 1.0 L min^{-1}. The outlet of the cell was connected to the torch of the ICP-MS using a laminar flow adapter where argon gas was added for stable operation of the ICP. The energy proportionated by each laser shot was measured daily in order to adjust the input energy of the laser system in a matter, that a fluence of 14.5 J cm^{-2} was achieved in all measurements. The ablation cell used in this study was built in the workshop of ETH Zurich and was made from polymethylmethacrylate (PMMA), so that its weight could be limited to 2.2 kg. It was developed to offer high spatial resolution of the sample, but also to host large samples (maximum dimensions: 230 x 34 x 16 mm (L x W x D)). Therefore, the samples are placed on a sled that allows a fast change of the respective sampling position without need to open the cell. A more detailed description can be found elsewhere [206]. Figure 4.5 shows a picture of the cell. The ablation window has a diameter of about 50 mm, thus allowing continuous line scans up to 40 mm without moving the sled manually. In contrast to other cells used in the laboratory, this cell offers a short washout time (between 1-3 s) and thus should enable the analysis of elemental distribution with high spatial resolution in a non-intermittent line scan.

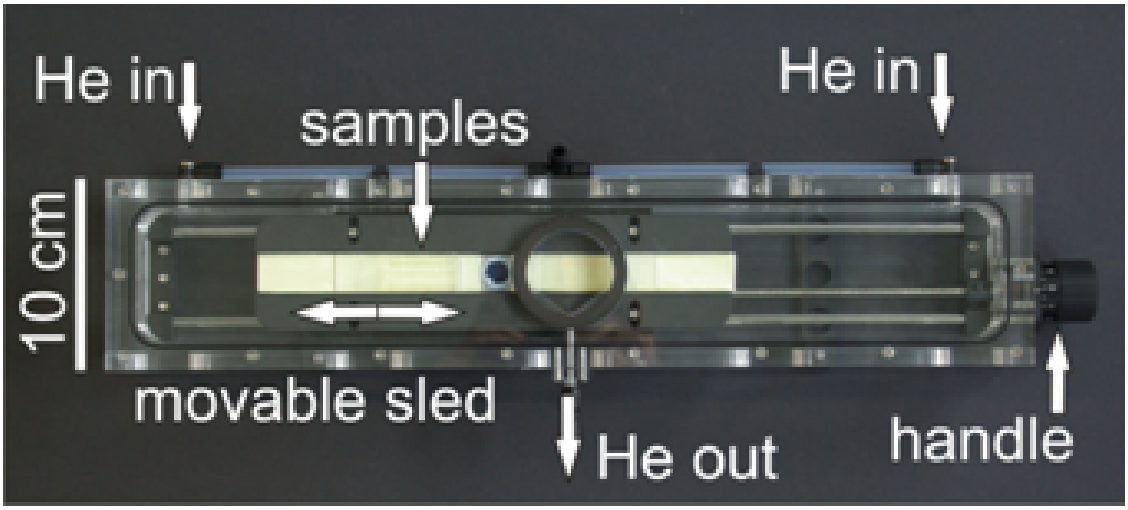


Figure 4.5: Photo of the ablation cell used in this study, [206]

Alternatively, for fs-LA, a chirped pulse amplification (CPA)-type Ti-sapphire-based laser system (Legend, Coherent Inc., Santa Clara, CA, USA) was used. The fundamental wavelength of the laser was in the near-IR range (795 nm), but it could also be operated on its third harmonic (265 nm) in the UV range. The crater diameter

was controlled by the distance between the focussing lens and the sample surface. For sample observation, a CCD camera equipped with a macro-objective was installed. The ablation gas used was also He with a flow rate of 1 L min^{-1}. Further details of the instrumental set-up can be found in reference [207].
Using ns-laser ablation, all measurements were carried out using an Agilent 7500cs ICP-MS instrument (Agilent Technologies, Japan), whereas an Elan DRC instrument (Perkin Elmer Sciex, Canada) was used for fs-LA. A more detailed summary of all instrumental parameters can also be found in table 4.3.

Table 4.3: Optimised operation parameters for LA-ICP-MS measurements

ICP-MS parameter using ns-LA-ICP-MS	
Instrument:	Agilent 7500cs
parameter	**typical value**
Forward power	1600 W
Nebulizer gas	0.75 L min^{-1}
Plasma gas	0.7 L min^{-1}
Cooling gas	13 L min^{-1}
Dwell time per isotope	50 ms
ICP-MS parameter using fs-LA-ICP-MS	
Instrument:	Elan DRC
parameter	**typical value**
Forward power	1380 W
Nebulizer gas	0.8 L min^{-1}
Plasma gas	0.7 L min^{-1}
Cooling gas	13 L min^{-1}
Dwell time per isotope	50 ms

Both instruments were optimized daily for high sensitivity using a NIST 610 glass standard. Besides sensitivity, also the ratios $^{238}U^+/^{232}Th^+$ and $^{248}ThO^+/^{232}Th^+$ were monitored to check for plasma temperature and oxide formation. $^{238}U^+/^{232}Th^+$ was optimized by adjusting the make up gas flow to a value near 1 while $^{248}ThO^+/^{232}Th^+$ under these conditions was typically below 0.3 %. For laser ablation, the following isotopes were measured: $^{13}C^+$ as matrix element and $^{34}S^+$ as indicator for the buffer front. During method optimisation only $^{199,200,202}Hg^+$ were monitored, whereas all mercury isotopes were monitored for isotope dilution analysis. Also here, spectral interferences on Hg were not expected by the sample matrix, so that the use of the collision cell was avoided. Although there are several spectral interferences which affect the detection of $^{34}S^+$, their presence was ignored, as the detection of $^{34}S^+$ only served for qualitative purposes.

4.2.5 Matrix Assisted Laser Desorption/Ionisation - Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-MS analysis was carried out with a Voyager DE-STR Workstation (Applied Biosystems, Langen, Germany) equipped with a N_2 laser (wavelength 337 nm, 3 ns puls width, 1600-1900 μJ laser energy). The observed mass range was between 5,000 and 100,000 Da for proteins, and between 700 to 6,000 Da for insulin and 500 to 5,000 Da for tryptic digestions. For proteins, 10 spectra with 100 shots per spectrum were averaged to achieve better ion statistics, whereas for peptides only one spectrum with 100 shots averaged was acquired to maintain sufficient mass resolution in the low mass range. For the MALDI-MS measurements in chapter 5.3, 250 single laser shots were averaged. Sinapinic acid (5 mg mL^{-1} in 7:3 water:acetonitrile, 0.01 % TFA) was used as a matrix for ovalbumin, α-cyano-4-hydroxycinaminic acid (10 mg mL^{-1} in 1:1 water:acetonitrile, 0.01 % TFA) was used for insulin and analysis of tryptic digestions. Other matrix substances applied were 2,5-dihydroxybenzoic acid (DHB, 5 mg mL^{-1} in 8:2 water:acetonitrile) and 2,4,6-trihydroxyacetophenon (THAP, 5 mg mL^{-1} in 8:2 water:acetonitrile and afterwards diluted 9:1 with 50 mg mL^{-1} diammonium hydrogen citrate). Sample preparation for MALDI-MS analysis was realized by mixing one aliquot of sample and matrix in a small vial, vortexing for 5 seconds, and afterwards pipetting 1 μL directly on the target (stainless steel hydrophobic target 96-2, Applied Biosystems) and drying on air. Calibration was achieved by bovine serum albumin for ovalbumin, and with a set of standard peptides (Applied Biosystems) in the mass range of 1,300 to 5,800 Da for peptides.

4.2.6 Electrospray Mass Spectrometry (ESI-MS)

For ESI-MS analysis, a QStar XL ESI-MS (Applied Biosystems) was used. The instrument was equipped with two linear quadrupoles and a time-of-flight mass analyser. For generation of full mass spectra, the quadrupoles were used as ion lenses whereas the TOF analyzer was used as mass analyzer. For the generation of MS/MS-spectra, the first quadrupole was used as a mass filter to select the parent ion, which was afterwards fragmented in the second quadrupole using nitrogen as collision gas. The resulting product ions were then analysed in the TOF. Figure 4.6 shows a scheme of the instrument.

The instrument could be operated using two ESI ion sources. For the analysis of intact proteins, the IonSpray source was used. Typically, an ionisation potential of 5.5 kV was applied, and nitrogen was used as nebulisation gas. For the coupling with μLC, the instrument was equipped with a NanoSpray ion source which was operated with an ionisation potential of 2.8 kV. Sample solutions could be introduced by a syringe pump included in the instrument (flow rate typically between 2 and 5 μL min^{-1}). The mass range scanned was between 400 to 4,000 Da for the analysis

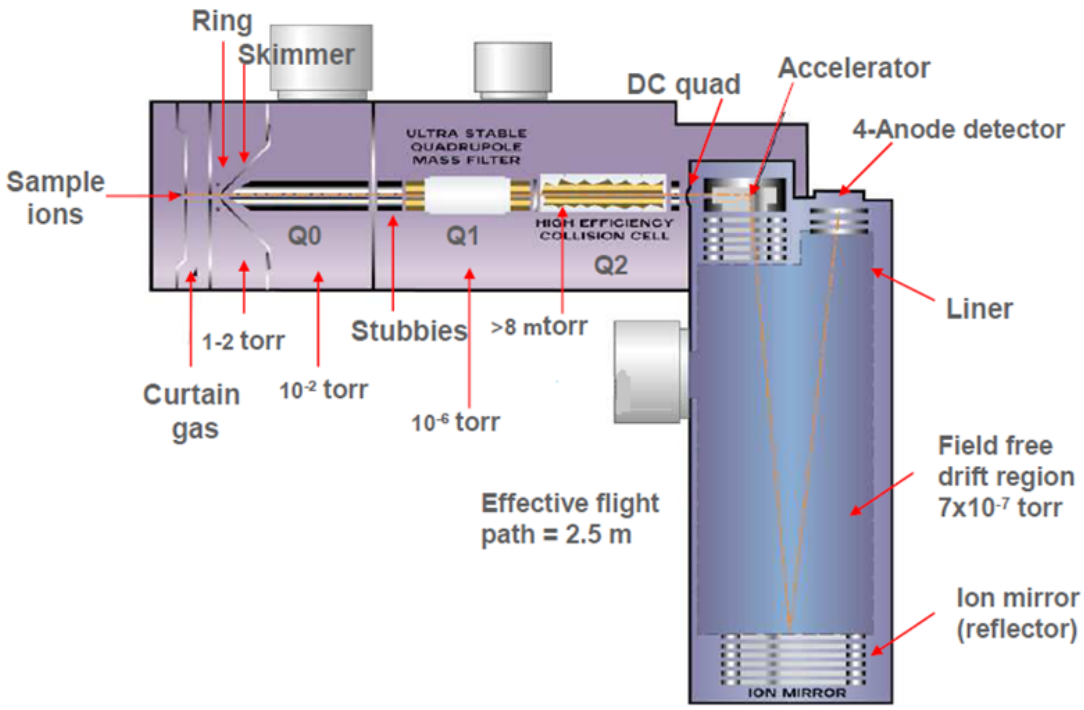


Figure 4.6: Scheme of the QStar XL instrument

of entire proteins. For mass spectra deconvolution, a Bayesian peak deconvolution algorithm included in the analyst software (Applied Biosystems) was used. Fragmentation spectra of peptides were generated by using collision induced dissociation (CID) using N_2 as collision gas. The observed mass range for peptides was between 400 and 2,000 Da in the TOF-MS mode, and between 100 and 1,600 Da in the MS/MS mode. For LC-ESI-MS/MS measurements, the column outlet was directly connected to the ion source at a flow rate of 2 μL min^{-1}. Using the data acquisition features of the instrument, first a full mass spectrum was recorded (measurement time 1 s), and then the 2 most abundant ions were fragmented using optimised collision energies (measurement time 2 s), if their m z^{-1} was between 600 and 1,400 Da, and their charge was between 2 and 4. Using these side conditions, the acquisition of fragmentation spectra of typical contaminants, as for example polysiloxanes or plasticizers, was reduced, whereas peptides were effectively fragmented.

4.2.7 Treatment of MS-data

Determination of mass bias

For all isotope ratio measurements with ICP-MS, the extent of mass bias was determined by measuring the respective isotope ratio of a solution/compound with natural isotopic abundance. For LA-ICPMS, mass bias was addressed using ^{nat}pHMB-derivatised ovalbumin. Isotope ratios were calculated after evaluation of the peak areas. Correction of the measured isotope ratios was accomplished using the ex-

ponential function as described in reference [187]. Mass bias was determined independently for all kinds of sample introduction systems used throughout this study, as voltages for ion lenses such as the extraction lens had to be varied in order to achieve optimum sensitivity. As a consequence, mass bias varied between 0.8 and 1.5 % per mass unit for the detection of Hg.

Identification of proteins and peptide sequences

Evaluation of MALDI-MS spectra of protein digestions and ion identification of product ion spectra generated by ESI-MS/MS was carried out using an open source software tool, mMass ([208], [209]). To generate an ion list, spectra were first slightly smoothed and baseline corrected. Afterwards deisotoping and the generation of a peak list was done using a minimum relative intensity of 1.5 % for MALDI-MS data and 0.5 % for ESI-MS/MS data. For the peptide identification from MS/MS spectra, the maximum charge state for fragment ions allowed was $[M+2H]^{2+}$. Protein identification via database search was accomplished using the Protein Pilot software from Applied Biosystems.

Evaluation of isotope ratios and abundances for isotope pattern deconvolution

Evaluation of peak areas for the calculation of isotope ratios and abundances of the respective ion clusters for isotope pattern deconvolution was done using Origin 8.0. After baseline creation, integration limits for all signals were set manually. In order to integrate equally for different isotopes, the integration limits as well as the adjustment for baseline creation were determined for the must intense trace of the data set (e. g. chromatogram) and then saved and re-used for other all isotopes in the same data set. For calculation of abundances, values were finally normalized to the sum of all peak areas.

4.3 Sample preparation

p-Hydroxymercuribenzoic acid was dissolved in 0.01 mol L^{-1} NaOH in a concentration of 25 mg mL^{-1} (70 μmol μL^{-1}). Reduction of disulfide bonds in the case of insulin was realized after denaturation with 6 mol L^{-1} urea with DTT for 1 h at ambient temperature. The reaction mixture was incubated with pHMB without further processing. As urea can lead to significant problems in molecular mass spectrometry, an alternative workflow was developed. For this, the proteins were dissolved in 50 mmol L^{-1} NH_4HCO_3 solution. Reduction of the disulfide bonds was accomplished if neccessary by incubation with DTT at 95°C for 5 minutes. After cooling down, the samples were incubated with pHMB without further processing. If not otherwise stated, incubation of the proteins with pHMB was also realized at

room temperature for 1h with a 10-fold excess of pHMB per cysteine. After the derivatisation had finished, samples were purified with Microcon centrifugation devices (Millipore, USA), using a cut-off membrane of 10 kDa in the case of ovalbumin and 3 kDa in the case of insulin. For comparison, cysteine residues were also derivatised with iodoacetamide for comparison of its binding behaviour. It was preceded likewise with respect to the molar ratios of the reagents and incubation times. As iodoacetamide decomposes under exposure to light, solutions were kept in the dark. For determination of the total Hg concentration of a sample or quantitative characterisation of ^{199}Hg-pHMB ovalbumin, the protein solution was diluted in 10 % nitric acid in order to decompose the Hg-protein complex. Afterwards, aliquots were either mixed with different amounts of the spike enriched in ^{200}Hg for quantification of total Hg present in the sample, or a standard solution with certified content of mercury for determination of the concentration of the spike.

4.4 Tryptic digestion

4.4.1 Digestion in solution

For tryptic digestion in solution, samples were reduced by incubation with DTT at 95°C for 5 minutes. If cysteine residues had to be derivatised, incubation with pHMB was conducted as described in chapter 4.3. Alternatively, a freshly prepared solution of iodoacetamide (concentration 100 mmol L^{-1}) was added and let to react for 20 minutes at room temperature and protected from light. Finally, trypsin solution (concentration 0.1 μg μL^{-1}) was added, so that the trypsin to protein ratio was in the range of 1:50 to 1:20. Digestion was accomplished overnight (15h) at 37 °C. For MS analysis, the solution was treated with ZipTips (Millipore, USA) to remove interfering salts, and finally eluted in 50 % acetonitrile/0.1 % formic acid.

4.4.2 Digestion in gel

For tryptic digestion of proteins previously separated in gel electrophoresis, the bands were carefully cut out with a razor blade. The bands were cut as close as possible to avoid unnecessary amounts of acrylamide. To remove salts and detergents, the bands were washed with 100 μL of 25 mmol L^{-1} NH_4HCO_3/50 % acetonitrile for 10 minutes. The supernatant was separated and rejected, and the procedure was repeated twice for 30 minutes. Finally, the gel bands were washed with 100 % acetonitrile to remove the remaining water completely. Afterwards, the gel pieces were dried in a vacuum centrifuge for 30 minutes.
For digestion, the gel pieces were rehydrated in freshly prepared trypsin solution (0.1 μg μL^{-1}) for at least 30 minutes. The excess of this solution was removed, and the pieces were covered with the least amount possible of 25 mmol L^{-1} NH_4HCO_3

solution. Digestion was accomplished overnight (15h) at 37 °C. The following day, the peptide containing solution was removed and collected, and the gel pieces were incubated with water (double volume in which the digestion took place). After 5 minutes shaking and ultrasonic bath each, the water was also removed and combined with the previous supernatant. For complete peptide extraction, one volume unit of extraction solution was added (50 % acetonitrile, 5 % formic acid), and the mixture was shaken for 30 minutes. The supernatant was collected and also combined with the previous one. This procedure was also repeated twice, followed by a final extraction in 100 % acetonitrile to extract also the most hydrophobic peptides. The volume of the combined solutions was reduced in a vacuum centrifuge, filtered by a 0.45 μm syringe filter and finally lyophilised. After reconstitution in 0.1 % formic acid, samples were analyzed using complementary mass spectrometric techniques.

4.5 Synthesis of ^{199}Hg enriched pHMB

The synthesis of pHMB with isotopically enriched mercury was realized according to a protocol yet published in 1901 [210]. Organic boron compounds react with inorganic mercury (II) chloride in a metathesis reaction giving the organomercury compound and boronic acid, as well as hydrochloric acid. Optimisation of the synthesis on the mg-scale was carried out using ^{nat}HgO. A boiling aqueous solution of 4-carboxy-boronic acid reacts immediately with $HgCl_2$, precipitating as a white solid. Figure 4.7 shows a scheme of the reaction.

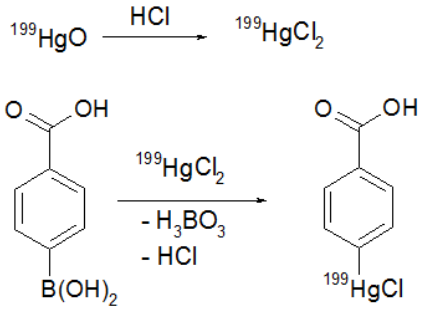


Figure 4.7: Synthesis of ^{199}Hg-pHMB starting from ^{199}HgO

As enriched mercury isotopes are normally distributed in the metal or oxide form, mercury (II) oxide had to be converted into its chloride by dissolving in 6 mol L^{-1} HCl. After evaporation of the liquid, the remaining solid $HgCl_2$ was redissolved in water for the final reaction. It seems crucial for the successful synthesis of the compound, that the amount of HCl is added exactly as required for the amount of HgO. If excess of HCl was used for conversion of the oxide into the chloride, no reaction in the following step of the synthesis was observed, probably due to the formation of chlorocomplexes of Hg^{2+}. To avoid the formation of doubly substituted

mercury (R-Hg-R), 4-carboxy-boronic acid was added in less than stoichiometric amount (0.95 equivalents). After separating the white solid by centrifugation and separation of the supernatant, the compound was washed by resuspending it first in 6 mol L^{-1} HCl and afterwards ultrapure water followed by repeated centrifugation. Finally, the compound was transferred to a glass plate to dry. The yield for both steps was in the range of 35 - 40 %, calculated for HgO. It has to be noted, that the compound obtained in this synthesis is p-hydroxychloromercuribenzoic acid, not the hydroxide used in experiments with pHMB with natural isotopic composition. By dissolving the solid product in diluted NaOH as usual, it is easily convertible in the hydroxide form.

5 Results and Discussion

In the following chapter, the results obtained during this project will be presented and discussed. Starting with the development of the labelling process and the characterisation of the resulting bioconjugate, examples for the use of isotope dilution analysis will be shown in combination with different analytical techniques commonly used for the separation of protein samples, such as liquid chromatography and gel electrophoresis. Additionally results will be presented on the application of isotope pattern deconvolution as a tool for the generation of quantitative data from mass spectra obtained by MALDI-MS. The combination of this technique with ICP-MS as a complementary tool for absolute quantification might be a powerful tool in the future. Finally, results are presented for studies on the binding behaviour of methylmercury towards proteins after incubation, and also under conditions *in vivo*, using tuna fish muscle tissue as a matrix.

5.1 Development of the protein labelling process

Up to now there are many reagents known which react with free cysteine residues. In protein analysis, one of the most applied compounds is iodoacetamide which is used to block reduced cysteine residues in order to avoid reformation of the intentionally broken -S-S-binding. The main purpose of this step is to completely destroy the structure in a protein (quaternary, tertiary and also secondary structure) and therefore also the activity of functional units. For tryptic digestions this is necessary to assure optimum accessibility for the enzyme to achieve a complete digestion of the substrate. The application of the labelling reagent p-(hydroxymercuri)benzoic acid (pHMB) should in principle follow a similar reaction scheme on the protein level and leads to the formation of a covalent bond between the Hg atom in pHMB and the sulfur atom of the sulfhydryl group under liberation of water. According to reference [22], a derivatisation reagent which is used for quantification purposes must fulfil some important preconditions, as for example selective (and quantitative) labelling for all proteins showing the respective binding site present in a sample. Further conditions are that a definite product is formed, and that sample preparation steps for derivatisation can be done with a minimum effort in order to avoid possible analyte loss during this step. In the following chapter, experiments are presented to prove, that pHMB can comply with these preconditions and hence can be applied

as a labelling agent for protein quantification. Ovalbumin was investigated as a first model protein, as previous reduction of cysteine residues was not necessary, as there are four of the total six cysteine directly available for derivatisation. As described by *Bramanti et al.* [179], pHMB was dissolved in 0.01 mol L^{-1} NaOH for easy and secure handling and dosage. The protein solution was incubated for 1 h at room temperature. In order to control if binding of mercury to the protein had taken place, the mixture was analysed by μLC-ICP-MS. As it can be seen in figure 5.1 a, injection of the reaction mixture without further purification of the labelled proteins resulted in a high background and considerable memory effects caused by the derivatisation agent. However, the derivatised proteins elutes as one single peak with a retention time of approximately 15 minutes. Further signals indicating partially labelled protein species or other Hg-containing side products are not observed. One possibility to overcome the drawback of elevated memory effects is to use ultrafiltration to separate the excess of reagent from the derivatised proteins. For ovalbumin, filters with a cut-off membrane of 10 kDa were used. Analysis of the purified labelled proteins with μLC-ICP-MS resulted in chromatograms with an improved signal-to-noise ratio and less memory effects, as it can be seen in figure 5.1 b.

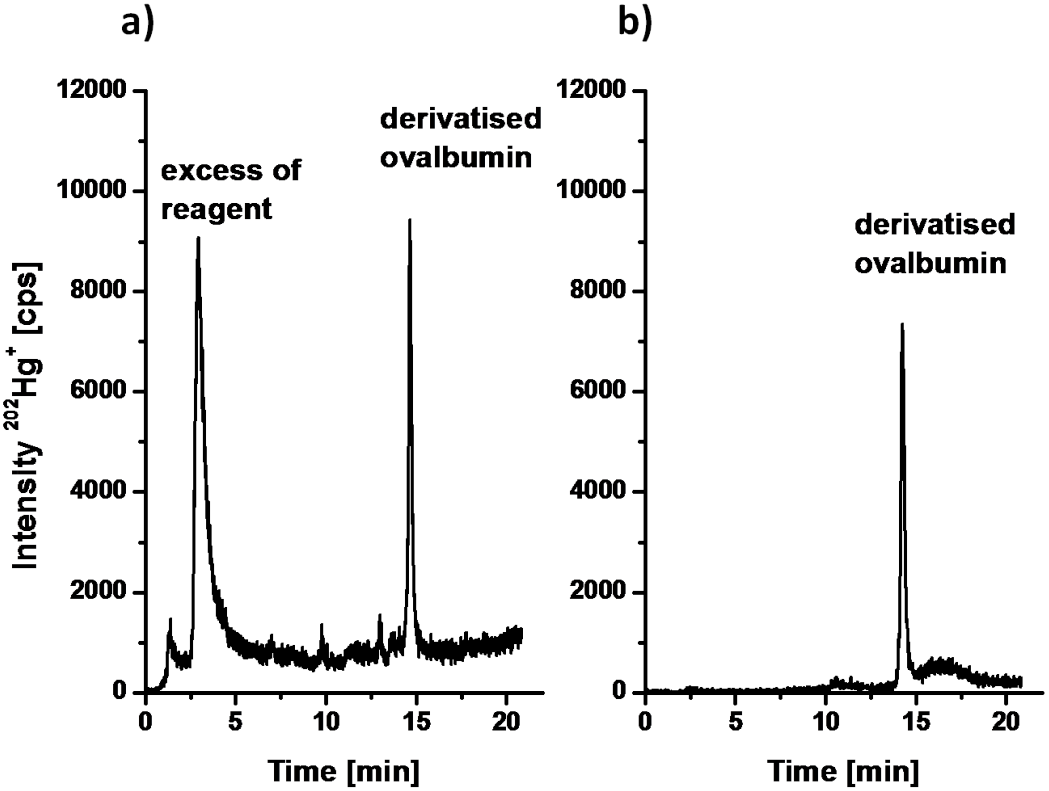


Figure 5.1: Typical LC separations of derivatised ovalbumin without (a) and after ultrafiltration with 10kDa cut-off (b)

5.1.1 Optimisation of the reaction conditions

The objective of the experiments conducted in the following was to investigate, if protein derivatisation with pHMB results in a well defined product also under exceptional reaction conditions, as might be necessary when a complex sample is to be investigated. The influence of the reaction conditions was investigated with ovalbumin as a model protein. Two different experimental series were performed in order to investigate the influence of the reaction time and the applied excess of derivatisation reagent. The standard derivatisation procedure was followed as usual, including the ultrafiltration step. The mercury content of a distinct amount of protein was determined by normal ICP-MS measurements using 10 ng g^{-1} of Rh for internal standardisation. Additionally, μLC-ICP-MS measurements were conducted to check for the purity of the labelled protein. The displayed values are the intensities measured on ^{202}Hg (compensated for instrumental drift by the internal standard Rh) normalized on the amount of ovalbumin (typically 1 pmol) in the sample. The reaction time was varied between 0.5 and 24 h, while the applied excess of reagent was varied between 1- and 50-fold with regard to the total amount of cysteine residues available for derivatisation.

Influence of the reaction time

Figure 5.2 a shows the effect of the reaction time on the stoichiometry of the derivatised protein. As it can be seen, labelling times between 0.5 and 24 h (with 10-fold excess of derivatisation agent with regard to the number of available cysteine residues) do not change the stoichiometry of the modified protein in a significant way, as only slight fluctuation ($\pm$ 8 %) of the signal intensity on mercury is observed. It can be concluded, that once the reaction with the specific binding sites has finished, no further binding of the derivatisation agent occurs, that would change the structure of the bioconjugate in an uncontrollable way. Furthermore, it was proven that the derivatised protein is stable for at least a few days when stored at 4°C.

Influence of the excess of derivatisation agent

In figure 5.2 b, the effect of the excess of reagent is shown. For higher molar ratios of pHMB with regard to the protein, no changes in the stoichiometry of the bioconjugate are observed. Therefore, once all the binding sites available are derivatised, no additional binding occurs, although there is still a huge excess of the derivatisation reagent present in the reaction solution, which could potentially also bind to other amino acid residues. However, the observed signal intensity on mercury is decreasing when lower excess than 10-fold with regard to the number of available cysteines is applied, so that a 10-fold excess of the derivatisation reagent is mandatory to achieve complete saturation of all cysteine residues.

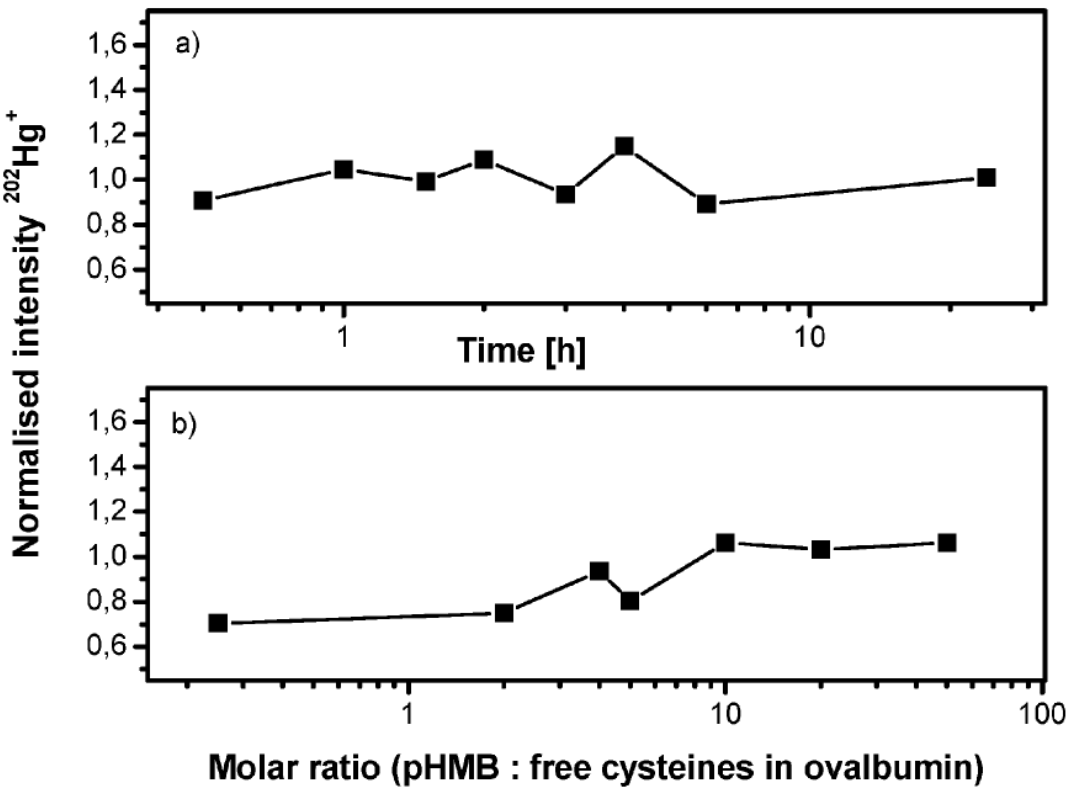


Figure 5.2: Influence of the reaction time (a) and the excess of reagent (b) on the derivatisation product

This is an important precondition for using any form of labelling to derivatise proteins in complex mixtures, as concentration of the target protein might be inferior in various orders of magnitude in comparison to other proteins in the sample, thus making the application of huge excesses of reagent mandatory to assure complete derivatisation of the target protein.

Selectivity for protein binding

In order to finally prove the association of mercury to ovalbumin, the concentration of derivatised ovalbumin was determined with two different methods:

- Bradford assay to determine the protein concentration
- Isotope dilution analysis to determine the total Hg concentration

After derivatisation and purification, the sample was split and treated independently. One aliquot was quantified using the Bradford assay as described in chapter 4.1.3 and quantified for its content of ovalbumin. The other aliquot was diluted in nitric acid, and the total Hg-concentration was determined using IDA with a spike containing ^{200}Hg. From the results, the concentration of ovalbumin was calculated under the condition, that the protein was derivatised with four units of the labelling reagent, as there are four cysteine residues available as binding sites. Contributions of unbound reagent are completely removed by previous ultrafiltration, so that they

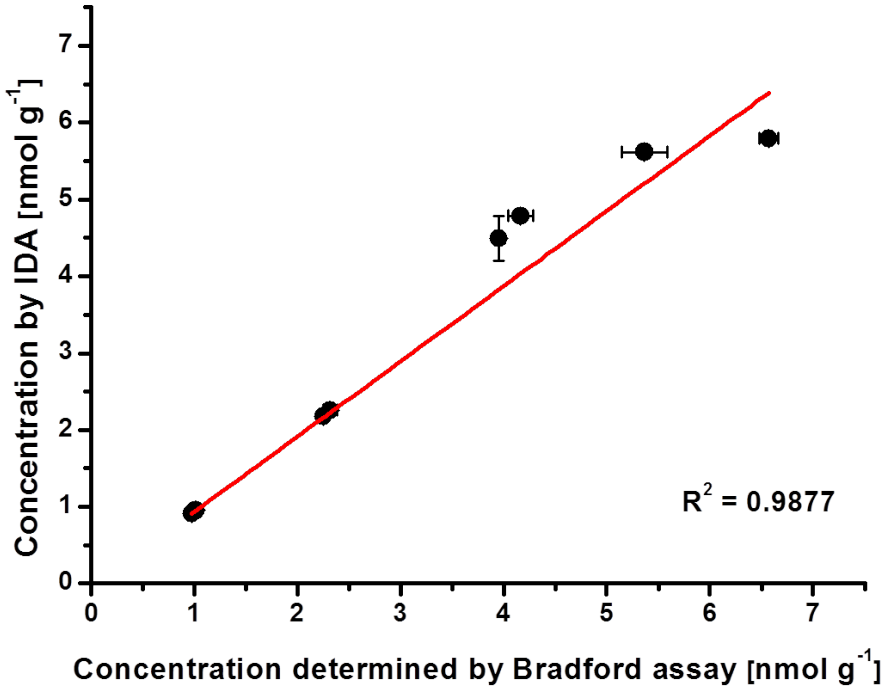


Figure 5.3: Determination of the protein concentration of derivatised ovalbumin using Bradford-assay and isotope dilution analysis of Hg

should not affect the results. If pHMB binds specifically to these residues, both methods should be in good agreement with each other. Figure 5.3 shows the obtained results for four independent protein derivatisations in different concentration ranges. Each derivatisation was carried out in duplicate. As it can be seen, both methods are in good agreement with each other, although there are deviations at higher concentration levels (above 5 nmol g^{-1}). For these levels, the calibration curve acquired for the Bradford assay already showed a slight non-linear behaviour. However, in the lower concentration range, both methods can be used for quantification of the derivatised protein. From the obtained results it can be concluded, that the number of molecules of pHMB binding to ovalbumin corresponds well to the number of free cysteine residues available and therefore it shows a specific binding behaviour.

5.1.2 Investigation of the adduct stoichiometry

To assure that the derivatisation of a protein with pHMB results in a definite product, but not in a mixture of different labelling states or the protein labelled in various binding sites within the amino acid chain, molecular MS techniques had to be applied. To determine the structure of the derivatised proteins, MALDI-MS as well as ESI-MS were used to characterise both model proteins, ovalbumin and insulin, in order to observe, if there are any differences in the results due to the systematically

different form of ionisation. Additionally, as both proteins are of different size and structure, also possible effects due to the previous reduction of cystine groups or steric effects in the smaller amino acid chain of insulin are to be scrutinized during these experiments.

As described in chapter 4.3, two possible ways for reduction of cystine groups in insulin were tested:

- Denaturation of the protein in 6 mol L^{-1} urea and incubation with DTT at room temperature for 1 h
- Denaturation and reduction of the protein in 50 mmol L^{-1} NH_4HCO_3 with DTT at 95°C for 5 minutes

Both methods lead to complete derivatisation of all cysteine residues available, as could be shown using MALDI-MS. The latter one was not only faster, but also more appropriate for the use with molecular mass spectrometry, as the use of urea could be avoided. For insulin filters with a cut-off membrane of 3 kDa were necessary, as the molecular weight is much smaller.

MALDI-MS of derivatised proteins

MALDI-MS was reported to be an ionisation source soft enough to ionize big molecules such as proteins without extensive fragmentation [53]. Analysis of ovalbumin and insulin under optimized conditions (see chapter 4.2.5) gave the results presented in the following chapter.
In the case of ovalbumin (figure 5.4) the ions typically observed for the underivatised as well as for the derivatised protein were $[M+H]^+$, $[M+2H]^{2+}$ and $[2M+H]^+$. In all cases a mass shift between the native and the derivatised protein could be observed. For the single charged ion of ovalbumin ($[M+H]^+$), the difference can be estimated to 916 ± 22 Da , whereas for the double charged ion ($[M+H]^{2+}$), this difference can be estimated to be 932 ± 15 Da [1], revealing a stoichiometry of roughly 3 molecules of pHMB per molecule of protein (molecular mass after binding 321 Da). This amount is less than expected, as there are 4 free accessible cysteines in ovalbumin. There are two possible explications for these findings:

- The protein was not completely derivatised, and therefore, the observed amount of reagent is less than expected. Additionally, other structural differences can come into play, as for example steric hindrance for one or more cysteine residues, as described by [182] and [183].

[1] The mass difference for both ions was determined in ten individual mass spectra. The effect of the charge is already included in the given numbers. For the signals corresponding to $[2M+H]^+$, the mass difference was not determined due to the elevated peak width, which leads to a high inaccuracies in its determination

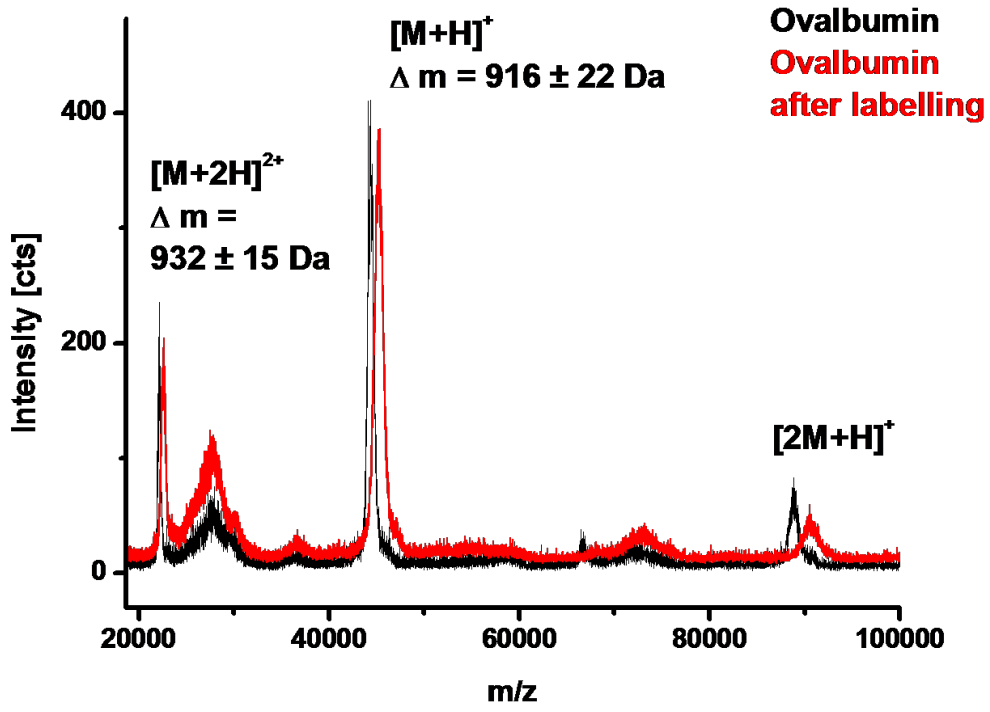


Figure 5.4: MALDI-MS spectra of labelled and not labelled ovalbumin

- The ionisation process was still too hard to maintain the integrity of the Hg-S-bound of the derivatisation agent to cysteine residues and therefore the label was partially lost during ionization.

It should be noticed, that the width of the signals obtained with MALDI-MS for derivatised ovalbumin are much broader than for native ovalbumin. In the derivatised protein, the signal width for $[M+H]^+$ at FWHM is about 900 Da, whereas in the case of the native protein it is only 300 Da. This observation might indicate that besides the micro-heterogeneities in terms of different structural isoforms of ovalbumin, the labelled form showed an additional mass distribution due to the label.
In the case of human insulin (shown in figure 5.5), the resolution of the obtained spectra is better, due to the smaller mass-to-charge ratio of the desired ion, as TOF mass analysers loose resolution with the square root of m/z [64]. Unfortunately, there are no signals observed corresponding to the derivatised a-chain of insulin. This behaviour may be related to the number of acidic amino acid residues in the polypeptide chain , as already previously observed [175]. In contrast, the derivatised b-chain gives various signals. The peak at m/z 4,071.8 Da corresponds to the two-fold labelled insulin b-chain, the peak at m/z 3,751.8 Da corresponds to the single labelled b-chain, whereas the peak at m/z 3,428.7 Da corresponds to the native b-chain. The peak at m/z 3,629.8 Da corresponds to the single labelled b-chain, which has also lost one terminal amino acid, threonine in particular. Other signals

could not be detected in the spectrum. All signals are summarised in table 5.1.

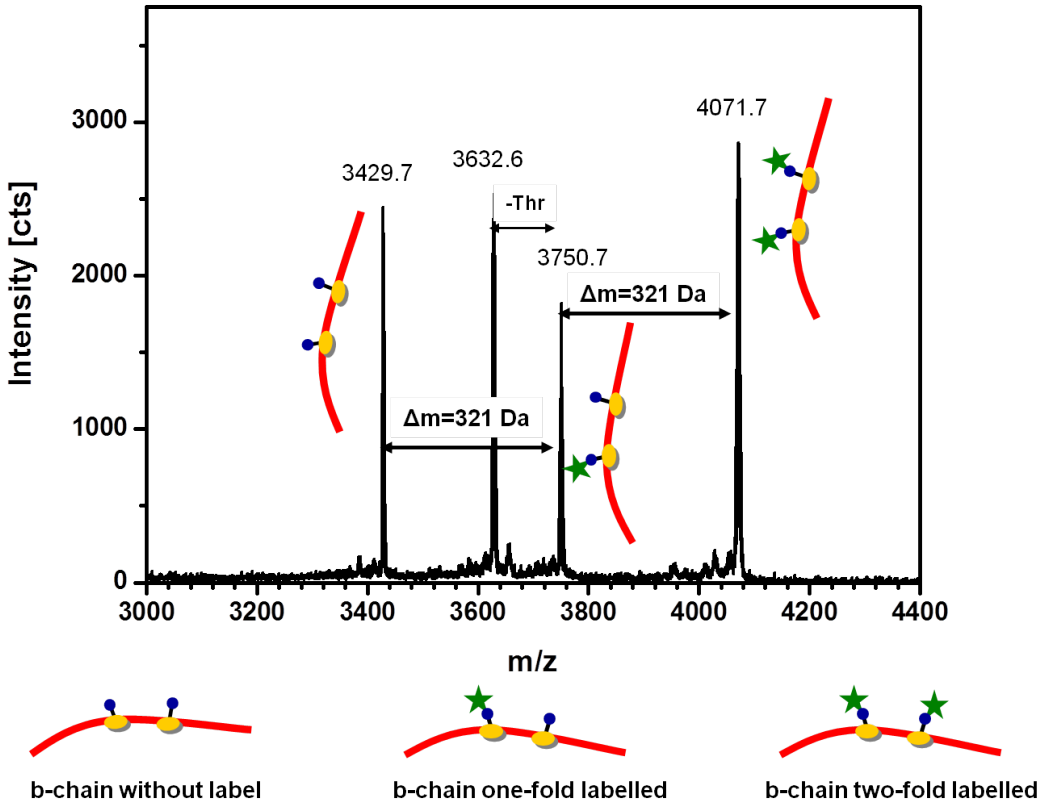


Figure 5.5: MALDI-MS spectrum of human insulin derivatised by pHMB

Table 5.1: Signals found in MALDI-MS for derivatised human insulin

Signal	Name	m/z expected	found	Δm (ppm)
A	B chain two-fold labelled	4072.7	4071.8	221.0
B	B chain one-fold labelled	3752.7	3752.3	106.6
C	B chain one-fold labelled - T	3631.7	3630.8	247.8
D	B chain without label	3429.9	3429.4	145.8

All signals correspond well to the calculated values within the limits of mass accuracy typically achieved.

ESI-MS of derivatised proteins

ESI-MS is in contrast to MALDI-MS considered to be an even softer ionisation source, as ionisation in an electrospray ion source can be understood as an oxidation/reduction process. There are many examples for the successful ionisation of biomolecules with intact binding of heteroelements or associated metals [211]. Both proteins were analysed in the ESI-MS unit under optimized conditions (see chapter

4.2.6) as well in their labelled form as in their native state. Ovalbumin samples were reconstituted after ultrafiltration in 80 % acetonitrile (0.01 % TFA), insulin was reconstituted in 72 % acetonitrile, 0.5 % acetic acid and 0.01 % TFA. All samples were introduced with a syringe pump to the ESI-MS unit equipped with the IonSpray source with a flow rate of 5 μL min^{-1}. A typical deconvoluted spectrum obtained for ovalbumin can be seen in figure 5.6.

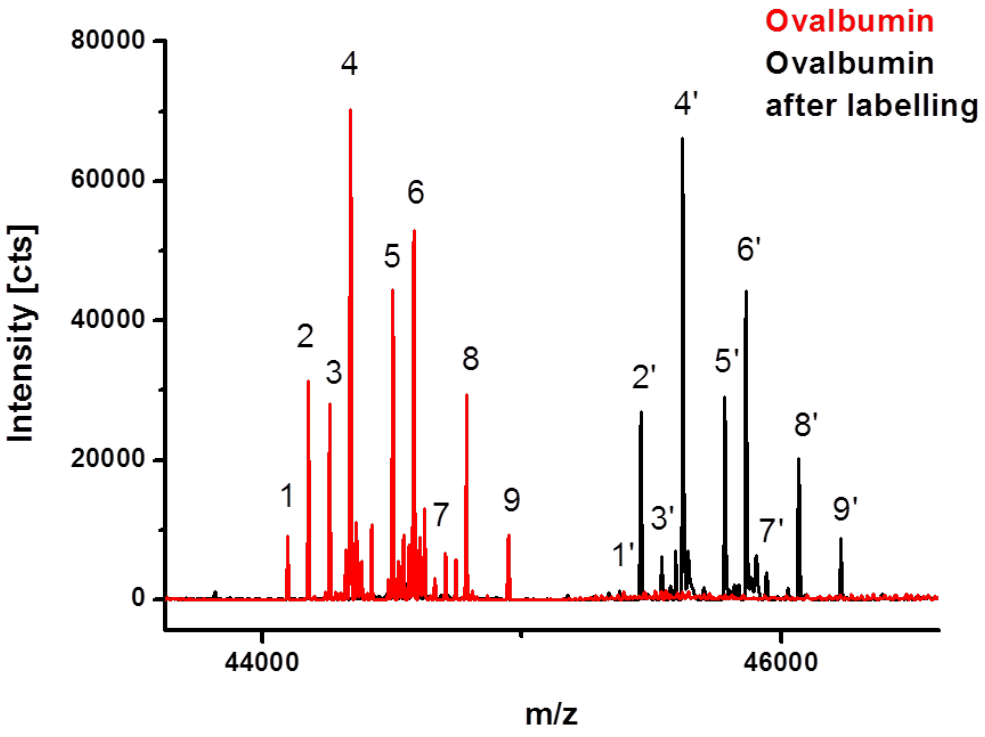


Figure 5.6: Deconvoluted ESI-MS-spectra of derivatised and underivatised ovalbumin

As it can be seen there is a series of signals found in both spectra (labelled and not labelled). Correlation of signals in similar position in both spectra always leads to a similar difference in mass between the labelled and the not labelled form which is in the range of 1280 Da. This corresponds very well to the theoretical value for 4 molecules of pHMB per molecule of protein (4 x 321 Da = 1284 Da). Mass accuracy was typically better than 50 ppm, and peak width was about 5 Da (FWHM). Reviewing the literature, it was reported before that this collection of signals found in the mass spectra corresponds to different glycoforms of ovalbumin [212]. Table 5.2 shows a list of the major signals in the spectra in figure 5.6, assigning the observed signals to the reported glycoforms of ovalbumin, also in the derivatised form. The theoretical masses were calculated from the mass of unprocessed ovalbumin (42221 Da), adding the mass of the corresponding oligosaccharide combination and subtracting 18 Da for the elimination of water during bond formation. The names of the observed glycoforms in table 5.2 are to be understood as

- Hex = number of hexose sugars added
- HexNAc = number of N-acetylhexoseamine added

also following reference [212].

Table 5.2: Identified glycoforms of ovalbumin

Nr.	name	mass theor.	found	mass labelled	Δ m
1	$Hex_5HexNAc_2$	44098	44097	45378	1281
2	$Hex_3HexNAc_4$	44180	44177	45458	1281
3	$Hex_6HexNAc_2$	44260	44259	45539	1280
4	$Hex_4HexNAc_4$	44342	44339	45619	1280
5	$Hex_5HexNAc_4$	44504	44502	45782	1280
6	$Hex_3HexNAc_6$	44587	44583	45863	1280
7	$Hex_6HexNAc_4$	44667	44665	45943	1278
8	$Hex_3HexNAc_7$	44790	44787	46068	1281
9	$Hex_4HexNAc_7$	44952	44948	46230	1282

Overall, 13 different signals could be identified according to the literature [212], although some were of very low intensity. It has to be noted that ovalbumin can potentially be phosphorylated, so that in some cases masses may also refer to a phosphorylated isoform instead of a different glycoform. Addition of a phosphate group would lead to an increase of the protein mass of 79 Da. Within the uncertainty of the determined mass values, this could for example explain the mass difference between glycoforms 2 and 3.
In contrast to MALDI, electrospray ionisation leads to multiple charged ions, so that the actual analysed value, the ratio of mass to charge (m/z) is lower, leading to higher mass resolution for the samples of ovalbumin, so that the broad signal corresponding to the protein in MALDI-MS results as a collection of signals in ESI-MS, that can be clearly identified as different glycoforms of the protein. Also, the binding between the mercury compound and the protein is conserved during ionisation, so that only the expected 4-fold labelled protein is found. Another important conclusion that can be drawn from these measurements is that labelling of proteins with pHMB is independent of protein isoforms, as all glycoforms (or phosphorylated forms) are labelled to the same extent by pHMB.
In the mass range between 43,900 Da to 45,100 Da (corresponding to unlabelled or partially labelled ovalbumin) only minor signals can be observed in the spectrum of the derivatised ovalbumin. Assuming the worst observed mass accuracy of about 100 ppm, none of these signals can be related to the signals observed in the previous spectrum of native ovalbumin. Hence it can be concluded that reaction yield is at least better than 97 %. As the absence of detectable signals in ESI-MS can also

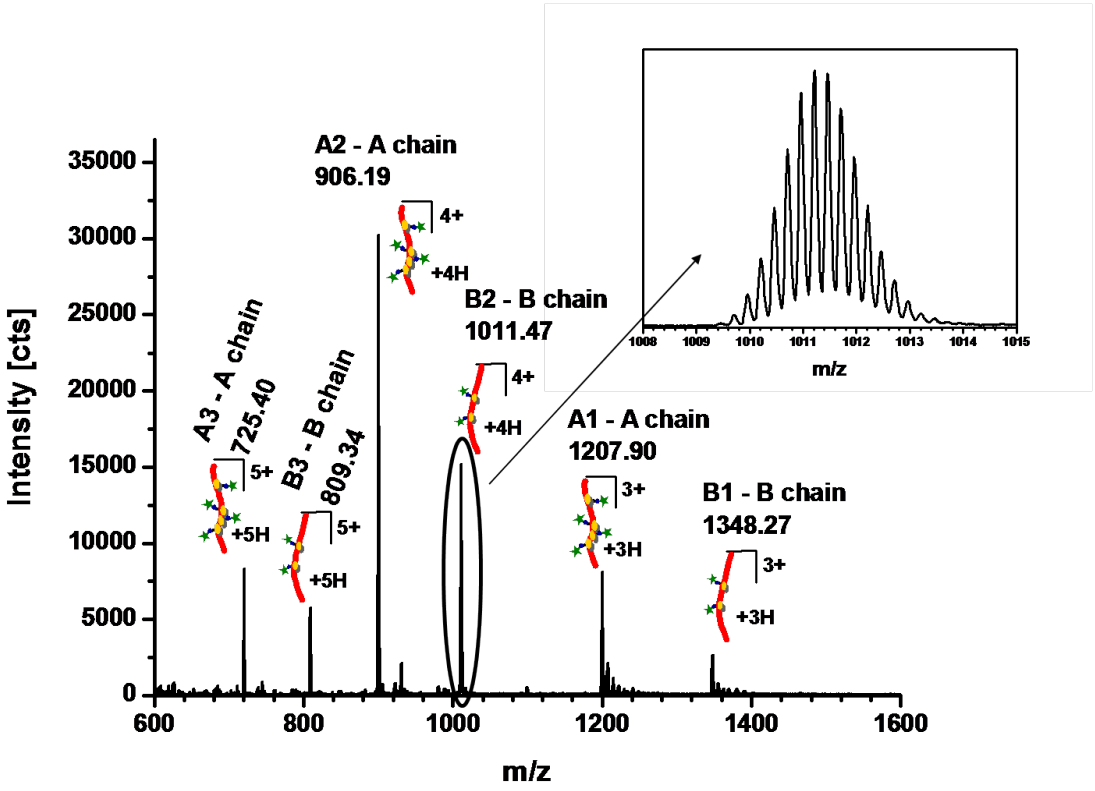


Figure 5.7: ESI-MS spectrum of labelled bovine insulin and the isotopic signature of the two-fold labelled b-chain as $[M+4H]^{4+}$

be caused by ion suppression effects, this was checked by addition of underivatised ovalbumin, but no evidence for ion suppression was found.

In the case of bovine insulin, the ESI-MS spectrum (figure 5.7) shows various multiple charged ions from the two-fold labelled b-chain, but as well from the four-fold labelled a-chain. Additionally, sodium adducts of all signals could be observed. Table 5.3 gives an overview over the expected and observed signals. Similar to the ESI-MS spectrum of ovalbumin, loss of the label was not observed as a consequence of ionisation. The isotope signature (shown as a detail in figure 5.7 on the example of the two-fold labelled b-chain as $[M+4H]^{4+}$) indicates the presence of two mercury atoms. Mass accuracy observed was below 100 ppm for all signals. As the number of labels in both chains corresponds well to the expected number of available derivatisation sites, it can be concluded, that insulin was also completely labelled.

Table 5.3: Ions found in the ESI-MS spectrum of labelled bovine insulin

Nr.	Name	charge	m/z expected	found	Δm [ppm]
A1	a-chain 4-fold labelled	3+	1208.32	1207.9	34.7
A2	a-chain 4-fold labelled	4+	906.74	906.19	60.6
A3	a-chain 4-fold labelled	5+	725.40	725.34	82.7
B1	b-chain 2-fold labelled	3+	1348.23	1348.27	29.7
B2	b-chain 2-fold labelled	4+	1011.42	1011.47	49.4
B3	b-chain 2-fold labelled	5+	809.34	809.36	24.7

ESI-MS/MS analysis of derivatised insulin

In order to confirm cysteine residues as potential binding sites, the ion with m/z 809.34 ($[M+5H]^{5+}$ of the two-fold labelled insulin b-chain) was subjected to MS/MS analysis using a collision energy of 25 eV. The obtained product ion spectrum can be seen in figure 5.8.

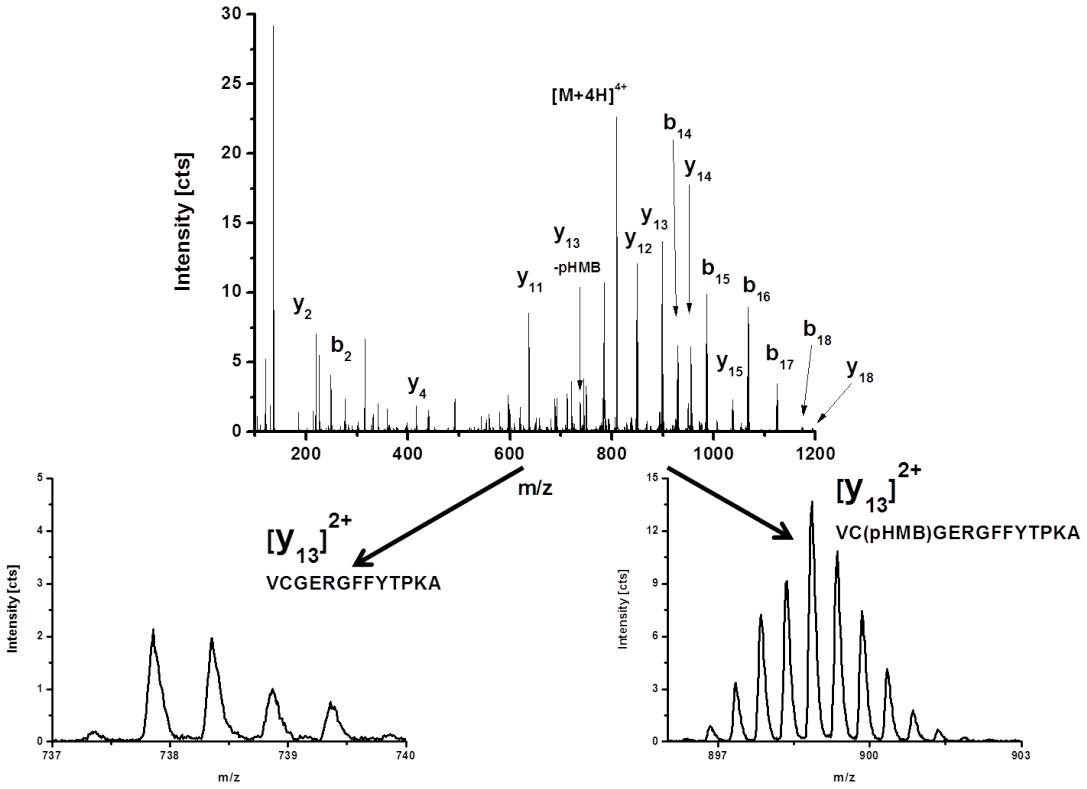


Figure 5.8: Product ion spectrum of m/z 809.34 fragmented with a collision energy of 25 eV, amplification of $[b_{13}]^{2+}$ in its labelled and unlabelled form

Table 5.4 indicates the fragment ions identified via matching of the mass spectrum to the sequence of insulin b-chain with and without pHMB. As the sequence of the b-chain of insulin has the sequence shown in figure 5.9:

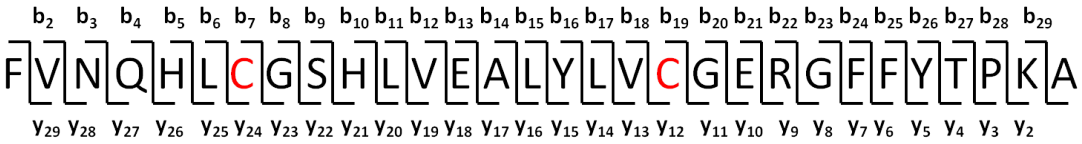


Figure 5.9: Amino acid sequence of the B chain of insulin together with possible product ions. Both cysteine residues which can carry the label are marked in red

all b-ions beginning with b_7 should show a mass shift of 321 Da and an isotopic signature corresponding to the presence of one mercury atom. Additionally. all b-ions from b_{19} should show a mass shift of 2 x 321 Da and an isotopic signature influenced by the presence of two mercury atoms. Vice versa, the same should be valid for all y-ions starting from y_{12} (mass shift 321 Da, one mercury atom) and y_{24} (mass shift 2 x 321 Da, two mercury atoms). As it can be seen in the table, various ions have been identified by software based matching using mMass. Besides being present as $[M+2H]^{2+}$, some of the identified fragment ions have also were present as $[M+3H]^{3+}$ due to the high charge state of the precursor ion. These ions were not included into the data evaluation process. The isotopic signature of mercury was clearly present in all of the ions that were supposed to carry it.

Table 5.4: Fragment ions identified by mMass

Ion	with pHMB	error (Da)	without pHMB	error (Da)
$[b_2]^+$	-	-	247.3129	-0.1653
$[b_3]^+$	-	-	361.4158	-0.2219
$[b_5]^{2+}$	-	-	313.8462	-0.1769
$[b_{12}]^{2+}$	829.6325	0.1910	-	-
$[b_{13}]^{2+}$	894.1896	0.1869	-	-
$[b_{14}]^{2+}$	929.7286	0.1440	768.8722	0.0059
$[b_{15}]^{2+}$	986.3076	0.1047	825.4512	0.0306
$[b_{16}]^{2+}$	1067.8944	0.0449	907.0380	0.0849
$[b_{17}]^{2+}$	1124.4734	0.0090	-	-
$[b_{18}]^{2+}$	1174.0390	0.0312	-	-
$[y_{18}]^{2+}$	1194.0478	-0.0431	-	-
$[y_{15}]^{2+}$	1037.3727	0.0530	876.5163	-0.0732
$[y_{14}]^{2+}$	955.7858	-0.0513	-	-
$[y_{13}]^{2+}$	899.2069	-0.0020	738.3505	0.0205
$[y_{12}]^{2+}$	849.6412	0.1824	688.7848	0.0553
$[y_{11}]^{2+}$	-	-	637.2128	0.1065
$[y_{10}]^{2+}$	-	-	608.6870	0.1291
$[y_9]^{2+}$	-	-	544.1299	-0.1998
$[y_6]^{2+}$	-	-	363.9241	-0.2239
$[y_4]^+$	-	-	416.4928	-0.2405
$[y_2]^+$	-	-	218.2733	-0.1207

However, also some of the corresponding ions without the derivatisation agent were identified by mMass. A direct comparison between ions belonging to the same fragment shows, that whereas the Hg-containing ions are the predominant signals in the spectrum, signals corresponding to the underivatised form show only little signal intensity. An example for these findings is also represented in figure 5.8, showing the b_{13}-ion as $[M+2H]^{2+}$ carrying the label (right) and without it (left). The binding between the label and cysteine residue therefore seems to be stable enough to be conserved during MS/MS analysis, although a small portion gets partially lost. Probably due to the huge size of the precursor ion, none of the identified fragment ions carried both units of the labelling agent. However, as b and y series are complementary to each other, both cysteine residues could be unambiguously identified as binding site for the label. From the results obtained up to now regarding the development of the protein labelling process and the characterisation of the resulting bioconjugates, it can be concluded, that pHMB fulfils all the conditions set to a labelling reagent for quantitative protein analysis. First of all, a product with a definite structure and stoichiometry was formed and all potential binding sites were populated by the reagent and were also unambiguously identified as being the supposed free cysteine residues. Second, the derivatisation reaction showed a quantitative yield and was realisable with little effort in terms of sample preparation. Consequently, in the following part of this work, the possibilities for protein quantification using the proposed method of protein labelling with pHMB is elucidated.

5.1.3 Analytical characteristics of the method

As quantitative analysis of proteins is one of the main objectives of this work, the developed method should have a highly linear dependence of the obtained signal from the concentration of analyte, but as well a high repeatability in terms of peak area and retention time. Furthermore, a significant reduction of detection limits is desired in comparison to the detection/quantification of proteins via elements being part of their constituting amino acids, such as sulphur or phosphorous (after enzymatic phosphorylation as post-translational modification). To check for these criteria, derivatised ovalbumin was analysed with the coupling of μLC to ICP-MS. The resulting calibration curve is shown in figure 5.10. The detection limit (table 5.5) was calculated after the 3σ criterion (LOD = 3 times signal noise of the background signal). The limit of quantification was calculated using the 10σ criterion (LOQ = 10 times signal noise of the background signal).

Detection limits obtained with labelling show significant improvement in comparison to the detection of sulphur. In the case of ovalbumin, the limit of detection was improved from 500 fmol [140] to less than 1 fmol without any previous enrichment. Additionally, it has to be noted, that for the detection of sulphur not only six cysteine residues, but also 16 methionine residues contribute to the overall sulphur content

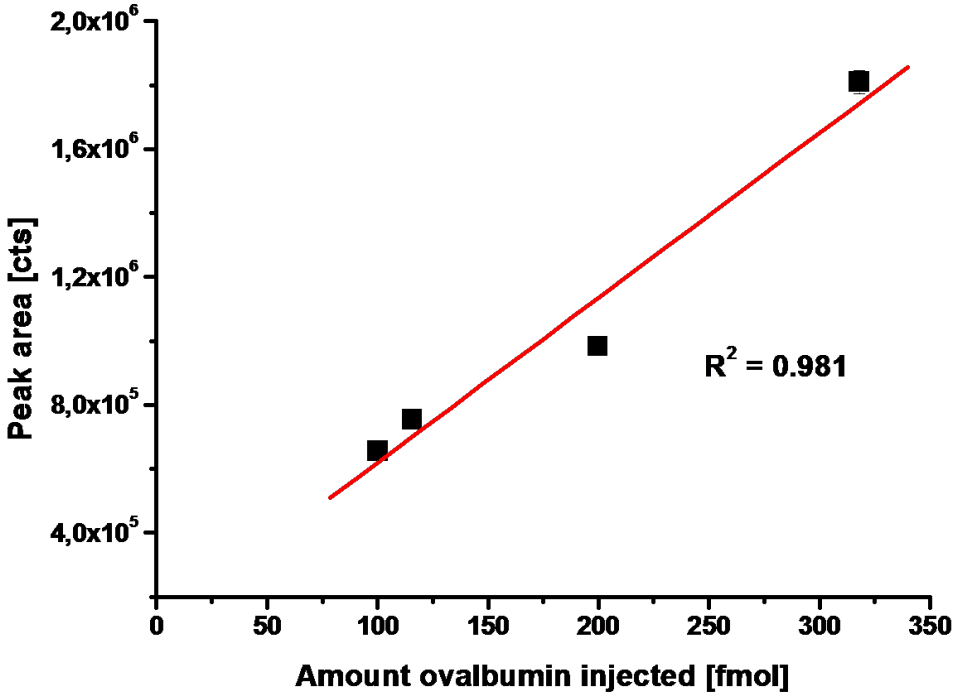


Figure 5.10: External calibration for derivatised ovalbumin obtained by μLC-ICP-MS. Typical RSD values observed were between 2 and 5 % (N=3)

of the protein, whereas in the case of mercury detection only four mercury atoms per protein are relevant for detection. As the detection of mercury is not prone to spectral interference, it can be easily conducted on a quadrupole instrument, whereas the detection of sulphur requires the use of collision/reaction cell technology or high resolution instruments. As ICP-MS detects elements independent of their chemical form, the above presented results show, that the quantitative analysis of derivatised proteins would be possible with commonly applied quantification strategies, as for example standard addition or external calibration using mercury standards. But as mercury has several stable isotopes, application of isotope dilution analysis is also possible, making quantitative analysis more accurate, as the internal standardisation

Table 5.5: Analytical characteristics of the proposed method for ovalbumin

LOD	700 amol absolute
LOQ	2000 amol absolute
sensitivity	5150 counts fmol^{-1}
repeatability	3.3 % peak height, 4.2 % peak area (N = 6)*

* Determination of the repeatability was realised via the repeated injection of approx. 320 fmol of derivatised ovalbumin

Table 5.6: Peptides identified using mMass after tryptic digestion of ovalbumin labelled with pHMB and iodoacetamide

Peptide sequence	Position [MC *]	mass [Da]	pHMB	IAC
GSIGAASMEFCFDVFK	2-17, [0]	1708.7709	√	√
DILNQITKPNDVYSFSLASR	86-105, [0]	2281.2800	√	√
LYAEER	106-111, [0]	780.3886	√	√
ELYR	124-127, [0]	580.2404	√	√
GGLEPINFQTAADQAR	128-143, [0]	1687.8398	√	√
ELINSWVESQTNGIIR	144-159, [0]	1859.1056	√	√
GLWEK	183-187, [0]	632.2878	√	√
AFKDEDTQAMPFR	188-200, [1]	1555.8230	√	√
DEDTQAMPFR	191-200, [0]	1209.5709	√	√
LTEWTSSNVMEER	265-277, [0]	1581.8231	√	√
LTEWTSSNVMEERK	265-277, [1]	1709.8163	√	√
VYLPR	281-285, [0]	647.3282	√	√
ISQAVHAAHAEINEAGR	324-340, [0]	1774.0337	√	√
EVVGSAEAGVDAASVSEEFR	341-360, [0]	2009.1037	√	√

* MC corresponds to the number of missed cleavages

for e.g. instrumental drift during gradient elution is accomplished by the target element itself.

5.1.4 Possible side effects caused by the label in mass spectrometry

To investigate the applicability of pHMB in proteomic studies, it is highly important, that the presence of the label does not have effects on the result of enzymatic digestions, e.g. reduction of trypsin activity due to steric hindrance, as MS-based identification is one of the main topics in today´s protein science. Similarly, it is as well as important that the fragmentation behaviour of labelled peptides is similar to conventionally derivatised ones, for example with iodoacetamide (IAC), so that software based peptide identification is not disturbed. For addressing the former point, ovalbumin was derivatised either with pHMB or iodoacetamide, and then subjected to gel electrophoresis and afterwards digested as described in chapter 4.4.2. The resulting peptide mixture was analyzed by MALDI-MS in order to compare the resulting peptide mass fingerprint. The identified peptides after evaluation of 10 independent mass spectra are summarized in table 5.6.
As it can be seen, there is no difference between the peptides found in pHMB-derivatised ovalbumin and ovalbumin which has been treated with iodoacetamide. This indicates that the presence of the label did not affect the enzyme in its activity.

However, in both cases, the overall sequence coverage was only 39 %. This might be a consequence of the missing reduction of the remaining disulfide bridge in ovalbumin, which leads to a partial maintenance of the protein structure and thus might affect trypsin activity. As the most abundant signals correspond to proteotypic peptides for ovalbumin, a MASCOT identification was unambiguously possible in both cases. The peptide ^{2}**GSIGAASMEFCFDVFK**^{17} is the only peptide found, which contains one of the four cysteine residues available for derivatisation. It has to be mentioned, that neither in the case of iodoacetamide treated ovalbumin nor in pHMB treated ovalbumin, this peptide was detected including the modification.
In the case of pHMB treated ovalbumin, software based peptide matching using mMass also lead to the identification of other peptides that potentially could carry the label, namely ^{62}**LPGFGDSIEAQC(pHMB)GTSVNVHSSLR**^{84} with m/z 2697 and ^{105}**LYAEERYPILPEYLQC(pHMB)VK**^{122} with m/z 2550. Both signals were rejected as potentially derivatised peptides, as the isotopic signature did not match to the expected isotopic signature for a Hg-containing peptide. Additionally, the cysteine residue ^{120}Cys should not be derivatised, as it was part of the cystine group originally present in ovalbumin. The latter signal could be assigned to a peptide resulting from self-digest of bovine cationic trypsin, whereas the origin of the former remains unknown.
Additionally, ovalbumin treated with iodoacetamide and pHMB after previous reduction was digested in solution as described in section 4.4.1. Also here, MALDI-MS of the digest did not lead to a significantly increased sequence coverage (about 44 % in both cases). Now however, the cysteine residue ^{120}Cys is available for derivatisation with pHMB, so that the peptide ^{105}**LYAEERYPILPEYLQC(pHMB)VK**^{122} was observed in the mass spectra together with the peptide ^{111}**YPILPEYLQC-(pHMB)VK**^{122} at m/z 1788, which includes the same residue. As for the in solution digestion the actual enzyme-to-substrate ratio could be better adjusted, no additional peptides due to self-digest of trypsin were found.
In order to compare the fragmentation behaviour, bovine insulin was reduced and afterwards incubated with pHMB and iodoacetamide. After tryptic digestion, the resulting peptides were analyzed using ESI-MS/MS, in order to investigate if there are any significant changes in the peptide pattern due to reduced trypsin activity. Second, the most abundant ions were fragmented using CID to investigate, if there are any differences in their fragmentation behaviour or possible drawbacks for software based identification.
Tryptic digestion was realized in solution as described in chapter 4.4.1. One aliquot was derivatised using pHMB as described before, the other was incubated with iodoacetamide in a similar amount and treated likewise to block the reduced cysteine residues. Theoretically, the peptides shown in table 5.7 are to be expected. There are two cleavage sites (arginine at position 46, lysine at position 54), so that

two peptides and a cleaved alanine residue (not considered) should be found, from which one should carry both pHMB-modified cysteine residues.

Table 5.7: Expected peptides from insulin b-chain

peptide	mass	cysteines
^{25}FVNQHLCGSHLVEALYLVCGER^{46}	2487.2271	2
^{47}GFFYTPK^{53}	859.4348	-

The obtained TOF-MS spectra can be seen in figure 5.11. Evaluation of the signals obtained in the spectra gave the results shown in table 5.8. Identity of the peptides was in all cases confirmed by MS/MS analysis.
As it can be seen in the figure, the smaller peptide formed (m/z 859.4135; GFFYTPK) is the predominant signal in both spectra. The digestion of pHMB-derivatised insulin leads to the formation of an additional peptide, which cannot be related to trypsin (m/z 717.9751). The peptide corresponds to the amino acid sequence ^{25}**FVNQHLC(pHMB)GSHLVEALY**^{40}, in which an additional cleavage after the ^{40}Tyr residue has taken place. The complementary fragment ^{41}**LVC(pHMB)GER**^{46} could not be found in the mass spectrum. Besides that, there are also ions which can be related to the tryptic peptide expected which has lost one or both units of the label during the tryptic digestion. Due to the previously observed conservation of the binding between mercury and sulphur during the ionisation process, this loss must have occurred during tryptic digestion. For iodoacetamide derivatised insulin, additional cleavages or loss of the derivatisation agent was not observed. However, intensity for the tryptic peptide which carries the derivatised cysteine residues is poor in both cases. This might indicate a generally reduced ion formation for peptides which contain modified cysteine residues. On the other hand, ionisation might be affected by the relatively big size of the tryptic peptide. In contrast, similar studies on lanthanide labelled peptides using the DOTA reagent indicated an increase in the signal to noise ratio for cysteine labelled peptides [168]. The authors also described a higher number of identified peptides containing cysteine residues for a tryptic digestion of BSA. However, there are fundamental differences in comparison to this study, as MALDI-MS was used for identification, and the investigated peptides were smaller (7 to maximum 13 amino acids).

5.1.5 Conclusions from this chapter

The experiments conducted in this part of the thesis clearly indicate, that pHMB as a derivatisation agent for proteins fulfils all preconditions for labelling purposes [22]. The reaction of proteins leads to the selective derivatisation of available cysteine residues. The experimental effort for derivatisation and purification could be limited to an additional ultrafiltration step. Additionally, it could be shown, that

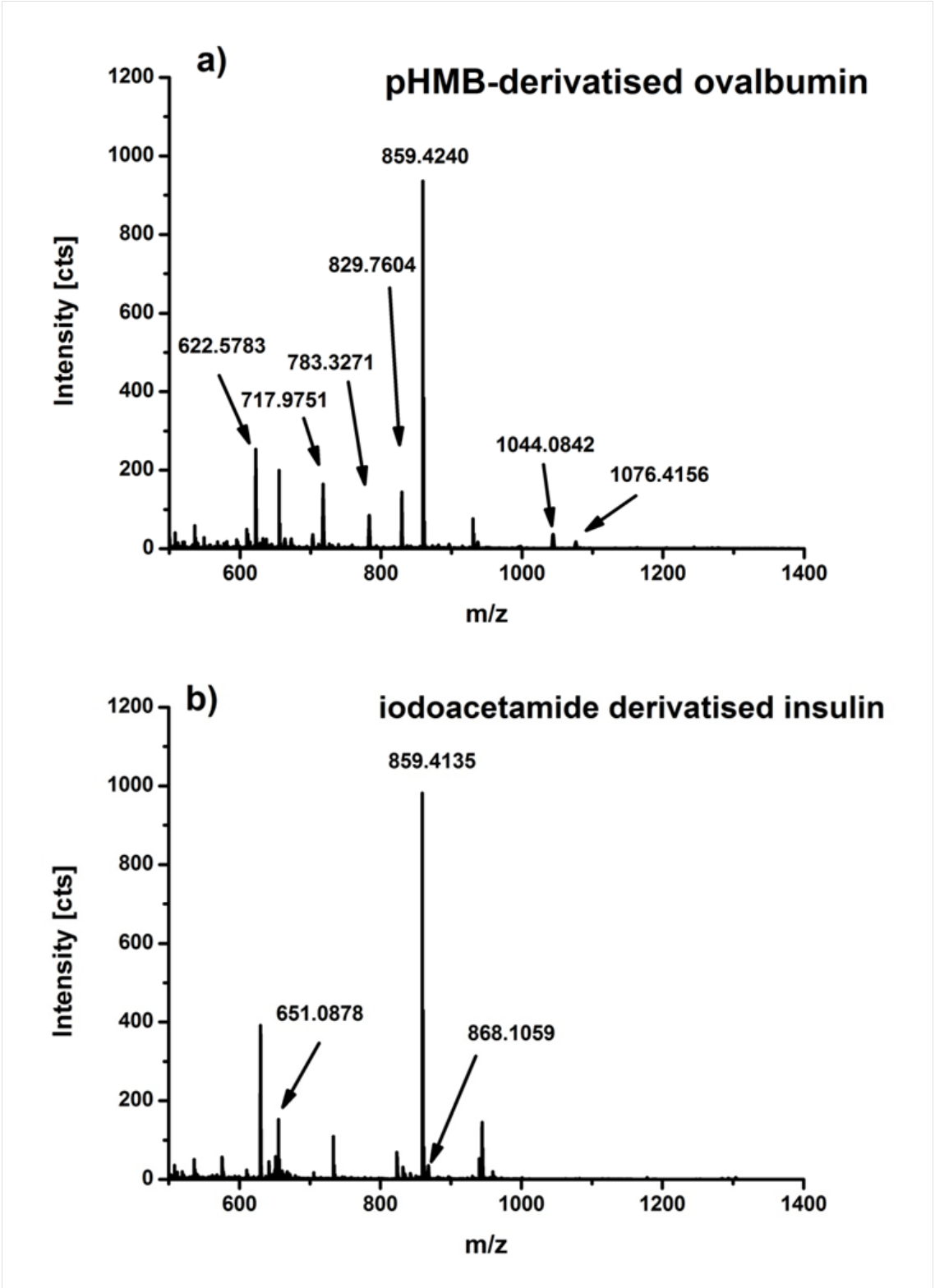


Figure 5.11: Comparison of observed ions after tryptic digestion of insulin; pHMB-derivatised (a) and iodoacetamide derivatised (b)

Table 5.8: Tryptic peptides identified by ESI-MS/MS in a digest of bovine insulin derivatised with pHMB and iodoacetamide

m/z	charge	peptide
pHMB derivatised		
622.5783	4	FVNQHLCGSHLVEALYLVCG ER
717.9751	3	FVNQHLC(pHMB)GSHLVEALY
783.3271	4	FVNQHLC(pHMB)GSHLVEALYLVC(pHMB)G ER
859.42	1	GFFYTPK
1044.0902	3	FVNQHLC(pHMB)GSHLVEALYLVC(pHMB)G ER
1076.4156	2	FVNQHLC(pHMB)GSHLVEALY
iodoacetamide derivatised		
651.0878	4	FVNQHLC(Ac)GSHLVEALYLVC(Ac)G ER *
859.4135	1	GFFYTPK
868.1029	3	FVNQHLC(Ac)GSHLVEALYLVC(Ac)G ER *

* (Ac) corresponds to the carbamylation of the cysteine residues caused by the treatment of the reduced protein with iodoacetamide

the reaction of the derivatisation agent with different proteins is quantitative and also independent of potential modifications of the protein such as phosphorylations or glycosylation. It could also be shown, that the presence of the label does not alter the possibilities neither for protein identification after tryptic digestion, nor for peptide identification by MS/MS analysis. Contrary, the unique isotopic pattern of Hg-containing peptides might help to specifically identify these peptides for sequence elucidation or for new quantification means, as to be presented in chapter 5.3. For quantification purposes as it is the objective of this work, the limits of detection could be reduced considerably in comparison to ICP-MS detection of other elements naturally present in proteins. However, calibration strategies based in external calibration might not be suitable for the analysis of real samples, as the potential pretreatment and complex separation strategies might compromise the accuracy of the results obtainable. Therefore, the use of isotope dilution analysis is preferable as a quantification strategy and its subsequent application in protein analysis is presented in the following.

5.2 Quantification of proteins using isotope dilution analysis

As pointed out in the introduction, isotope dilution analysis is considered to be an attractive method for absolute quantification of biopolymers such as proteins. In the following, its application for the quantification of proteins after labelling is demonstrated in combination with different types of sample separation techniques, such as chromatography and gel electrophoresis. As the moment of the addition of the spike is crucial for the accuracy attainable, the focus was set on species- (or label-) specific isotope dilution analysis, as the realisation of the isotope dilution step can be accomplished in an early stage of sample treatment. However, in order to conduct this type of analysis, the spike has to be available in the same chemical form as the analyte. This is one of the limiting factors for the broader applicability of quantification approaches based in species-specific IDA. In the case of labelled proteins or peptides, this means, that the labelling reagent pHMB has to be synthesised using enriched mercury isotopes, and that the products of its reaction with the target proteins must have the same structure, and they have to be obtainable under maintenance of the isotopic information of the spike. As a general remark to this part of the work it has to be mentioned that there is a lack of appropriate certified reference materials available on the market to ultimately validate new methods in the field of quantitative protein analysis. Therefore, all methodologies presented in the following are to be understood as a proof of concept for protein quantification using the proposed labelling approach.

5.2.1 Synthesis and characterisation of ^{199}Hg-pHMB

The synthesis of pHMB with enriched ^{199}Hg was performed with 13 mg of ^{199}HgO as described in chapter 4.5. 8.9 mg of the pure compound were obtained, corresponding to a yield of 37 %. ESI-MS in the negative mode was used to identify the desired compound (m/z theor. 353.95 for $[C_7H_5O_2Hg - H]^-$, found 353.97, data not shown). The isotopic pattern of the compound also corresponded exactly to the pattern expected from the enriched mercury used for the synthesis. The ESI-MS spectrum also showed, that a small amount of doubly substituted mercury was formed (m/z theor. $[C_{14}H_{10}O_4Hg - H]^-$ 440.8, found 439.98) although 4-carboxy-boronic acid was added in a less than stoichiometric amount. As the doubly substituted compound is hindered in its reactivity towards proteins, it should not disturb in the derivatisation reaction. After derivatisation, it is removed together with the remaining excess of the derivatisation agent by ultrafiltration and should not affect quantitative analysis. Therefore, its formation as a side-product is negligible. To prove the maintenance of the isotopic composition of the enriched

mercury, part of the supernatant liquid of the reaction mixture was diluted to an appropriate concentration, and all mercury isotopes were measured in ICP-MS to assure, that no contaminations with natural mercury occurred during the synthesis of the compound, that would alter the isotopic abundances of the spike. Additionally, ^{199}Hg-pHMB derivatised ovalbumin and insulin were analysed with the coupling of SEC (ovalbumin) and μLC (insulin) to ICP-MS to prove that the isotopic composition is also maintained after derivatisation. Due to the low concentration injected into the LC, the measured abundances are less accurate due to overestimation of the low abundant isotopes. The obtained data for the isotopic composition are shown in table 5.9.

Table 5.9: Abundances in % of the mercury isotopes found in ^{199}Hg-pHMB

isotope	**theoretical***	**found in pHMB**	**found in pHMB-ovalbumin**
196	0.092 ± 0.003	0.006 ± 0.001	0.015 ± 0.006
198	1.49 ± 0.008	1.55 ± 0.02	1.67 ± 0.01
199	91.97 ± 0.02	91.70 ± 0.03	90.6 ± 0.02
200	4.86 ± 0.02	4.91 ± 0.01	5.18 ± 0.02
201	0.68 ± 0.01	0.72 ± 0.01	0.92 ± 0.03
202	0.79 ± 0.01	0.90 ± 0.02	1.37 ± 0.04
204	0.115 ± 0.002	0.21 ± 0.05	0.25 ± 0.01

* Theoretical values were supplied by the local distributor

Figure 5.12 shows the chromatogram obtained for the separation of ^{199}Hg-pHMB derivatised insulin. As it can be seen, only one peak for the b-chain of insulin is found. Furthermore, the isotope ratio of ^{199}Hg to ^{200}Hg reveals the expected ratio for the spike (found 18.1 ± 0.05, expected 18.8, natural ratio 0.73). It can be concluded that derivatisation results in an equal product for both forms of pHMB.
A final characterisation was achieved by incubating human insulin with the synthesized pHMB and its comparative analysis with MALDI-TOF-MS. As it is the same compound, the same ions should occur, but with an isotopic pattern mainly determined by the isotopic composition of the Hg spike. Figure 5.13 shows the obtained mass spectrum for the derivatised insulin b-chain. All characteristic ions (two-fold labelled b chain, single labelled b chain and native b chain due to fragmentation processes) were found in the spectrum. Figure 5.14 shows the isotopic pattern found in the ion of the two-fold labelled b-chain (peak at m/z 4069 Da). For comparison, the ion cluster of the b chain labelled two-fold with ^{nat}pHMB was also included in the figure. It can be seen, that the molecular mass for the most abundant signal in the ion cluster of the two-fold labelled b chain and the single labelled b chain are shifted to lower mass in comparison to insulin labelled using pHMB with natural isotopic composition as a consequence of the altered isotopic distribution of the spike.

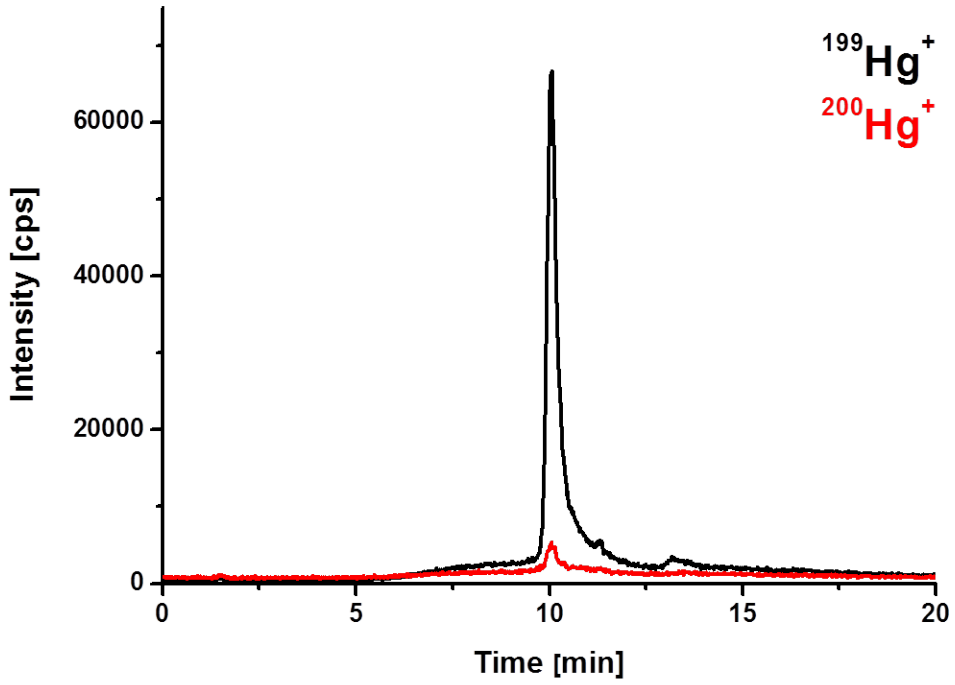


Figure 5.12: Chromatogram of the b-chain of human insulin derivatised with ^{199}Hg-pHMB

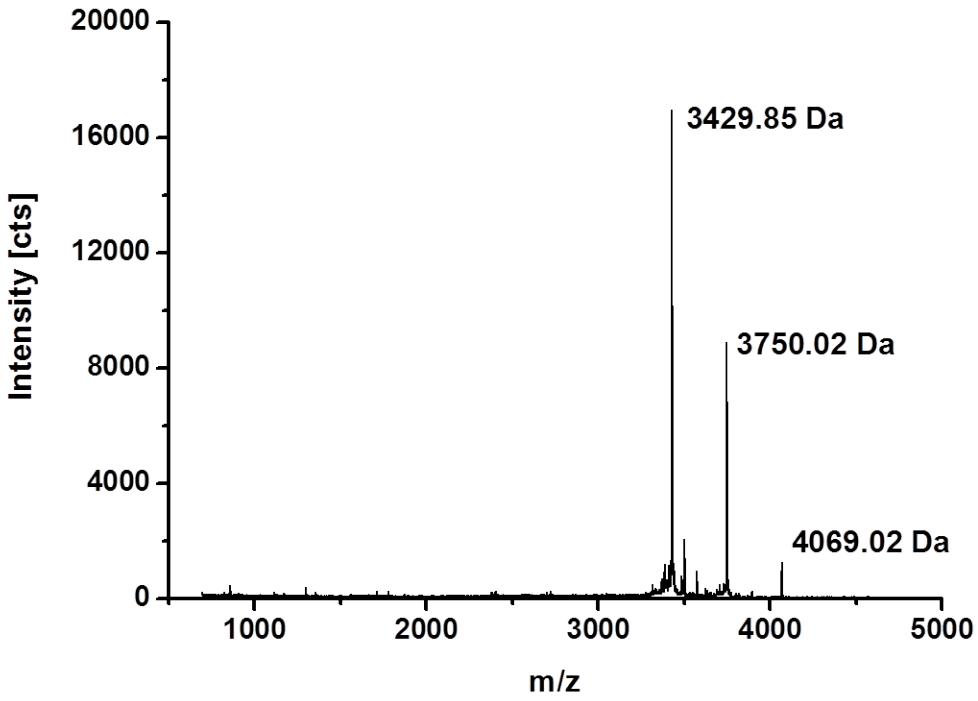


Figure 5.13: MALDI-MS spectrum of human insulin derivatised with ^{199}Hg-pHMB

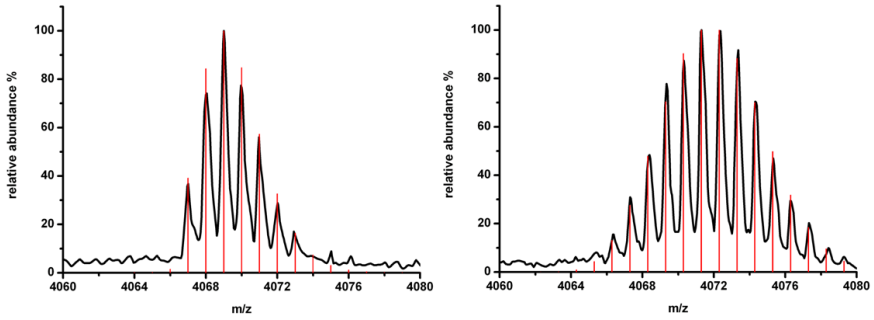


Figure 5.14: MALDI-MS spectrum of human insulin derivatised with ^{199}Hg-pHMB (left) and natural pHMB (right). Black lines reveal the measured spectra, red columns the theoretically predicted abundances

5.2.2 Protein quantification using HPLC-ICP-MS

Due to the potential complexity of protein samples, analytical methodologies based on HPLC-ICP-MS typically involve more dimensional separation protocols to effectively separate the target protein(s) from all other components. As a first dimension, only a rough fractionation is applied, as for example size-exclusion chromatography (as shown for example in [213]). Here, fractions can be collected that contain the sought protein, but probably also other components of the sample or different isoforms of the same protein. These fractions are then subsequently further separated using a second chromatographic separation, typically reversed-phase chromatography due to its high separation potential for proteins and peptides. This methodology however has certain drawbacks, as there are various sources for analyte loss and hence potential error sources for accurate quantification. First of all, incomplete column recoveries for both columns can be a major source of error and have to be determined, but also additional steps for preconcentration of the collected fractions by means of e. g. ultrafiltration can significantly contribute to the possible bias between the analytical result and the real concentration value (see fig. 3.16). As pointed out in chapter 3.6, isotope dilution analysis can in principle compensate for these error sources when conducted in the label-specific mode due to the early realisation of the isotope dilution step. As in the case of label-unspecific isotope dilution (post-column isotope dilution) the addition of the spike is realised only just before detection, the same effects as mentioned above become important and limit its potential for accurate quantification. In order to prove the potential of label-specific isotope dilution analysis for absolute protein quantification, ovalbumin was separated using a two-dimensional separation protocol using size-exclusion chromatography as a first dimension, followed by fraction collection and preconcentration using ultrafiltration (cut-off 10 kDa) and injection into reversed-phase μLC. Figure 5.15 gives an overview on the applied methodology.

After derivatisation of ovalbumin using ^{nat}pHMB, different mixtures were prepared

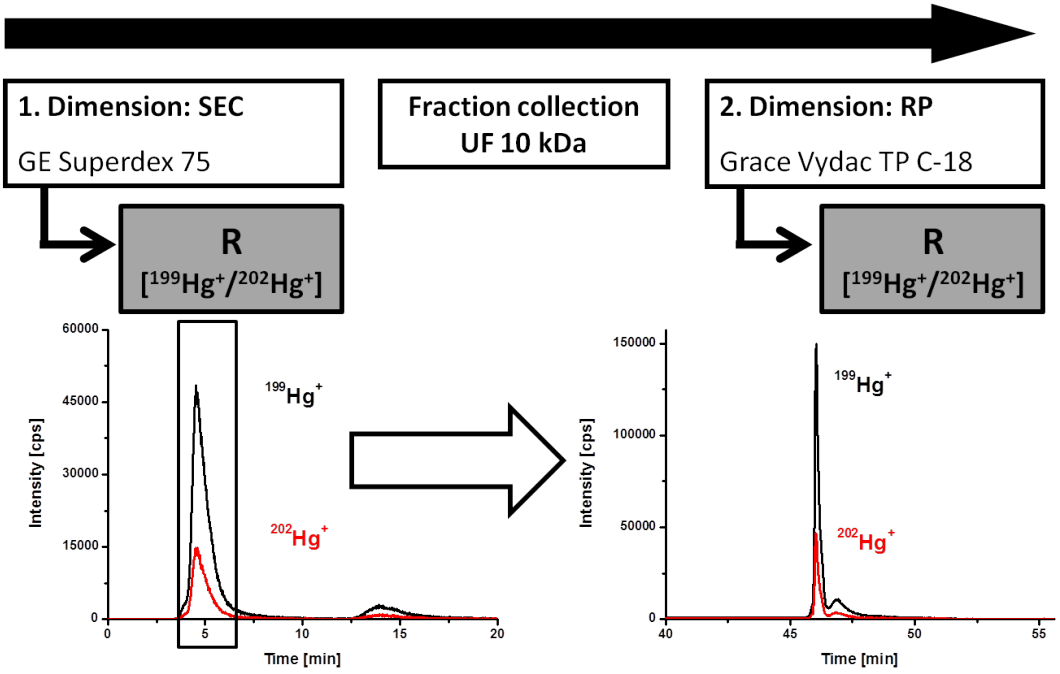


Figure 5.15: Workflow applied for a two-dimensional chromatographic separation

with ovalbumin independently derivatised using ^{199}pHMB as a label-specific spike. All mixtures were analyzed using SEC-ICP-MS in order to determine their initial isotope ratio. After fraction collection and preconcentration, the samples were submitted to μLC-ICP-MS to determine their isotope ratio after completion of all sample separation steps. After each injection in SEC, a blank was run in which 10 μL of 5 % 2-mercaptoethanol were injected to remove eventually remaining traces of Hg. As for label-specific isotope dilution the isotope ratio is the only measurand experimentally determined, it should have the same value in both chromatographic separations, if no contaminations have occurred during the previously realized sample separation steps. For the analytical result, maintenance of the initial isotope ratio corresponds to a recovery of 100%. The results obtained for the proposed methodology can be seen in table 5.10. Three individually prepared mixtures with different spike to sample ratios were analyzed. The results indicate that label-specific isotope dilution can overcome potential analyte loss during the sample separation protocol. Additionally, it becomes clear, that there is no apparent contamination with natural mercury observed during the entire separation, which might affect the measured isotope ratio and thus lead to an overestimation of the protein amount in the sample.

However, the use of more dimensional separation protocols based on chromatography is limited. Especially as a technique for sample fractionation, there are much more powerful techniques available for protein samples, such as polyacrylamide gel electrophoresis (PAGE). Due to its easy coupling to ICP-MS, methods based on chromatography only are still quite favoured in speciation studies, where the amount of individual components of interest is limited, and detection of a specific compound

Table 5.10: Results obtained using a two-dimensional chromatographic approach for the determination of labelled ovalbumin using label-specific isotope dilution analysis

Sample	ratio Spike/Sample	R ^{202}Hg/^{199}Hg * (SEC-ICP-MS)	R ^{202}Hg/^{199}Hg * (RP-μLC-ICP-MS)
1	1.01	0.3110 ± 0.004	0.3149 ± 0.006
2	3.03	0.1305 ± 0.003	0.1379 ± 0.005
3	0.34	0.6459 ± 0.003	0.6458 ± 0.005

* Three individual chromatographic runs were evaluated for the isotope ratio

can be reduced to detection via a heteroelement detectable with ICP-MS. As a consequence, the complexity of the resulting chromatograms is reduced. Identification of a compound can be done by comparison of the retention time to retention times previously determined with standards if available. For complex protein mixtures as for example blood derived samples, labelling will lead to a high number of species carrying the elemental label, and thus only partially resolved signals in chromatography. Also, proteins which are present in a higher concentration might lead to chromatographic peaks with increased width or extensive peak tailing, which can overlap with other signals in the chromatogram. Therefore, other methods for sample fractionation such as gel electrophoresis might be more suitable for the analysis of complex protein samples. Besides the high resolving power of denaturing SDS-PAGE, it also offers the advantage that each sample is located in a different lane of the gel, and that every gel is prepared freshly for analysis. This can help to reduce memory effects, which are especially critical in size-exclusion chromatography as partial adsorption of trace elements on the stationary phase normally occurs, although the separation mechanism is considered to be free of interaction between analytes and stationary phase.

5.2.3 Quantification of labelled ovalbumin after electrophoretic separation using LA-ICP-MS

Polyacrylamide gel electrophoresis (PAGE) is one of the benchmark techniques in biochemistry and molecular biology due to its simplicity and low costs, thus offering high potential for the separation of complex protein samples according to their isoelectric point or molecular mass. As pointed out in the first part of this work, staining of the gels using different dyes like coomassie blue or silver, are still widely applied means to visualize the separated proteins. However, the information they deliver is of only unspecific character and their sensitivity is limited. An appealing alternative for the detection and quantification of proteins is the use of elemental mass spectrometry. In recent years, its potential for protein quantification has

attracted a lot of attention. It cannot only give information about the spatial distribution of an element of interest within the gel, but also further reduce the limit of detection for a given protein, and therefore enables the sensitive detection of a protein within a complex matrix of other proteins. Besides heteroatoms like phosphorous and sulphur, metals were frequently detected and quantified in different types of proteins. Due to the multielement capacity of ICP-MS, even multiplexed analysis of different proteins in one run would be realisable, if the proteins under scrutiny contain different heteroatoms. Laser ablation (LA) coupled to ICP-MS is nowadays an established technique for the analysis of solid samples, and has also frequently been applied to the analysis of trace metal distribution in electrophoresis gels since its first introduction by McLeod and colleagues [214]. Since then, many examples for its application have been published [215]. Typical analytes of interest are for example metals like Cu and Zn [216], but also other trace metals, as for example Cd [217] and Pt [218] have been investigated using this technique. Also Se-containing proteins are of scientific interest, as there might be a relation between Se uptake and the development of cancer [219].

However, LA is prone to elemental fractionation and other matrix effects, so that accurate quantification still remains difficult [220]. Several approaches for quantification of proteins previously separated by PAGE have been published in recent years. Already the first publication on this technique suggested the use of element doped gels as standards for external calibration [214]. Although the authors achieved a good calibration precision of 6% RSD, this calibration approach does not take into account the possibility of inhomogeneous distribution of the protein within the gel, and therefore, may lead to greater uncertainty and inaccuracy of the method. Also the use of liquid standards has been suggested [221]. This however, besides the aforementioned problems of the heterogeneous distribution, the different characteristics of a nebulised solution and the laser generated aerosol in an ICP leads to another significant source of uncertainty. Another approach frequently used for LA is to use the ion signal of a matrix element as internal standard, in the case of PAGE $^{13}C^{+}$ [220]. However, the internal standard and the analyte have to enter the ICP in the same form, which might not always be given in the case of carbon and other analytes. Therefore, different results using C as internal standard have been observed [222]. On the other hand, isotope dilution analysis (IDA) was demonstrated to be a useful tool for accurate quantification with LA-ICPMS sampling [223]-[225]. Recently, the use of species-specific isotope dilution analysis was described by *Deitrich et al.* for the quantification of superoxide dismutase (SOD) using non-denaturing GE-LA-ICPMS [226]. As the isotopically enriched spike, in this case SOD enriched in ^{65}Cu and ^{68}Zn, was chemically identical, instrumental drifts, e.g. during the ablation process and the aerosol transport, could be compensated. However, their results indicate, that diffusion and loss of metals during separation might still be a major

issue, if non-covalently bound metals are to be investigated. In contrast to the weak binding between metals incorporated in coordination complexes in proteins (Fe, Cu, Zn etc.), metals attached covalently to proteins also remain attached to the protein under denaturing conditions. Therefore, the full separation potential of GE can be capitalized for labelled proteins, as also shown with iodinated proteins [173]. As an alternative, separated proteins can be blotted onto membranes to be incubated with labelled antibodies [159]. Also here, denaturing gel electrophoresis can be conducted without the risk of uncontrolled distribution of the element which is likely to be detected. For isotope dilution analysis, this methodology is not favourable, as the addition of the spike is accomplished in a late part of sample treatment. Also, the use of label-specific IDA is not possible, so that only the species-unspecific addition of the spike can be done (e. g. addition as nebulised solution to the laser generated aerosol), that is related with the same problems as mentioned above, as missing compensation for distribution effects or the occurrence of uncertainties due to aerosol transport phenomena. In order to explore the potential of mercury labelling in combination with label-specific isotope dilution analysis for the analysis of PAGE gels, a methodology for the sensitive detection of labelled ovalbumin was developed using ns-LA-ICP-MS. The laser ablation process was first optimized in order to achieve high sensitivity and spatial resolution of separated compounds. In order to explore the potential of label-specific IDA for accurate quantification, it was compared to external calibration using standard solutions of the labelled protein.

Optimization of the operation parameters

Preparation of the gels for laser ablation was done as described in chapter 4.2.3. First of all, a comparison of ns-LA to fs-LA was conducted in order to evaluate if one the two techniques is better suited for the desired application. For further method optimisation, a gel containing approximately 40 pmol of labelled ovalbumin (corresponding to 32 ng of Hg) in each of the total eight lanes was used. The obtained sensitivity in LA-ICPMS is dependent on the amount of ablated material containing the analyte of interest. Therefore, the most influential parameters such as laser repetition rate, spot size and scan velocity were carefully optimised. This optimisation aimed to find conditions that enable a complete in-depth ablation of the entire protein amount present in one line scan with a sufficient spatial resolution to separate signals coming from different spots together with short analysis time.

Evaluation of fs-LA for the ablation of proteins from PAGE gels

Ballihaut et al. [219] described recently, that the use of NIR fs-LA (wavelength 1030 nm) for the ablation of Se-containing proteins from a gel leads to an increased sensitivity when compared to 266 nm ns laser ablation system. They applied a special ablation strategy (moving the laser parallel to the protein band, whereas the

gel advanced perpendicular), that allows them to ablate a greater volume per unit time (about 27-fold). Surprisingly, the increase in sensitivity was about 40-fold in comparison to ns-LA ablation. They attributed this to a difference in the particle size formed by fs-LA, in detail that the particles generated are smaller and are hence transported to and evaporated/ionized in the ICP-MS more efficiently. In order to verify whether this is also the case for 193 nm ns-LA, a comparison to fs-LA with 265 nm and 800 nm was done. As both modes of the fs laser systems delivered different amounts of energy per laser shot (0.14 mJ for UV operation, 0.92 mJ for IR operation), adjustment of the fluence received by the gel was done by changing the spot sizes to 80 μm (UV-265 nm) and 180 μm (IR-800 nm). Afterwards, also the laser repetition rates had to be adjusted to 50 Hz for the UV mode and 40 Hz for the IR mode in order to achieve a similar in-depth ablation of about 85 % of the total protein amount in one single line scan. Finally, a gel containing different amounts of labelled ovalbumin (between 15 to 35 pmol) was analysed using all laser systems. The resulting slopes of the calibration graphs do not indicate a pronounced gain in sensitivity by the use of fs lasers. Contrary, taking into account the peak areas obtained after normalisation to $^{13}C^+$, fs-LA seems to be dramatically less sensitive than ns-LA. If absolute intensities (for example for one point of the calibration curve) are evaluated, it could be seen that in all cases a similar signal intensity is observed. The results of this comparison are summarized in table 5.11.

Table 5.11: Resulting slope of the linear calibration using different laser ablation systems after normalisation to $^{13}C^+$ and absolute countrate on $^{202}Hg^+$ obtained for one of the samples analysed for generation of the calibration

	ns-193 nm	**fs-265 nm**	**fs-800 nm**
Slope of calibration [fmol^{-1}]	0.1141 ± 0.0049	0.0908 ± 0.0082	0.0079 ± 0.0010
Absolute intensity [^{202}Hg$^+$, cts]	63,700 ± 5,700	68,500 ± 4,800	87,700 ± 16,600

Although care was taken that ablation took place in the center of the gel band, a slight deviation caused by different amounts of sampled Hg cannot be excluded. This could explain the slightly higher signal intensity in the case of fs-800 nm LA. As due to normalization not only the Hg-intensity, but also the amount of $^{13}C^+$ introduced into the plasma contributes to this value, this can also be an effect of the ablated amount of matrix. In order to estimate this amount, the traces on the surface of the gel caused by the laser were evaluated with an optical microscope (Olympus BX51). The width of the lane was measured using the microscope software tool, depth was estimated from the difference in focus position of the lane bottom and the gel surface (approximately 5 μm accuracy). The depth estimated for the different laser systems was 80 μm in the case of ns-193 nm, 180 μm in the case of fs-266 nm and 300 μm in

the case of fs-800 nm laser ablation. Figure 5.16 shows fotographs of the resulting craters on the surface of the gel together with their dimensions from the different laser systems. Additionally, also the beam profiles of the respective laser systems are shown in a simplified form.

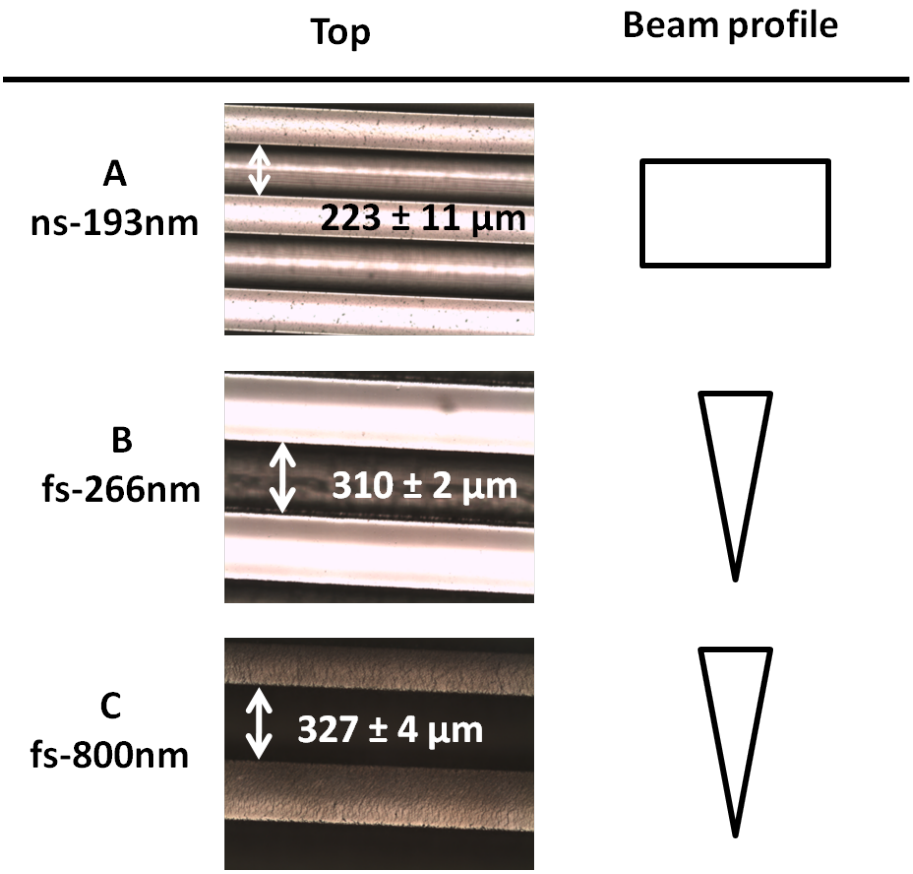


Figure 5.16: Resulting craters after ablation using UV-ns, UV-fs and IR-fs-LA together with their estimated dimensions

Both wavelengths of the fs-LA system have led to an increased impact on the gel surface, leading to a deeper penetration into the gel. The reason for this can be found in the beam profile of the fs-laser system, which is of a gaussian shape, whereas the ns laser has a homogeneous energy distribution, so that in all cases a similar amount of protein was ablated, although the fs laser had ablated a higher amount of the matrix. Additionally, the IR-fs laser lead to serious burn damage of the gel surface. As the absorption of the gel is not so effective for radiation in the IR-range than it is for the UV range, the transmitted energy caused this damage. As a consequence, the results obtained do not support the findings reported by *Ballihaut et al.* [219]. One reason for their findings is surely the fundamentally different ablation strategy leading to an increased amount of sampled material. As the element analysed in their study (selenium) is also prone to the so-called carbon enhancement effect (increased sensitivity of the detector response in ICP-MS, [130]), the augmented sensitivity they reported may be attributed to this effect caused by the increased introduction of organic matter to the plasma. Within this work, further experiments were therefore

conducted using ns-193 nm LA.

Repetition rate of the laser

As a first optimization step, the fraction of total mercury ablated in one line scan was determined using different laser repetition rates. A higher repetition rate provides a higher number of laser pulses that hit the gel per time unit, therefore ablating more material and penetrating deeper into the gel matrix. In order to achieve highest sensitivity, the depth penetration necessary for a complete ablation of the protein was determined. After each line scan with a given laser frequency (160 μm spot size, 50 μm s^{-1} scan velocity), the surface of the ablation crater was refocused and another line scan was acquired. This procedure was repeated until no further Hg signal was detectable above the instrumental background. After peak integration and normalization to the sum of all signals, the fraction of the respective ion signal for each line scan was determined. The obtained results are shown in figure 5.17. Besides improved sensitivity, the complete ablation of the protein is also desirable to reduce possible errors during calibration due to different amounts of ablated material in independent samples. Using a repetition rate of 5 Hz, four consecutive line scans were necessary to remove the majority of the Hg-containing protein. With a repetition rate of 10 Hz, the same amount was removed in two line scans, whereas consistently a repetition rate of 20 Hz was able to remove the same amount in one single line scan. For this experiment, also $^{29}Si^{+}$ was monitored as an indicator whether the laser beam had removed the entire gel matrix and thus started to ablate on the microscope slide. However, complete ablation of the gel was not observed. The maximum depth penetration necessary for complete removal of the protein from the gel was estimated using light microscopy to be 170 $\pm$ 5 μm, whereas the gel matrix had a thickness of 450 μm. It is also important to note that the highest concentration of the protein is found close to but not directly at the surface of the gel, which would be removed when using lower frequency or higher scan rates.

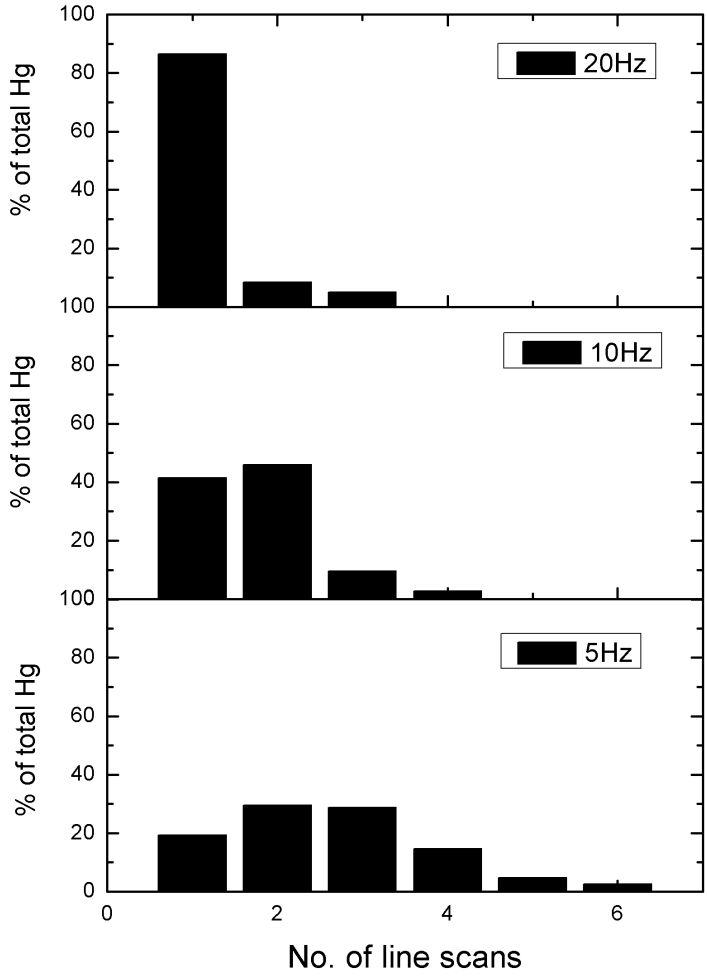


Figure 5.17: Comparison of Hg signal intensity distribution with different laser repetition rates. Difference in the observed peak area was below 5 % in all datasets evaluated (N = 3).

Spot size

The beam diameter used has a direct influence on the amount of ablated material and thus on the sensitivity of the method. Otherwise, a bigger laser spot does reduce the spatial resolution, so that a compromise between both influences has to be found. A series of measurements was carried out using different spot sizes to compare the achieved value of the ^{13}C-normalized integral of the ^{202}Hg-signal. The obtained results can be seen in figure 5.18.

It was found that a larger beam diameter leads to an increase in signal intensity with approximately quadratic dependency, so that a polynomial fit of 2^{nd} order was used to fit the data points, giving a good correlation to the experimental values ($R^2 = 0.973$). Figure 5.19 shows that the resolution of the protein bands in the transient signals was not improved with a smaller beam diameter, so the largest spot size available on the laser system was used throughout this work. It needs to

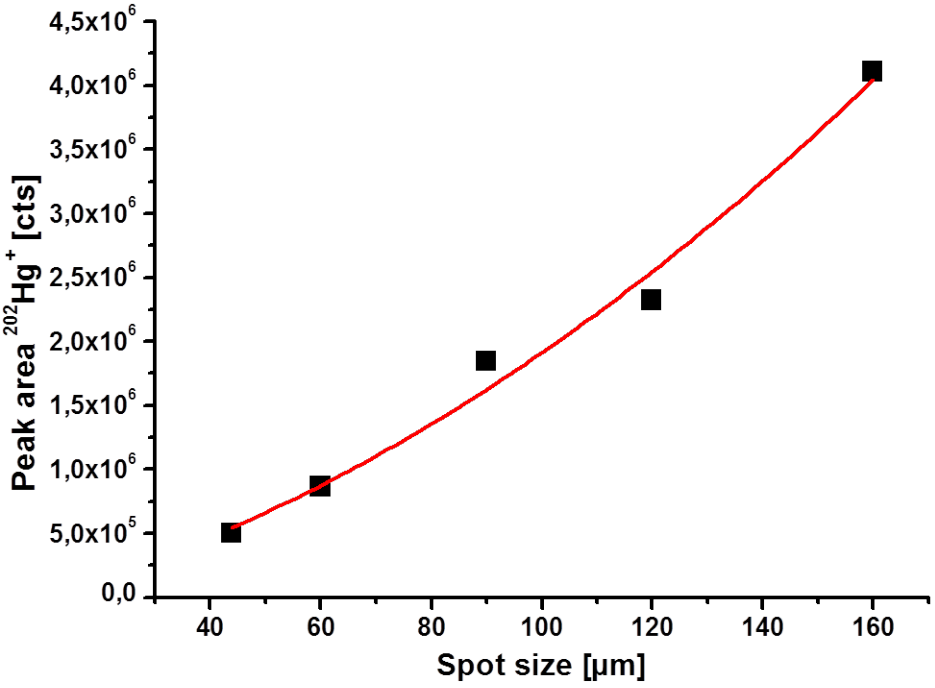


Figure 5.18: Comparison of the signal intensity of $^{202}Hg^{+}$ with different laser spot size

be noted, however, that the actual width of the scan was significantly wider than the beam diameter employed. Typical ablation lines showed a lane width of 225 ± 5 μm (with a nominal spot size of 160 μm), which is most likely due to additional thermal desorption of material during the ablation event.

Scan velocity

A decrease of scan velocity increases the number of laser pulses that will hit the sample while moving a distance unit, hence leading to a deeper penetration of the sample surface and higher sensitivity. Also, spatial resolution can be increased, as the time, which the laser needs to arrive at the next spot containing the analyte of interest is longer, and thus meanwhile a complete washout of the previous spot can be accomplished. However, decreased scan velocity does also increase the total time which is necessary to completely analyze a sample. Therefore, a reasonable compromise had to be found. Figure 5.20 shows the intensity of the obtained mercury signal using different scan velocities, but also the time in s needed to conduct a complete line scan. A more detailed explanation of the presence and origin of two signals will be given in chapter 5.2.3. Similar to the investigations regarding the spot size, no improvement of the resolution between the main signals was observed, so that it can be concluded, that the resolution is limited by the distribution of the protein in the gel, and not by the laser ablation process. Although a scan velocity of 25 μm s^{-1} would further increase the sensitivity of the method, 50 μm s^{-1} was

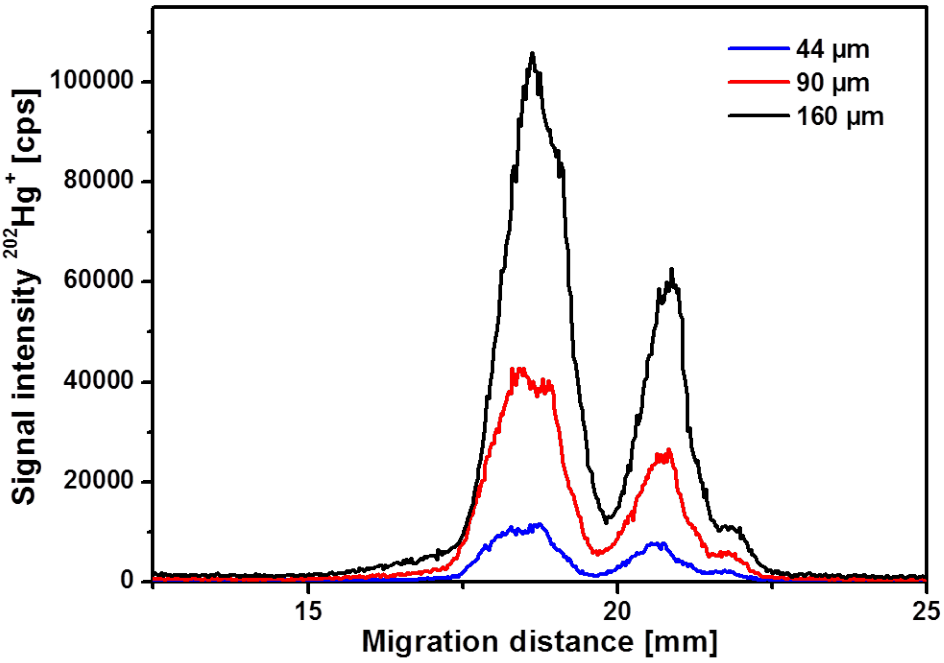


Figure 5.19: Transient signals obtained with different beam diameters

chosen for further measurements. Under these conditions, a typical analysis time of 750 s is required for the ablation of one line scan (3.6 cm length).

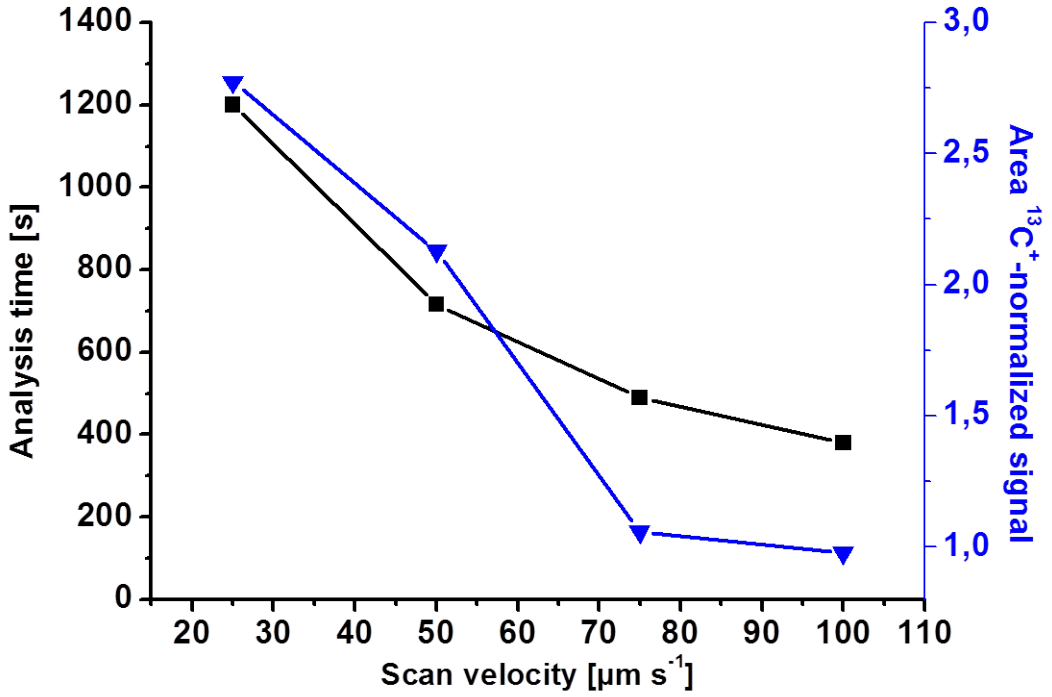


Figure 5.20: Dependence of the signal intensity and the analysis time necessary from the applied scan velocity. Typically observed RSD´s were below 5 % in all cases.

Transport mechanism of carbon and mercury

As pointed out before, different elements might follow different transport mechanisms to the ICP after being ablated by the laser, such as solid matter (particles with a certain size distribution), or gaseous species. The nature of the aerosol formed seems to be dependent on the element and of its chemical surrounding in the sample. In both cases, effects can occur that lead to differences in the response of the ICP-MS, as for example preferential ablation of volatile species, or signal loss due to the deposition of bigger particles during the way to the ICP torch [227]. Such phenomena can induce fractionation effects and hence limit the applicability of internal standardisation by the use of matrix elements such as $^{13}C^+$. In order to probe the transport mechanisms involved for the analyte (Hg) and the internal standard (C) in this study, a gas exchange device was placed between the outlet of the ablation cell and the torch. This device and its characteristics is thoroughly described in reference [228]. Briefly, the carrier gas and all gaseous components are exchanged to Ar by diffusion, whereas particles are not affected and pass through the device. In a first experiment, the extent of analyte loss caused by the gas exchange device was estimated. Therefore, NIST 610 was ablated using single hole drilling with a laser frequency of 10 Hz and 90 μm spot size with and without the device connected with a carrier gas (He) flow rate of 0.63 L min^{-1}. The flow rate was limited by the exchange capabilities of the unit. After the acquisition of 30 laser shots, the obtained signal intensities for the isotopes ^{7}Li, ^{27}Al, ^{140}Ce, ^{232}Th and ^{238}U were compared to each other. For the heavy elements, signal loss in the range of 25 % was observed, whereas ^{7}Li and ^{27}Al remained almost unaffected. Additionally, an unchanged ratio of $^{238}U^+/^{232}Th^+$ as an indicator for the plasma temperature, and a lower ratio of $^{248}ThO^+/^{232}Th^+$ as an indicator for oxide formation was observed. It can therefore be concluded that the use of the gas exchange device leads to a partial analyte loss due to particle deposition. After that, a gel containing $\approx$ 40 pmol of labelled ovalbumin was ablated under optimised conditions. Here, no signals for neither Hg nor C were detected using the gas exchange device, whereas the normal configuration gave similar signal intensities as typically observed. Therefore, it can be concluded, that both elements must be transported as gaseous species, and C could be used as internal standard in this case. In the case of C probably CO or CO_2, whereas Hg due to its high volatility might be transported as Hg(g). However, detailed investigations on the species were not conducted. Additionally, it has to be mentioned that this must not be necessarily for other metals related to proteins, as for example Cu, Zn or Fe.

Investigation of possible side effects of sample treatment prior to LA

In order to estimate whether the treatment of the gel (e. g. staining with coomassie blue for protein visualisation) prior to analysis had any effect due to loss of mercury or unspecific contamination due to staining solutions, two gels were loaded with the same sample (ca. 40 pmol of ovalbumin derivatised with ^{199}pHMB) and electrophoresis was carried out as usual. Afterwards, one gel was treated as usual (exchange of water with glycerine and drying), whereas the other gel was stained using coomassie blue before drying. If any of the above mentioned effects should play a role for the determination of Hg-labelled gels, changes in the relative signal intensity and the isotope ratio should be observed. In contrast to the study of *Raab et al.* [205], a loss of mercury during the staining procedure was not observed, as ablation of protein spots from both gels gave similar ($^{13}C^+$ normalized) signal intensity. This is due to the highly covalent binding of mercury in contrast to metals, which can be naturally incorporated into proteins. The stability of the bioconjugate is not affected neither by using denaturing separation conditions nor by coomassie staining. Also, as ^{199}pHMB was used for derivatisation, the isotope ratio indicates, that there is no contamination with natural mercury during the staining process, as reported by other authors [229]. The isotope ratio measured in protein spots from both gels gave values corresponding to the isotope ratio of the pure spike (expected ratio $^{202}Hg^+/^{199}Hg^+ = 0.022$, measured 0.02238 ± 0.0028 in the stained gel, 0.02353 ± 0.0044 for the unstained gel, four individual line scans were evaluated [2]. However, only coomassie blue was tested as a staining reagent, so that this does not necessarily have to be valid in case of silver staining.

Protein distribution in the gel

A typical line scan acquired under optimized conditions (20 Hz repetition rate, 160 μm spot size, 50 μm s^{-1} scan velocity) is shown in figure 5.21. The distribution of the elements shown can give different information. Carbon as a matrix element indicates the beginning and the end of the ablation process. Ideally, its signal intensity should remain constant throughout the entire distance of the scan. The peak in the sulphur trace indicates the end of the electrophoresis, as the increase of the signal is due to the sulphur-containing compounds (e. g. bromophenolblue [3]) in the buffer front. Unfortunately, the sulphur detection was not sensitive enough to also respond to the sulphur content present in ovalbumin. Finally, there are two well-resolved signals visible in the $^{202}Hg^+$ trace. Although only ovalbumin was presumed to be present in the sample, two distinct peaks are clearly separated and can be observed in both, the

[2] The given values for the isotope ratio show slight deviations from the values given in table 5.9 on page 98. Due to the indicative character of this experiment, a thorough compensation of mass bias was omitted, which explains the deviation.

[3] Bromophenolbue = 3´,3´´,5´,5´´-Tetrabromophenolsulfonephthalein

ICP-MS based data and also the coomassie blue stained gel. As both peaks were also observed in untreated ovalbumin after electrophoresis (data not shown), the second signal corresponds probably to impurities or degradation products present in the ovalbumin standard. Analysis of the protein standard with MALDI-MS indicated the presence of a second protein species with a molecular weight of approximately 40 kDa, which fits well to the molecular weight of the second peak, when considering the respective migration distances. A tryptic digest of the protein sample followed by MALDI-MS analysis of the generated peptides did not indicate the presence of other proteins as ovalbumin, so that the unknown species is probably a degradation product of ovalbumin.

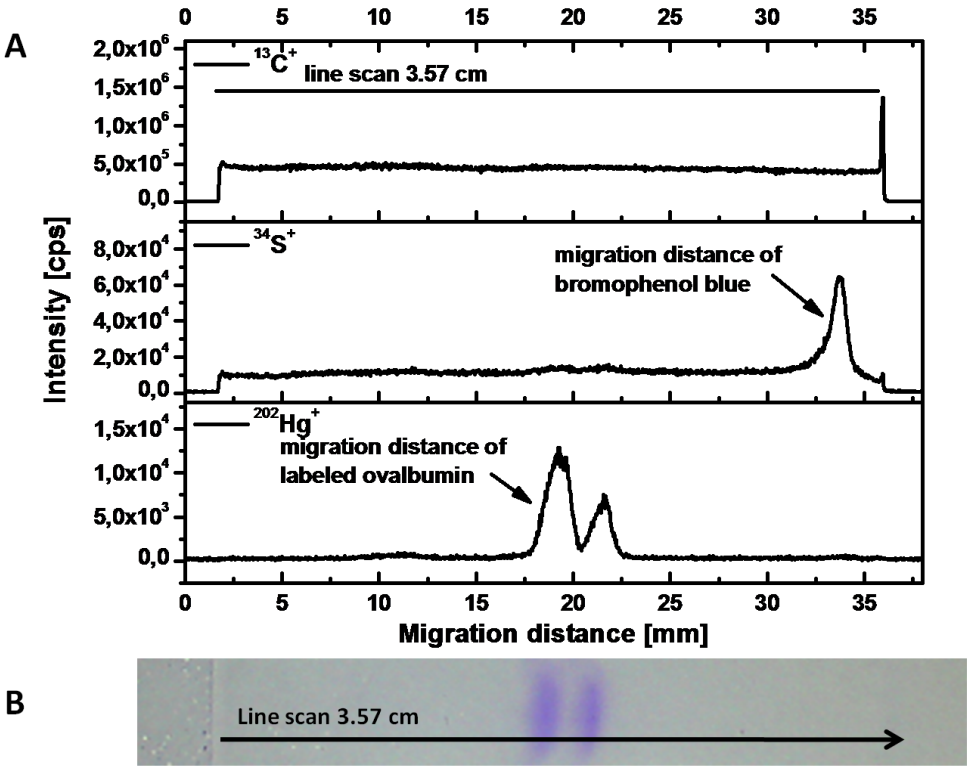


Figure 5.21: Typical electropherogram obtained with a line scan under optimized conditions (20 Hz repetition rate, 160 μm spot size, 50 μm s^{-1} scan velocity); **A**: Data derived from LA-ICP-MS; **B**: stained gel for visual comparison

External calibration as quantification strategy

Using the optimized parameters for laser ablation, a calibration using different amounts of derivatised ovalbumin was carried out. For each lane five independent line scans were acquired and the mean value of the integrated signal was used for calibration. As can be seen in figure 5.22 a, the correlation coefficient is rather poor when only the signal intensity of mercury is used for calibration. This is due to the strong matrix dependency of the laser ablation process. After normalization of the signal to the signal intensity of $^{13}C^+$ as a matrix element (figure 5.22 b), correlation

is improved. However, the relative standard deviation for individual line scans of the same lane is high.

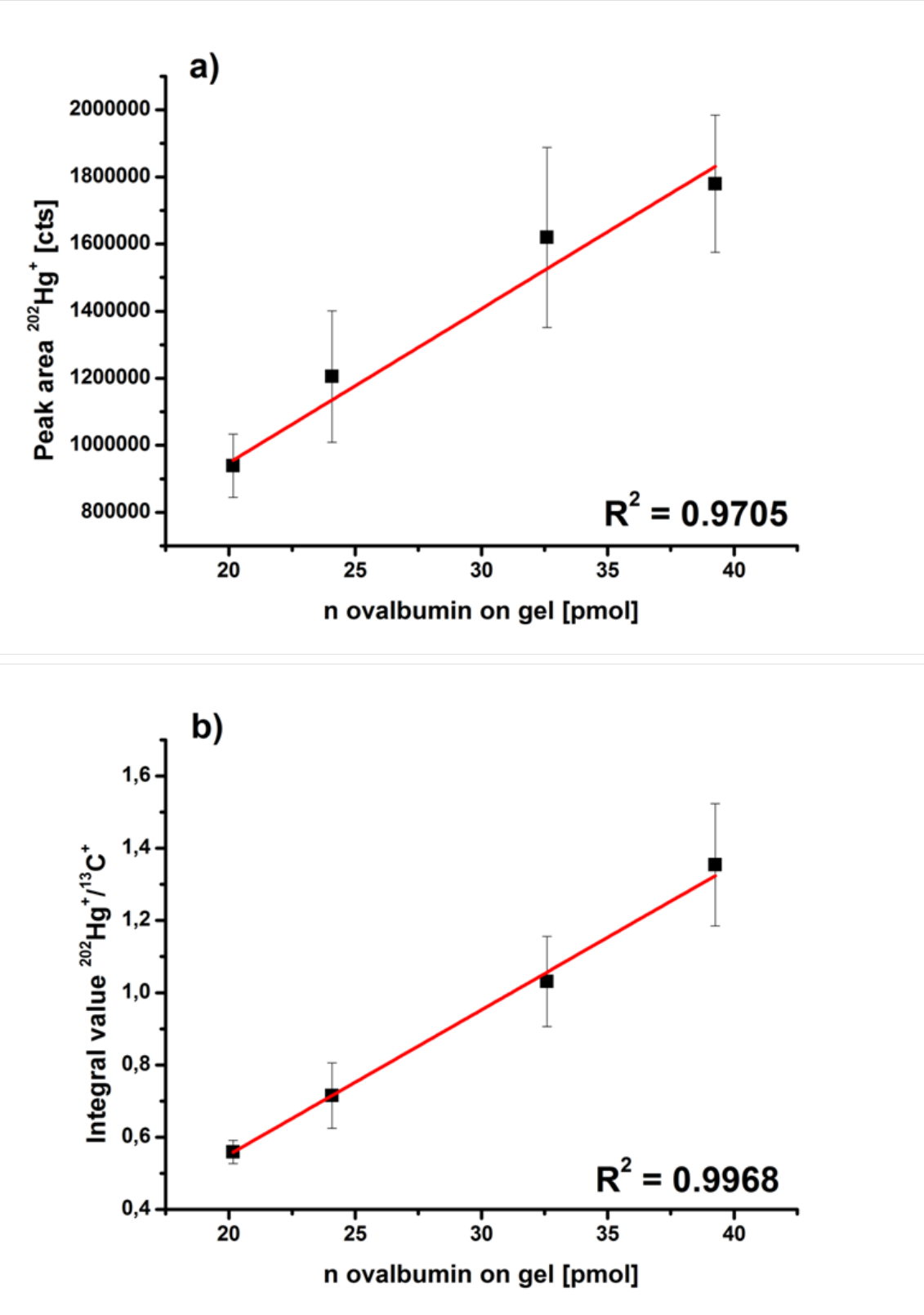


Figure 5.22: Linear calibration using $^{202}Hg^{+}$ signal intensity (a) and $^{13}C^{+}$-normalized signal intensity (b)

One reason for this may be the distribution of the protein within the lane. Appli-

cation of the sample was carried out using combs that transfer a certain amount of the liquid sample from the reservoir to the gel. These combs are then pierced into the surface of the gel. Due to the surface tension of the drop, an inhomogeneous distribution of the protein over the width of one lane can be expected. In order to confirm this explanation, a line scan was made in an angle of 90° relative to the band. The result is shown in figure 5.23.

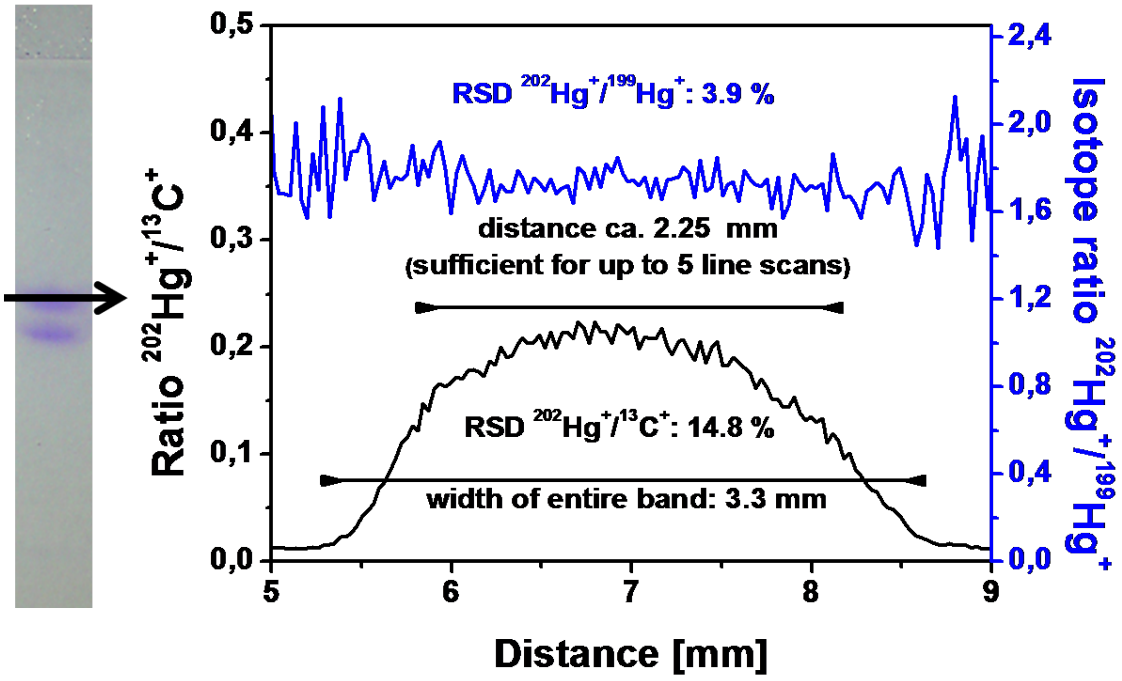


Figure 5.23: Line scan in an angle of 90° relative to the protein band, $^{13}C^+$ normalized signal intensity and isotope ratio $^{202}Hg/^{199}Hg$

Taking into account a width of 2,250 μm for the protein spot, which corresponds to the area needed to conduct up to 5 line scans, the concentration of the protein across the band varies by 15% (see figure 5.23), which corresponds well with the standard deviation observed during the line scans, which was in the range of 5-20%. However, the isotope ratio $^{202}Hg^+/^{199}Hg^+$ is independent of the absolute signal intensity, and it remains practically constant over the entire width of the lane (RSD below 4 %). Therefore, isotope dilution analysis results should be more accurate and precise, as the high standard deviation of different line scans should not be observed.

Analytical figures of merit

Using the above described parameters, the figures of merit of the method were determined. Limit of detection was calculated to be 8 ng of mercury according to the 3 σ criterion, corresponding to 160 fmol of ovalbumin. For external calibration, repeatability was between 5 and 20% in case of peak area, and between 1 and 3 % in case of retention time/migration distance. A comparison to other studies (see

Table 5.12: Comparison of the instrumental conditions and obtained detection limits to other studies

Protein	Element	Laser conditions	Reference	LOD of the sought protein
SOD	Cu, Zn	ns-266nm (3.5 J cm^{-2} 20 Hz, 250μm spot)	[226]	16 pmol
β-casein *	P	ns-213nm (55% 20 Hz, 120μm	[230]	16 pmol
α-casein	P	ns-266nm (0.7 mJ 8 Hz, 100 μm)	[231]	5 pmol
BSA *	I	ns-266 (3 mJ 15 Hz, 500 μm)	[173]	150 fmol
CYP1A1 *	Eu	ns-266 (3 mJ 15 Hz, 500 μm)	[158]	140 fmol
GSHPx **	Se	fs-1030nm (13 J cm^{-2} 40 μm)	[219]	low fmol levels
Mre 11 ***	Au	ns-213nm (4.2 J cm^{-2} 10 Hz, 100 μm)	[232]	0.2 amol
Ovalbumin	Hg	ns-193nm 14.2 J cm^{-2} 20 Hz, 160 μm	this study	160 fmol

***** Proteins were first blotted onto a PVC membrane and then ablated

****** LOD for fs-LA most intense band, a 2 mm wide lane was ablated

******* Protein was incubated with gold-labelled antibody and blotted onto membranes. One antibody contained roughly 53.000 gold atoms

table 5.18) is difficult, as besides instrumental parameters like spot size also the number of metal atoms attached to the investigated protein is of importance for the detection limit. Superoxide dismutase (SOD, [226]) does only contain one atom of Cu and Zn, resp., whereas ovalbumin investigated in this study does contain 4 mercury atoms after labelling. Similarly, this detection limit cannot be generalized, as other proteins may contain a different number of mercury atoms. In comparison to liquid analysis using μLC-ICP-MS, the achieved detection limit by laser ablation was about 100 times higher. However, an increase of sensitivity is still possible, for example by using bigger spot size in order to ablate more material. Using 160 μm spot size, only a fraction of the exploitable width of the protein band (estimated in the range of 2 mm) was ablated, to that there would be at least a factor of 10 to 20 possible, if the entire width of a protein containing band is ablated.

Application of label-specific isotope dilution analysis as quantification strategy

As pointed out before, isotope dilution analysis can help to compensate for matrix effects during the ablation and the distribution of the protein within the lane, and thus can also help to achieve more accurate results when complex separation protocols are necessary. In order to prove its applicability, different quantities of naturally derivatised ovalbumin were quantified using ^{199}pHMB-derivatised ovalbumin. Also different ratios of spike to sample were analyzed. The concentration of the spiked protein was accurately determined before the analysis using inverse isotope dilution analysis (see section 4.3).
The samples were then subjected to gel electrophoresis. Each sample was analyzed in duplicate, with one empty lane between two samples, in order to avoid potential cross contamination by diffusion, as described elsewhere [226]. The empty lanes were filled with sample buffer in order to assure isotropic conditions during separation. The gels were prepared and ablated as described in section 4.2.3. The isotope ratio was determined by integration of both signals typically present. For statistical reasons, four independent line scans were evaluated for each lane. The obtained results can be seen in table 5.13.

Table 5.13: Results for the quantification of labelled ovalbumin using label-specific isotope dilution analysis as a quantification strategy

No.	R [^{202}Hg/^{199}Hg]	R [^{202}Hg/^{199}Hg] after GE	n exp. [pmol]	n found [pmol]	recovery [%]
1	0.2858 ± 0.0083	0.2818 ± 0.0088	3.04	3.03 ± 0.17	99.6 ± 5.6
		0.2815 ± 0.0166		3.03 ± 0.21	99.8 ± 8.9
2	0.3761 ± 0.010	0.3780 ± 0.0243	3.24	3.32 ± 0.17	102.7 ± 5.2
		0.3770 ± 0.0153		3.31 ± 0.22	102.3 ± 6.6
3	0.2819 ± 0.0070	0.2767 ± 0.0097	10.06	9.72 ± 0.66	96.7 ± 6.8
		0.2771 ± 0.0018		9.74 ± 0.62	96.9 ± 6.4
4	0.2173 ± 0.0063	0.2165 ± 0.0052	13.02	13.25 ± 1.05	101.8 ± 7.9
		0.2154 ± 0.0042		13.17 ± 0.68	101.1 ± 5.2
5	0.5642 ± 0.0186	0.5610 ± 0.0093	21.47	20.65 ± 1.25	96.5 ± 6.1
		0.5545 ± 0.0073		20.29 ± 1.19	94.5 ± 5.9

As it can be seen, the determined amounts of ovalbumin correspond very well to the expected values. The analysis was quantitative with accuracy between 95 and 103 %, whereas the precision was between 5 and 9 %. However, the precision obtained here takes into account not only the isotope ratio measurement, but also possible errors occurring during sample preparation. Therefore, it is slightly higher than the values obtained for the RSD of the isotope ratio measurements (about 4 %). In comparison to external calibration the use of isotope dilution analysis was not only superior in terms of precision, but less prone to instrumental drifts or matrix effects. The limit

of detection (160 fmol for ovalbumin) were identical to those obtained using the external calibration. A graphical representation of the determined quantities of the samples versus the expected quantities shows linear correlation, as shown in figure 5.24. Also, the spike-to-sample ratio does not have any pronounced effect on the recovery.

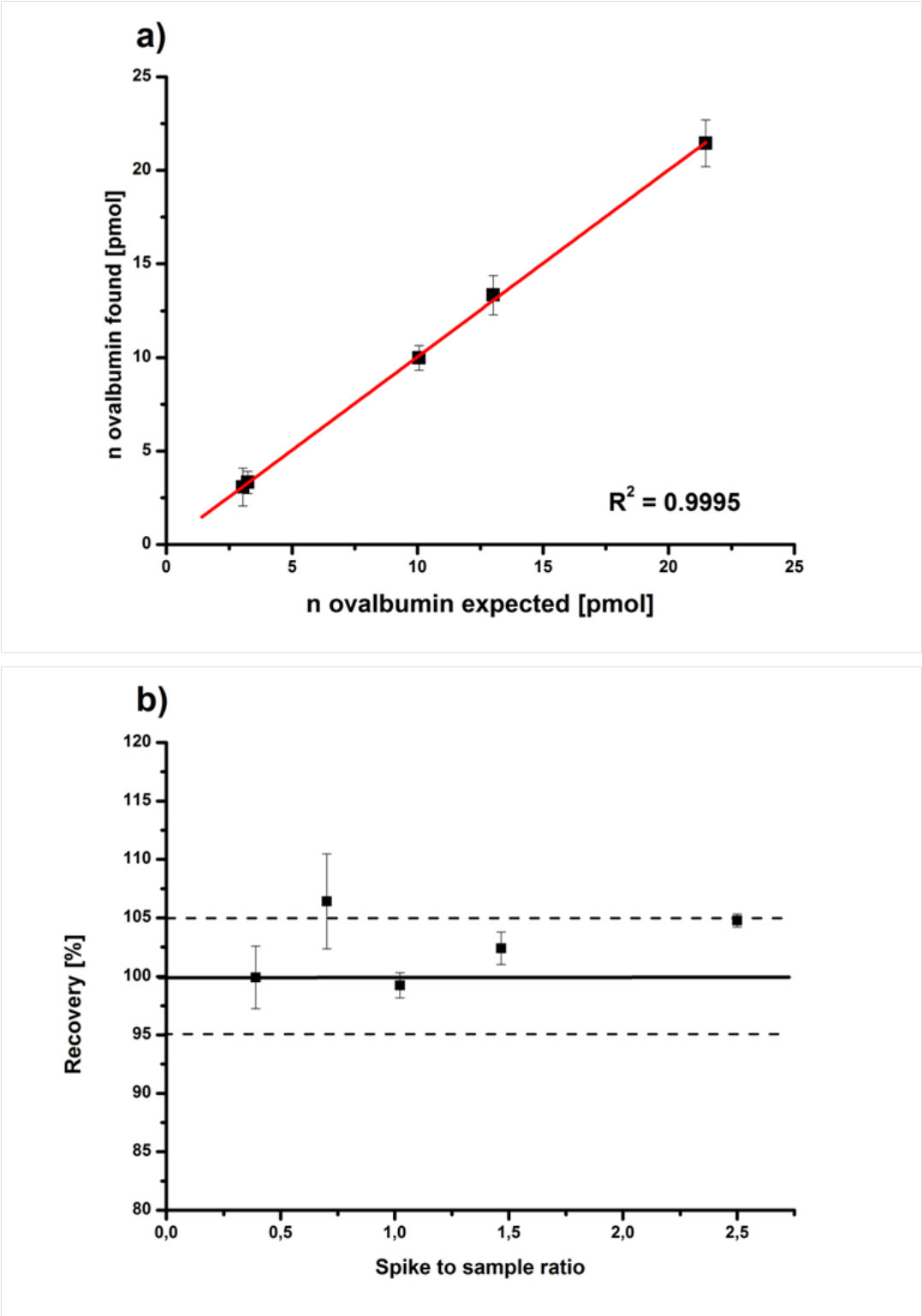


Figure 5.24: Results obtained for the quantification of pHMB derivatised ovalbumin using label-specific IDA, a: Amount of ovalbumin experimentally determined vs. expected amount; b: Dependence of the recovery from the isotope ratio in the mixture.

From the results obtained with both quantification approaches, it can be concluded that the use of label-specific isotope dilution analysis can improve precision and accuracy of the results. The main reason for this is that once the isotope dilution step is accomplished, the analytical result becomes independent of the amount of analyte which is finally sampled. In the case of GE-LA-ICP-MS, this can correct for the inhomogeneous distribution of the protein spot in the gel, which is of special importance when two dimensional gels are to be studied. Here, the small size of the spots will impede uniform distribution in any direction; therefore external calibration might be probably prone to errors. However, the application of the direct protein quantification from gels is probably not feasible when real samples are investigated. In this case, more sophisticated separation protocols are mandatory which may require additional sample fractionation, e.g. 1D gel electrophoresis followed by tryptic protein digestion and peptide analysis, or two-dimensional GE. Due to the high stability of the labelled protein, the isotope dilution step can already be accomplished in an early point of sample preparation, which can help to improve accuracy of the quantitative data obtainable throughout the entire analytical process.

5.2.4 Absolute protein quantification after tryptic digestion and peptide analysis

As mentioned above, the complexity of e. g. plasma samples will overburden the resolution potential of one dimensional gel electrophoresis, so that for a complete separation at least a second separation dimension is necessary. If for example GE-LA-ICP-MS is applied for direct quantification of proteins from such a sample, the results could be seriously biased due to the possible presence of other components in a sampled spot or contributions from other spots by diffusion. In proteomics, sample separation is commonly followed by in-gel tryptic digestion in order to generate peptides corresponding to the proteins present in the spot. The main advantage of this procedure is, that peptides as smaller molecules can be separated more efficiently, and the precursor protein can be identified via its set of peptides using database search. All labelling approaches presented in chapter 3.3 follow this proceeding. As a consequence, the specifity of the detected signal for the targeted analyte is further increased by the introduction of additional separation dimension. From the analytical point of view, there are certain drawbacks which have to be kept in mind:

- The protein spot must be excised precisely in order to obtain the entire protein amount. On the other hand, the quantity of gel, which does not contain protein should be kept as low as possible.
- The amount of protein which is digested should be quantitative (or at least as high as possible). This amount is hard to determine and at least not to be considered repeatable, as it depends on various factors such as enzyme activity.

- All peptides should be quantitatively extracted from the gel (or at least to a high degree).

All of these preconditions can be overcome by the use of label-specific isotope dilution, as similar for the determination of entire proteins using GE-LA-ICP-MS, the amount of analyte actually detected in the sample only affects sensitivity, but not the result of the analysis. Therefore, the applicability of labelling with pHMB and the use of label-specific isotope dilution analysis should be elucidated in the following in combination with a multidimensional workflow typically applied in proteomics. Figure 5.25 shows a scheme of the workflow applied.

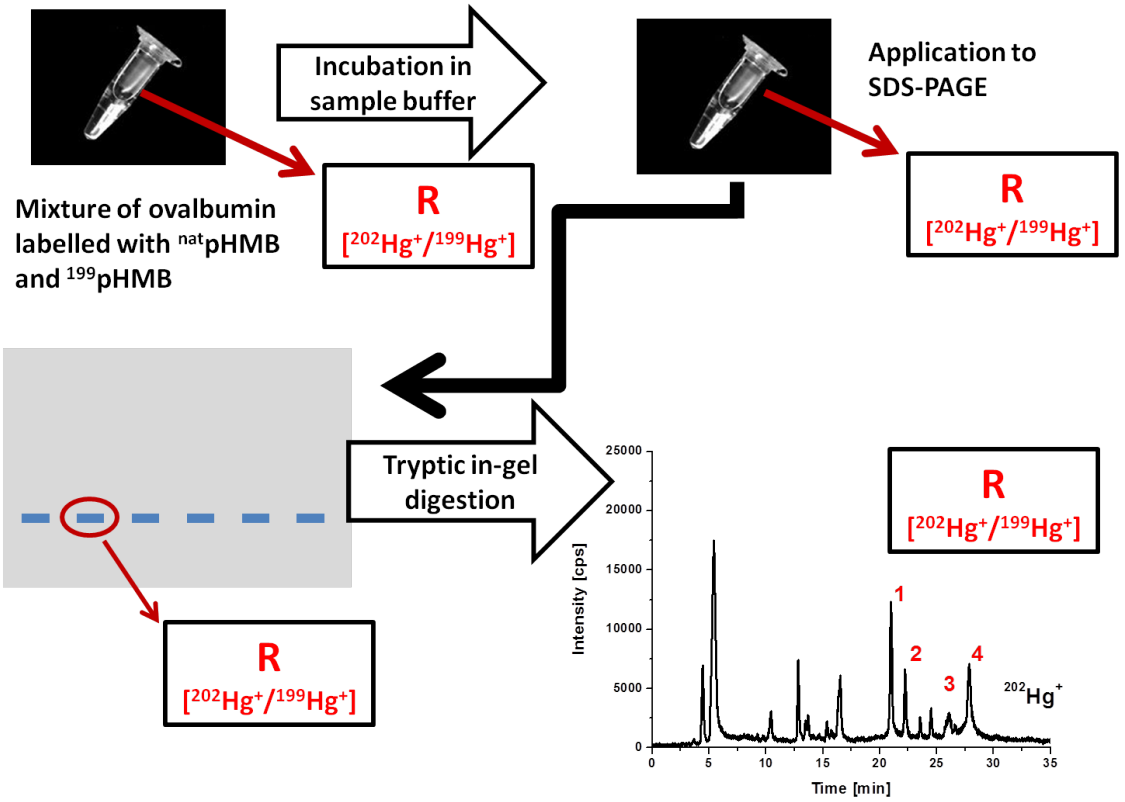


Figure 5.25: Workflow applied for the analysis of proteins using gel electrophoresis followed by tryptic digestion and peptide analysis using μLC-ICP-MS

For method development, a mixture of ovalbumin labelled with ^{nat}pHMB and ^{199}pHMB was prepared, which was then incubated in sample buffer and subjected to gel electrophoresis. After staining of the gel, the protein band corresponding to ovalbumin was excised and digested using trypsin for the generation of peptides and also using HNO_3 for determination of the isotope ratio $^{202}Hg^+/^{199}Hg^+$ using ICP-MS. The tryptic digest was then injected into the μLC-ICP-MS coupling for the separation of Hg-containing peptides. The isotope ratio is determined in all steps of sample treatment in order to identify possible sources of contamination, and thus potential sources of error. Preliminary results obtained for the quantification of ^{nat}pHMB ovalbumin using ^{199}pHMB ovalbumin gave in principle satisfactory results. For analysis,

a mixture of equal amounts of both components was prepared. Table 5.14 shows the determined isotope ratios for the individual steps during sample treatment together with a virtually calculated recovery.

Table 5.14: Results obtained for the quantification of ^{nat}pHMB-ovalbumin using ^{199}pHMB-ovalbumin following the multidimensional workflow as shown in figure 5.25

Step in sample treatment	**R ^{202}Hg/^{199}Hg**	**Recovery * [%]**
1 Untreated sample	0.3151 ± 0.0038	100
2 Incubated in sample buffer	0.3201 ± 0.0058	102 ± 2 %
3 Excised spot	0.3281 ± 0.0073	105 ± 2 %
4 Peak in μLC separation		
Peak Nr. 1	0.3427 ± 0.0030	111 ± 1 %
Peak Nr. 2	0.3545 ± 0.0079	116 ± 2 %
Peak Nr. 3	0.3437 ± 0.0135	112 ± 4 %
Peak Nr. 4	0.2972 ± 0.0310	93 ± 10 %

* For calculation of the recovery an arbitrarily chosen concentration for the spike was used (C_{Sp} in equation 3.4 on page 52, 10 nmol g^{-1}) and used for calculation in the isotope dilution equation. The resulting concentration (C_S) of the untreated sample was set as 100 % recovery and used as a reference value. Three individual injections were evaluated.

From the obtained results it can be concluded that in principle absolute quantification of proteins using the proposed multidimensional separation approach combined with label-specific isotope dilution analysis is feasible. However, a slight tendency for overestimation of the quantity of the analyte is clearly visible. As mentioned before, also potential contaminations of the element of interest contribute to the isotope ratio and hence to the analytical result, so that extreme care has to be taken to avoid all kinds of contamination. One possible source of this type of contamination could be identified as the bromophenolblue used as a dye in the sample buffer. After a strong increase of the ^{202}Hg signal in comparison to the signal of ^{199}Hg was observed between step 1 and 2 of the sample separation protocol in an earlier analysed gel, the amount of bromophenolblue in the sample buffer was reduced to 0.05 %, which significantly improved the results. Although the binding between pHMB and cysteine residues should be stable, it cannot be excluded, that the effect of contamination might be caused by more reactive Hg species [4] such as Hg^{2+} which were unintentionally introduced into the sample. These species might react with cysteine residues which have become additionally available due to the digestion of the protein, or insert in disulfide bridges [175]. However, analysis of a tryptic digest of a gel piece where no ovalbumin was found and posterior incubation with pHMB

[4] Reactivity in this case refers to the reactivity of the compound towards sulphur containing species.

indicated that peaks 1, 2 and 4 are artefacts owed to cysteine containing peptides from trypsin. As a small part of the label is lost during tryptic digestion (as shown in chapter 5.1.4), this quantity can afterwards derivatise the peptides formed by autoprotolysis of the enzyme. It has to be stated here, that the observed extensive autoprotolysis was only observed with a certain batch of the trypsin provided by Sigma Aldrich. This was also proven by comparison of MALDI-MS analysis of tryptic digests of ovalbumin using trypsin from another (earlier delivered) batch, where no peptides owed to self-digest were observable.

Quantification of ovalbumin spiked in human serum

As already mentioned in the introduction, there is only a very small number of certified reference materials available to validate analytical methods for protein quantification. In the case of the model proteins used in this study, ovalbumin and insulin, no such material is available, so that no real validation of the methods presented is possible. On the other hand, one protein for which a certified reference material is available (CRM-393, certified for apolipoprotein A1, human) does not contain cysteine residues, so that it is not analyzable with the proposed method. However, as a proof of concept for the applicability of labelling with pHMB in combination with label-specific isotope dilution analysis for real samples, human serum was spiked with ovalbumin and then derivatised. Due to the complexity of the matrix human serum, the combination of gel electrophoresis and in-gel tryptic digestion followed by peptide analysis using μLC-ICP-MS was applied. First, human serum was diluted 1:10 with 50 mmol L^{-1} NH_4HCO_3 in order to reduce the concentration of albumin and immunoglobulin proteins. Afterwards, underivatised ovalbumin was added to an aliquot of the diluted serum, so that its final concentration was in the range of 25 - 43 nmol g^{-1}. This spiked serum was derivatised using ^{nat}pHMB as described in chapter 5.1. After reconstitution of the original volume after ultrafiltration (cut-off 10 kDa) with 50 mmol L^{-1} NH_4HCO_3, different aliquots of the derivatised serum solution were mixed with ovalbumin independently derivatised with ^{199}pHMB, representing the label-specific spike and thus realisation of the isotope dilution step. The solutions were incubated in sample buffer and subjected to gel electrophoresis. After the electrophoretic separation was finished, the gel was first stained to then excise the separated protein spots of the immunoglobulins (IgG), albumin and ovalbumin and subsequently digest them as described in chapter 4.4.2. The reconstituted digest was injected into the μLC-ICP-MS system, and isotope ratios were evaluated via peak integration.

A photograph of the gel can be seen in figure 5.26. As demonstrated in the figure, the spiked ovalbumin was successfully separated from all other abundant protein fractions. Besides ovalbumin, also the spots corresponding to albumin, transferrin and the IgG proteins could be separated. Instead of a molecular weight marker, an

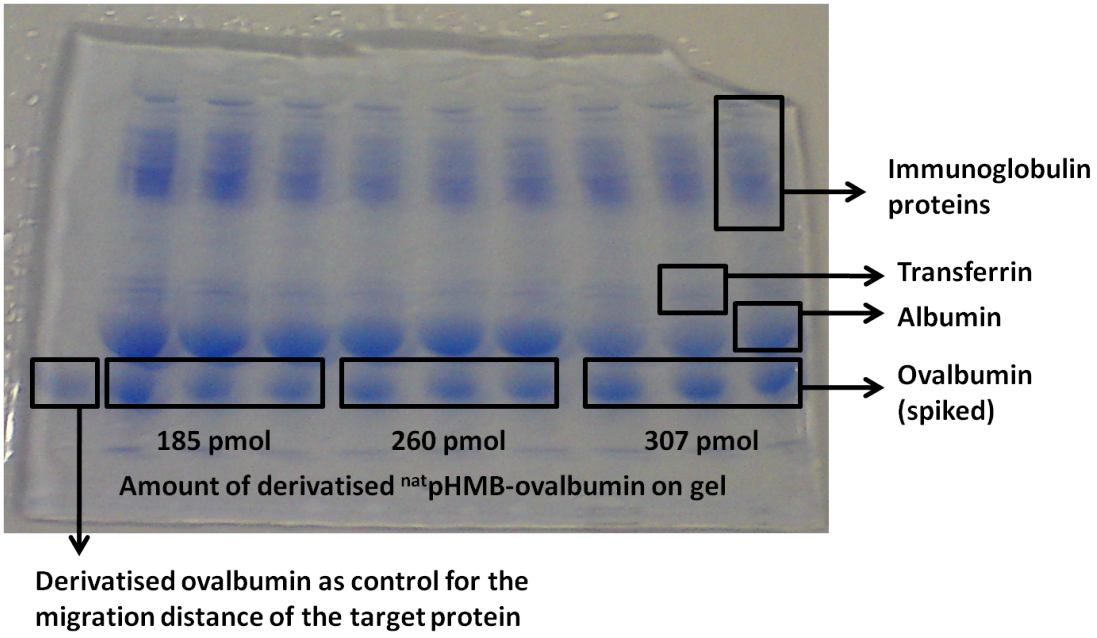


Figure 5.26: Photograph of a gel containing derivatised serum samples together with previously spiked ovalbumin. The most important protein spots are indicated.

aliquot of derivatised ovalbumin was applied to one of the lanes in order to control the migration distance of the target protein. Previous electrophoretic separation of unspiked serum indicated that there are no further protein spots visible in the zone to which ovalbumin had migrated under the conditions applied for electrophoresis. However, the absence of visible spots does not necessarily indicate the absence of other (labelled) proteins.

The size of the squares in figure 5.26 highlighting the different protein fractions corresponds to the size of the excised gel pieces for the respective protein fraction.

The evaluation of the isotope ratio $^{202}Hg/^{199}Hg$ for the spot corresponding to the immunoglobulin proteins and the albumin fraction gave a value of 0.9499 ± 0.0156 and 0.9229 ± 0.0265 resp.. Both isotope ratios do not correspond to the isotope ratio of natural mercury ($R_{th.}$ $^{202}Hg/^{199}Hg = 1.7700$), as could be expected from the plasma sample derivatised with ^{nat}pHMB, but neither correspond to the isotope ratios expected for the different mixtures. In the spot corresponding to ovalbumin, a similar isotope ratio was found. A possible explanation for this finding could be the high amount of other proteins in the sample, which might lead to hindered migration of a small amount of derivatised ovalbumin when co-migrating with more abundant proteins such as albumin and IgG. Therefore, a small contribution of ^{199}Hg is found in each of the spots. Partial loss of the label during electrophoresis can be excluded as a potential reason, as this should have been previously observed in the laser ablation experiments. Here, no elevated background levels of mercury in areas of the gel, where no protein was found have not been observed. Additionally, ICP-MS measurement of such spots in gels containing only derivatised ovalbumin did neither reveal the presence of Hg. The high contribution of ^{nat}Hg in the spot

corresponding to ovalbumin can be explained by the high concentration of unbound labelling reagent in the plasma samples after ultrafiltration. In order to completely derivatise all proteins in the sample, a high amount of the derivatisation reagent had to be added to achieve the necessary 10-fold excess of reagent in all cases (approximately 300 nmol of the reagent were added). Therefore, a complete separation of the labelling reagent during the electrophoresis cannot be guaranteed. Estimation of the relative amount of the spike present in this spots using isotope pattern deconvolution (see chapter 5.3 for further details on the proceedings) revealed a relative amount of 13.9 ± 0.4 % of ^{199}Hg in the spot of the IgG and 14.9 ± 0.8 % in the case of albumin. In both cases however, the absolute signal intensity, and hence the absolute of Hg present in the spots is rather poor if compared to the signal intensity in the spot of the target protein ovalbumin, although the colour of the spots indicates, that at least similar amounts of protein should be present. This is further illustrated in table 5.15. Calculations of the respective protein amount for albumin was done using the value for its concentration (55 mg mL^{-1}) published in reference [2].

Table 5.15: Estimated amount of substance for the different protein spots together with their respectively observed signal intensity on $^{202}Hg^{+}$ after digestion in nitric acid

	Ovalbumin	**Albumin**	**IgG**
Estimated amount [μg per lane]	16.9 *	18.2	**
Signal intensity [$^{202}Hg^{+}$, cps]	≈ 190,000	≈ 26,000	≈ 5,600

* Corresponding to the spot containing 185 pmol of derivatised ovalbumin and including the amount of label-specific spike previously added.
** For the IgG-proteins the theoretical concentration was not calculated as due to the huge number of proteins which can be present in this fraction no accurate reference values were found.

The results underline the inherent selectivity of pHMB as a labelling agent when used under non-reductive conditions in sample preparation. Although there are other proteins present in the sample in at least similar concentration, they are labelled to a significantly lower extent. In the case of e. g. albumin, only one cysteine residue is available for derivatisation under the applied conditions. Also for the IgG proteins, high numbers of free cysteine residues are not expected. This also reduces the impact of the amount of ^{199}Hg found in other protein spots, as although e.g. the spot corresponding to the IgG proteins contains about 14 % of ^{199}Hg, its overall amount with respect to the target protein ovalbumin can be estimated to less than 3% without taking into account the potential distribution of the enriched Hg within the ovalbumin spot. Finally, the digested ovalbumin was injected into the μLC-ICP-MS system in order to separate all Hg containing peptides and to

determine their isotope ratio. Previously to HPLC analysis, MALDI-MS spectra for every sample were acquired in order to confirm the presence of ovalbumin in the spot. The spectra did not indicate the presence of any other protein besides the target protein. One of the resulting chromatograms is shown in figure 5.27.

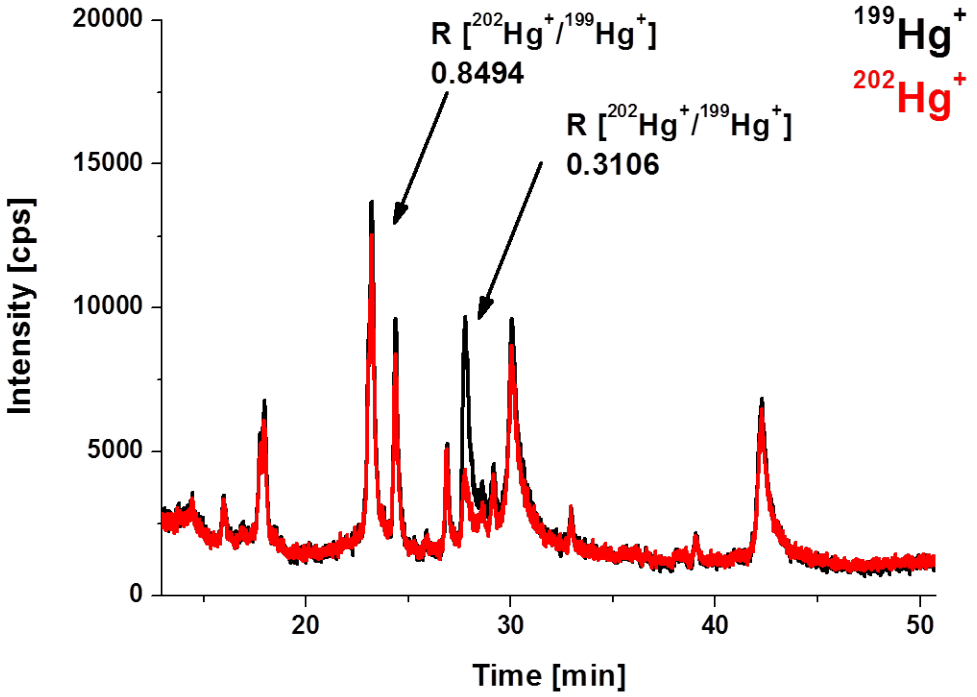


Figure 5.27: μLC-ICP-MS chromatogram of a tryptic digest of the ovalbumin containing spot

As it can be seen in the figure, that whereas all signals appearing in the chromatogram show a more or less similar isotope ratio corresponding to the isotope ratio previously measured in the protein spot digested with nitric acid (indicated in the figure for the signal with retention time of approximately 23 minutes), the signal with a retention time between 27.5 to 28.5 minutes shows a distinctly different isotope ratio of 0.3106. This isotope ratio indicates the presence of a considerable amount of the enriched Hg coming from the ^{199}pHMB-ovalbumin. The same signal was previously identified to come from a tryptic peptide of ovalbumin, whereas the other signals could be possibly attributed to peptides formed due to self-digest of trypsin, which have been unintentionally derivatised with pHMB liberated during the digestion procedure. As already shown in chapter 5.1.4, tryptic digestion of ovalbumin under the applied conditions only lead to a small number of peptides, so that out of the (at least) four potentially Hg-containing peptides only one was successfully generated and liberated from the gel matrix. Bigger fragments and underivatised protein residues have been precipitated as a consequence of the acid content in the staining solution and cannot be released from the gel afterwards. Contrary, the excess of labelling reagent eventually also present in the spots is also precipitated

Table 5.16: Results obtained for the quantification of ovalbumin previously spiked into human serum after two-dimensional separation using gel electrophoresis and tryptic digestion/chromatographic separation

Sample	**Concentration Ovalbumin [pmol on gel]**	**Recovery [%]**
1	185	86.2 ± 5.3
2	260	76.2 ± 11.4
3	307	77.5 ± 10.5

by the low pH value in the staining process, but can be resolvated during the digestion process in 25 mmol L^{-1} NH_4HCO_3, so that it can react with newly appearing sulphur containing molecules. Subsequent evaluation of all chromatograms (each digestion was injected three times) for the three different quantities of ovalbumin previously spiked into the serum sample gave the results shown in table 5.16. Overall, reproducibility of the retention time of the evaluated signal between all samples was better than 2 %. In order to obtain a measure for the potential of the method, the recovery of the amount of ovalbumin spiked into the human plasma samples was calculated. Although probably a considerable quantity of the analyte will be lost during the sample separation procedure (gel electrophoresis and tryptic digestion), the early moment of the addition of the spike can help to significantly compensate these losses.

As it can be seen in the table, the results obtained indicate incomplete recovery of the ovalbumin spiked into the human serum (between 76-86 %). This might be caused by the presence of the matrix in case of derivatisation in diluted human serum. Especially during the ultrafiltration step in sample preparation, this can lead to elevated analyte losses in comparison to the preparation of samples containing only ovalbumin as a standard protein. These losses however are not compensated by label-specific isotope dilution analysis, as the addition of the spike occurs after this step. Furthermore, other authors reported increased degradation of a protein originating from a different organism spiked into biological human samples ([200], in their study bovine SOD was spiked to haemolyzed red blood cells). However, taking into account the complexity of the applied workflow and the probably high amount of analyte which has been lost during gel electrophoresis and tryptic digestion, the potential of label-specific isotope dilution strategy is clearly demonstrated. These losses occurring due to the above mentioned restrictions caused by the enzymatic digestion step exclude other quantification strategies such as external calibration or internal standardisation. On the other hand, the sensitivity of the proposed method is hampered this way as well, as the amount of substance finally detectable diminishes, although it does not compromise the analytical result. One of the crucial

points for this methodology is clearly evaluation of the peak areas from the chromatograms, as due to the low absolute signal intensities especially for $^{202}Hg^{+}$ strong deviations might occur, as already visible in the relatively high standard deviation for the individual samples. Additionally, the contribution of other Hg-containing species to the evaluated isotope ratio cannot be excluded, as the use of ICP-MS as a detection system leads to the loss of all structural information. Therefore, it would be desirable to be able to detect the species of interest using molecular mass spectrometry in order to exclude influence of other co-eluting components.

5.2.5 Conclusions from this chapter

The results obtained in this part of the thesis clearly point out the superiority of label-specific isotope dilution analysis in comparison to other calibration strategies. Due to the high chemical stability of the bioconjugates, even sample preparation protocols involving various steps with potential "not quantitative" character can be tackled in combination with isotope dilution analysis to compensate for all analyte loss occurring before the final detection.

The use of the proposed methodology for LA-ICP-MS might be restricted to less complicated samples or targeted approaches, as for the analysis of real samples in a proteomic study, more sophisticated separation protocols are mandatory which may require additional sample fractionation, e.g. 1D gel electrophoresis followed by enzymatic digestion. Although also direct quantification from the gel is of potential interest, the presence of other components also carrying the label can potentially interfere in the results. However, for well defined samples with largely known composition, as for example control and quantification of the expression of a target protein in bacteria or yeast, this methodology can be interesting. Alternatively, as some proteins are also used as active substances in the pharmaceutical industry, this procedure can also be used as a first step of quality control of their synthesis, e. g. quantification of main and potential side products.

One the other hand, the proposed labelling approach in combination with label-specific isotope dilution analysis was applied for the quantification of a protein spiked into human serum. Although there are still certain drawbacks to be overcome in the proposed methodology, the first application of such a labelling approach to the analysis of real samples was proven.

5.3 Application of isotope pattern deconvolution (IPD) for the quantification of labelled proteins

As pointed out in the previous chapter, peak purity is one of the prerequisites for accurate quantification of species using label-specific IDA with ICP-MS detection after chromatographic (or electrophoretic) separation, as other compounds which carry the element of interest alter the analytical result due to their contribution to the isotope ratio. As all structural information about the analyte is destroyed during ionisation in an inductively coupled plasma, differentiation between different compounds is not possible using this ionisation technique. As shown in the previous chapter, not only peptides that originally belong to the components of the sample, but also unintentionally formed peptides can lead to this undesired contribution. However, the signal obtained from a peptide modified with pHMB in mass spectrometry contains all necessary information about the stoichiometry of the adduct and isotopic composition, so that in principle also quantification should be feasible using molecular mass spectrometry, if not only the isotope ratio between two distinct isotopes is used, but the entire ion cluster. In this chapter, the applicability of molecular mass spectrometry (MALDI-MS) for peptide quantification should be elucidated using isotope pattern deconvolution (IPD) as a tool for generating information about the relative contribution of the sample and the spike in an ion cluster. As a proof of concept, insulin b chain was used as a model peptide due to its relatively small size, making interpretation of mass spectra more easily.

5.3.1 Introduction to IPD

Although quantification using isotope dilution analysis using ICP-MS is very well recognized as an accurate method for quantification, its application in routine analysis is (with some exceptions) not practised commonly. Besides the above mentioned drawback of contributions of other components, also other reasons may lead to preferential use of other MS techniques, such as GC-EI/CI-MS or LC-ESI-MS. Unfortunately, determination of isotope ratios from typical molecular ion clusters of a given compound is not straightforward, as a signal at a certain m/z value may also contain contributions from other isotopes of the same element, but also contributions from other polyisotopic elements, in the case of peptides for example carbon, nitrogen, oxygen or sulphur. However, in recent years many examples for the application of IPD as a means for compound quantification using molecular MS techniques have been published, for example for the quantification of butyltin compounds in environmental samples ([233], [234]), polybrominated diphenylethers [235] or the determination of alkylated mercury compounds [236]. But also for the use in combination with ICP-MS and speciation analysis, this methodology can open

new possibilities, as for example correction for spectral interferences ([237], [238]) or internal mass bias correction [239]. There are also examples for its application in biological questions, as for example for the determination of clinical iron status in blood [197] or to differentiate between endogenous and exogenous Se in rat urine [114]. Although already known in mass spectrometry [240], isotope pattern deconvolution was adapted to the mass spectrometric analysis of proteins and peptides in order to differentiate between different substance classes in MALDI-MS spectra [241]. Using an algorithm to deconvolute isotopic abundances in the mass spectra, the authors were able to significantly reduce the number of signals not corresponding to peptides (noise level of the spectrum) and to increase the signal-to-noise level of the signal corresponding to the monoisotopic peptide. Short after, the fundamental concept and applicability of IPD was comprehensively explained and applied to the analysis of dimethyl selenide using GC-MS [237]. The authors described two ways of generating contribution factors from the experimentally determined ion cluster, in the intensity based domain and in the mass based domain. Whereas the intensity based domain approach is already feasible at relatively low resolution, the mass based domain takes into account the shift of the mass centroid and its width of the compound caused by the presence of an isobaric interference, and thus requires high resolution instrumentation. As MALDI-MS was chosen for the experiments, only the intensity based approach was investigated in the following due to the relatively high mass of about $\approx$ 4070 Da of labelled insulin b-chain in MALDI-MS and hence reduced mass spectrometric resolution. However, also ESI-MS and ESI-MS/MS techniques are suitable for this procedure, which will further increase the selectivity of the detected signal for the analyte which is the target for quantification.
A complete description of the theoretical background for the calculations done in the following can be found in reference [242]. In case that equal compounds with different isotopic composition contribute to an ion cluster, its resulting shape follows the superimposition of the isotope distribution of all individual components. In other words, the abundance (relative amount of the signal intensity compared to the sum of all signals) measured for each signal which forms part of the cluster can be expressed as:

$$A_j = \sum_{i=0}^{n} x_i C_{ij} \tag{5.1}$$

with:

A_j **:** Abundance of signal at m/z j

x_i **:** molar contribution factor of the compound i

C_{ij} **:** abundance of m/z j in the pure compound i

For an ion cluster of n individual signals at different m/z values, which results from i equal components with different isotopic abundances, a matrix results with n rows and p columns:

$$\begin{bmatrix} A_1 \\ A_2 \\ \vdots \\ A_n \end{bmatrix} = x_1 \cdot \begin{bmatrix} A_{1,1} \\ A_{1,2} \\ \vdots \\ A_{1,n} \end{bmatrix} + x_2 \cdot \begin{bmatrix} A_{2,1} \\ A_{2,2} \\ \vdots \\ A_{2,n} \end{bmatrix} + \ldots + x_i \cdot \begin{bmatrix} A_{i,1} \\ A_{i,2} \\ \vdots \\ A_{i,n} \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ \vdots \\ e_n \end{bmatrix} \tag{5.2}$$

The elements e_1 to e_n corresponds to an error vector, which can be introduced in the calculation if there are more parameters (nominal masses) than unknowns (molar contribution factors, number of contributing components). Systematic application of multiple linear regression of abundances of the underlying components can give their molar contribution coefficients to the experimentally determined abundances of the ion cluster (corresponding to A_1 to A_n). Therefore, the relative contribution of the individual components can be estimated. This mathematical operation can be conducted using a spreadsheet calculation program such as Microsoft Excel. Calculation of the equation array in equation 5.2 will give an exact solution if the number of equations corresponding to the number of individual signals evaluated) is higher than the number of unknowns (number of compounds contributing to the ion cluster). Depending on the ion source used, also different contributions of changes in the ion cluster have to be included into the calculation, as for example due to the formation of $[M\text{-}H]^+$ ions in EI-MS [242]. However, their presence can be estimated calculating their contribution to the measured spectrum by computing the respective equation array. If experimentally determined abundances are used for calculation, the effect of this type of spectral overlap can be reduced.

5.3.2 Relative protein quantification using IPD

The general concept for relative quantification studies involves principally the comparative analysis of two different and independent samples (e.g., cell tissues, body fluids) with respect to the abundance of one or more proteins. One of the widely known approaches for relative protein quantification after chemical addition of a label is the ICAT approach [91]. A similar workflow was developed for the use in combination with elemental labelling with pHMB. Two aliquots of human insulin as model analyte were derivatised as described in chapter 4.3. Both samples were treated equally with regard to reduction, incubation conditions (similar amount of derivatisation reagent added in both forms, ^{nat}pHMB and ^{199}pHMB) and purification using ultrafiltration. After purification, different mixtures of sample and spike were prepared (realisation of the isotope dilution step) and analyzed by MALDI-MS. A scheme of the proposed workflow is shown in figure 5.28.

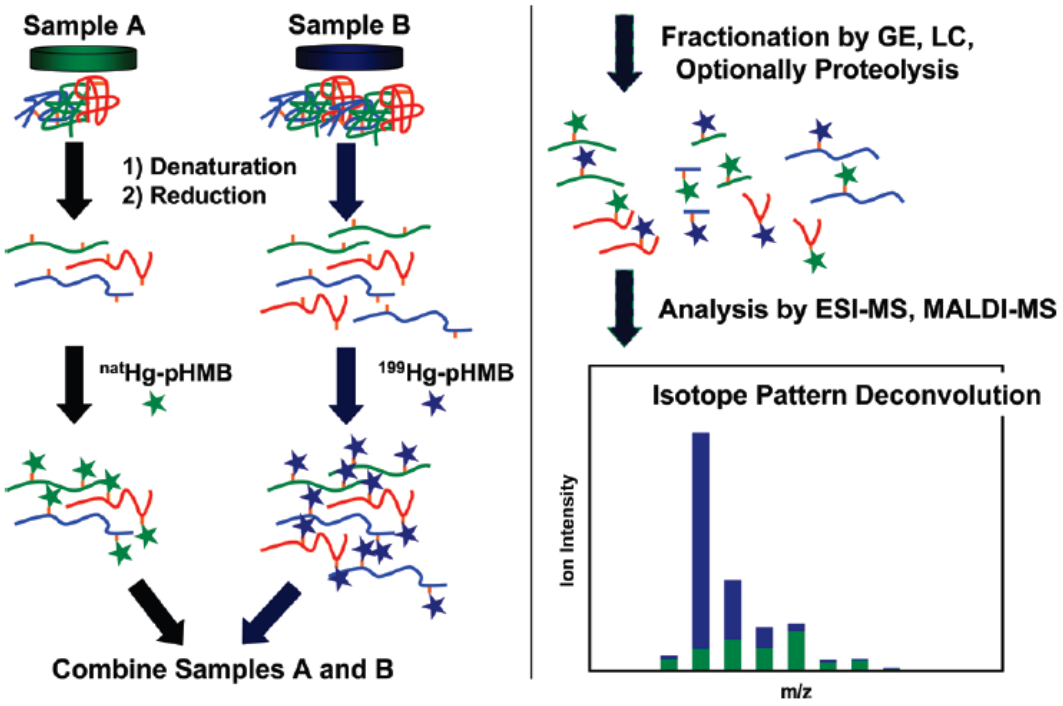


Figure 5.28: Workflow for relative protein quantification using pHMB labelling

Previous to these experiments, different matrix substances were tested for their performance with respect to the analysis of labelled insulin in MALDI-MS. For the proposed workflow, it would be desirable to reduce the extent of fragmentation of the two-fold labelled b chain in order to improve the sensitivity of the method. On the other hand, good mass resolution of the ion clusters is mandatory for evaluation of the abundances of the individual signals. Besides the classical matrix substances for peptide analysis, α-cyano-hydroxy-cinnaminic acid (CHCA) and 2,5-dihydroxy--benzoic acid (DHB), also 2,4,6-Trihydroxyacetophenone was tried as a matrix. It was reported that using this matrix improved ionisation of phosphorylated peptides and adducts of cis-platin to DNA could be observed [58], [134]. In principle, these findings could be confirmed in the case of pHMB-labelled peptides, as the ion corresponding to the two-fold labelled b chain was the base peak in the mass spectrum. However, the overall signal intensity was drastically reduced in this case, and the peak shape was distorted, so that evaluation of signals would be highly erroneous. Therefore, CHCA was used as a matrix substance for further experiments.
As already shown in chapter 5.2.1, the product of insulin b chain and ^{199}pHMB results in an equally derivatised peptide as in the case of ^{nat}pHMB, with abundances which correspond perfectly to the expected isotopic signature for the presence of the enriched mercury. In figure 5.29, the respective ion clusters for the two-fold ^{nat}pHMB and ^{199}pHMB-labelled intact insulin b-chain are shown (m/z 4070). A mixture of equal quantities of both gave the ion cluster shown below. It can be clearly seen in the figure that the resulting ion cluster is a composite of the two

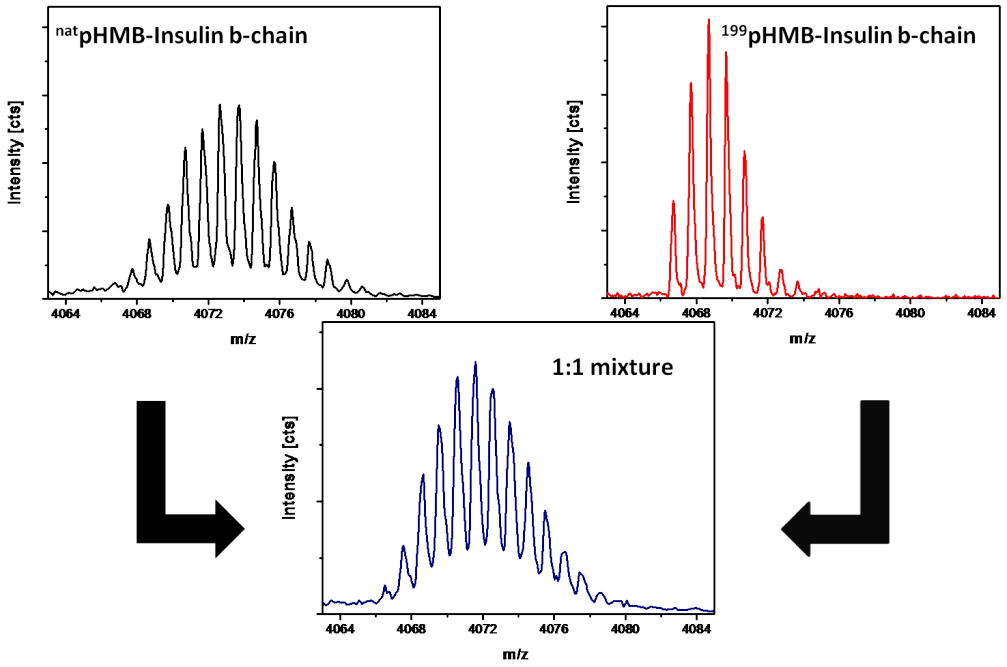


Figure 5.29: Scheme of the IPD principle with ^{nat}pHMB insulin, ^{199}pHMB insulin and a 1:1 mixture

differentially labelled species. Quantitative evaluation of the spectrum using IPD revealed a sample-to-spike ratio of 0.89 with a standard deviation of 10 %, which agrees well with the theoretical value. Further results are summarized in table 5.17.

Table 5.17: Results obtained for relative quantification of insulin b chain using IPD after detection with MALDI-TOF-MS

ratio (sample:spike)	determined ratio (n=3)	RSD (%)
1:1	0.89	10
1:2	0.52	5
1:3	0.39	7
1:10	0.11	10

The results indicate that the proposed workflow might serve as an appealing tool for relative protein quantification. The elevated standard deviation for the obtained results is mainly owed to the bad shot-to-shot repeatability in MALDI-MS and the manual evaluation of peak areas. The formation of artefactual contributions due to the formation of ion types other than $[M+H]^+$ has not been observed in MALDI-MS. Although the MALDI process induced cleavages of the mercury-sulphur bonds within the labelled insulin B chain, they should not influence the quality of the results as the isotope dilution step occurred before. Thus, isotope pattern deconvolution could be applied not only to the quasi-molecular peak for the generation of quantitative information, but also to the single labelled species at m/z 3750 commonly observed in the mass spectra. In principle the evaluation of both signals

Table 5.18: Comparison of the ion clusters for the single labelled and the two-fold labelled insulin b-chain with respect to their characteristics for the evaluation of quantitative data with IPD

	[b-chain + 2 pHMB]	**[b-chain + 1 pHMB]**
Sum formula	$[C_{172}H_{242}N_{40}O_{46}S_2Hg_2]$	$[C_{165}H_{238}N_{40}O_{44}S_2Hg]$
Monoisotopic mass	4071.67 Da	3749.67 Da
Mass fraction of Hg	0.0985	0.0535
Error mag. factor	0.1174	0.1829

indicated consistency, but the quality of the data was considerably worse when evaluating the signal of the single labelled b-chain. The reason for this can be found in the number of mercury atoms present in the species. Whereas there are two Hg atoms dominating the shape of the ion cluster of the two-fold labelled peptide, there is only one left in the fragmented ion. This leads to a "dilution" of the isotope effect in the latter, resulting in a lower correlation between the mass fraction of Hg compared to the entire molecule. This effect has been described previously for the presence of different numbers of ^{13}C-atoms as labels in organic compounds with increasing number of total carbon atoms [242]. Similar to elemental isotope dilution analysis, a factor called error magnification factor can be calculated for IPD, taking into account the correlation of the theoretical isotope compositions. This factor is therefore dependent on the nature of the scrutinized compound. Calculation of this factor can be done following reference [242] by calculation of the (matrix) term $(A^T \text{ x } A)^{-1}$, where A corresponds to the matrix representing the theoretical isotope abundances. This value has been calculated for both ion signals evaluated. From the results it becomes obvious, that quantitative evaluation of the ion cluster of the single labelled b chain of insulin is associated with a higher systematic uncertainty as the evaluation of the ion cluster of the two-fold labelled b chain. Table 5.18 illustrates these findings. In the table, the respective sum formula and monoisotopic mass for both ions are shown together with the mass fraction of mercury in the ion and the calculated error magnification factor for a mixture containing equal amounts of sample and spike, for which its value is minimized.

This effect has also to be kept in mind for the possible application of this procedure to the evaluation of quantitative data from MS/MS spectra. As shown in chapter 5.1.2, the formation of fragment ions containing more than one unit of the label can be hindered, as shown with the example of the b chain of insulin. Therefore, the evaluation of bigger fragment ions may lead to increased error magnification. In figure 5.30, this finding is illustrated using the peptide ^{25}FVNQHLC(pHMB)GSHLVEALY^{40} resulting from a tryptic digestion of bovine insulin. In the figure, the error magnification factor is shown as a function of the ratio between sample and spiked compound for a selection of fragment ions typically

observed in the product ion spectrum. For comparison, also derivatised glutathione is included into the figure, representing a small peptide consisting of only three amino acids including one cysteine residue for derivatisation (EC(pHMB)G).

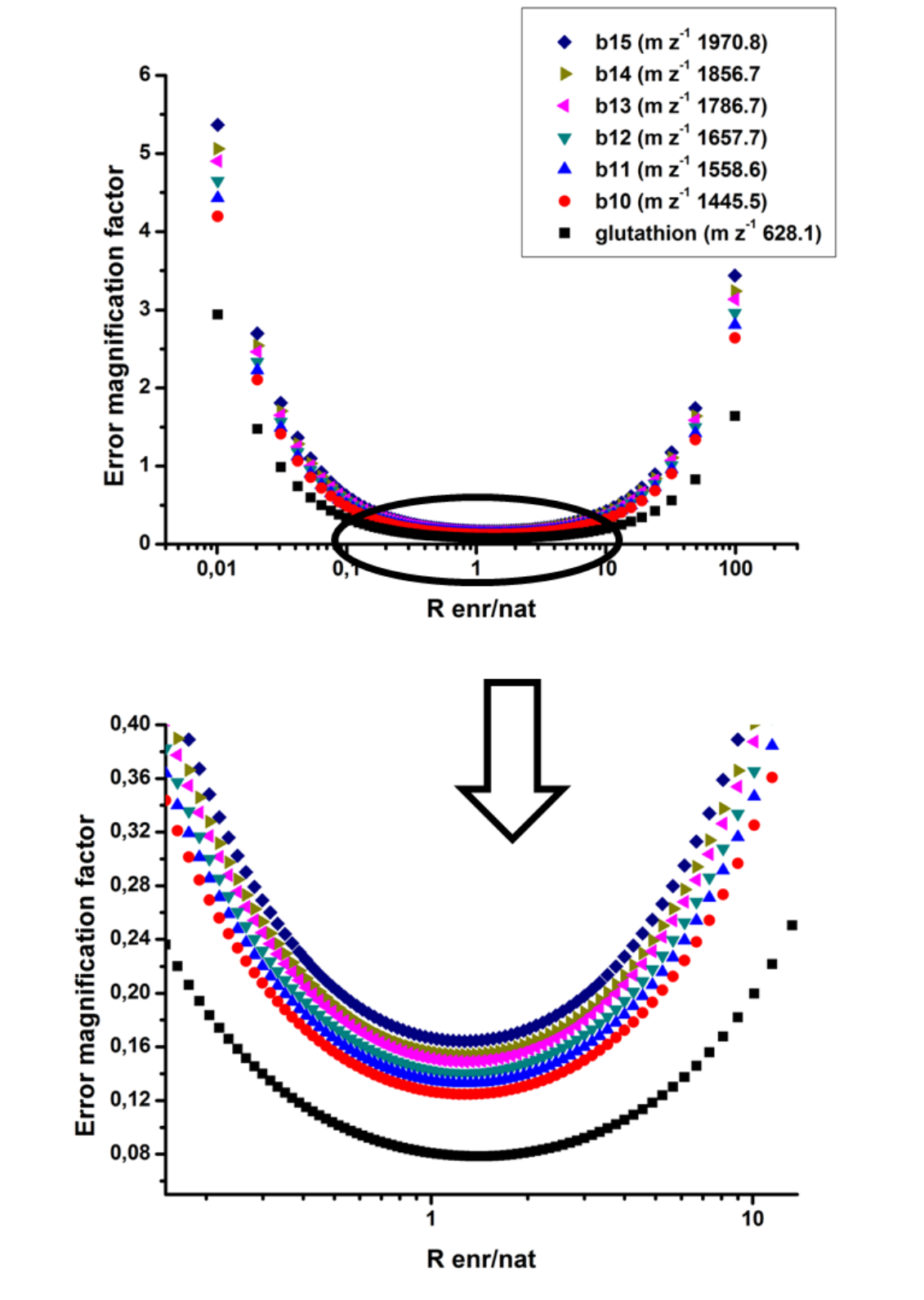


Figure 5.30: Error magnification factor as a function of the ratio of spike to sample in the mixture for different fragment ions found in a tryptic peptide from bovine insulin and glutathione

The figure shows clearly that the error for the determination of the isotope ratio

increases with the molecular weight of the fragment ion. Even under optimum conditions regarding the spike to sample ratio, the standard error for the smallest ion, glutathione is in the order of 8 %, whereas it increases considerably for heavier ions, such as the fragments of the tryptic peptide investigated. Therefore, the use of IPD should be restricted to smaller ions or ions which possibly contain more than one unit of the labelling reagent.

5.3.3 Absolute protein quantification using IPD

Using the above mentioned workflow, only relative quantification of proteins is possible. However, as pointed out in the introduction, relative quantification is only applicable in a restricted number of cases, as a control sample is always necessary for comparison of the obtained results. Therefore, the development of analytical methods for absolute quantification of proteins was chosen as one of the fundamental objectives of this work. The use of chemical labelling with pHMB permits the production of labelled standard proteins with known Hg/protein stoichiometry and isotopic composition. Furthermore, the derivatisation of standard proteins was demonstrated to be possible with high reaction yield and high purity, which is especially important in the case of derivatisation with enriched mercury isotopes. Using the outstanding quantitative characteristics of ICP-MS as a complementary mass spectrometric technique, the ^{199}pHMB labelled protein can be characterised with regard to the absolute concentration and further used as an internal standard following the principle of label-specific isotope dilution analysis. Consequently, the isotopic information embedded in a given protein fragment after addition of a certified amount of a ^{199}pHMB labelled protein is obtainable with mass spectrometry. Following the principle of isotope dilution analysis, the analytical result is independent of potential analyte losses occurring during the sample separation protocol, so that once complete mixture between both components is achieved, only the isotopic information is sufficient for absolute quantification. Together with the above presented possibility of using IPD for the calculation of the molar fractions between sample and spike, absolute protein quantification is feasible using molecular mass spectrometry instrumentation such as MALDI- or ESI-MS as a detector. The relation between the calculated molar contribution factors (x_{nat}, x_{enr}) and the amount of substance in moles (N_{nat}, N_{enr}) in the sample is given by:

$$\frac{N_{nat}}{N_{enr}} = \frac{x_{nat}}{x_{enr}} \tag{5.3}$$

As the amount of N_{enr} is known, the calculation of N_{nat} is straightforward. Figure 5.31 illustrates a possible workflow for the proposed methodology.

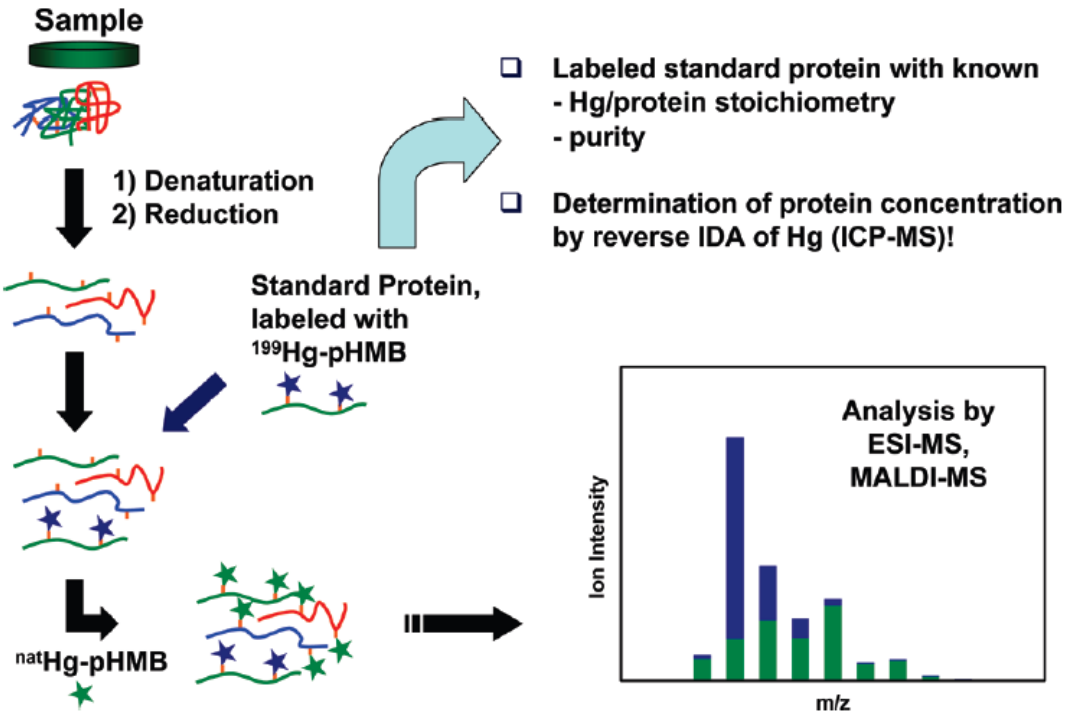


Figure 5.31: Workflow for absolute protein quantification using complementary mass spectrometric techniques

In order to demonstrate the potential and applicability of such complementary approaches, the above presented methodology was adopted to absolute protein quantification. Therefore, the ^{199}pHMB-labelled insulin b-chain was characterised by reverse isotope dilution analysis against a standard solution with natural isotopic composition of mercury. After preparation of samples with quantities of insulin in the low pmol range, MALDI-MS was used to generate mass spectra which were then once again evaluated using IPD. The obtained results are represented in figure 5.32.

In the investigated concentration range, good agreement between the expected and the determined quantity of insulin was observed. Also here, the precision of the method is basically dependent on the quality of the mass spectra which are evaluated. The fundamental advantage of label-specific isotope dilution analysis is emphasized, namely the compensation for analyte losses which can occur during all steps of sample separation. Although the detection limit attainable for MADLI-MS detection is one order of magnitude higher than the detection limit for ICP-MS detection, it offers the principle advantage, that the compound which is to be quantified can be separated from other Hg-containing compounds in the mass spectrum, which could potentially alter the isotope ratio in ICP-MS detection and therefore bias the analytical result.

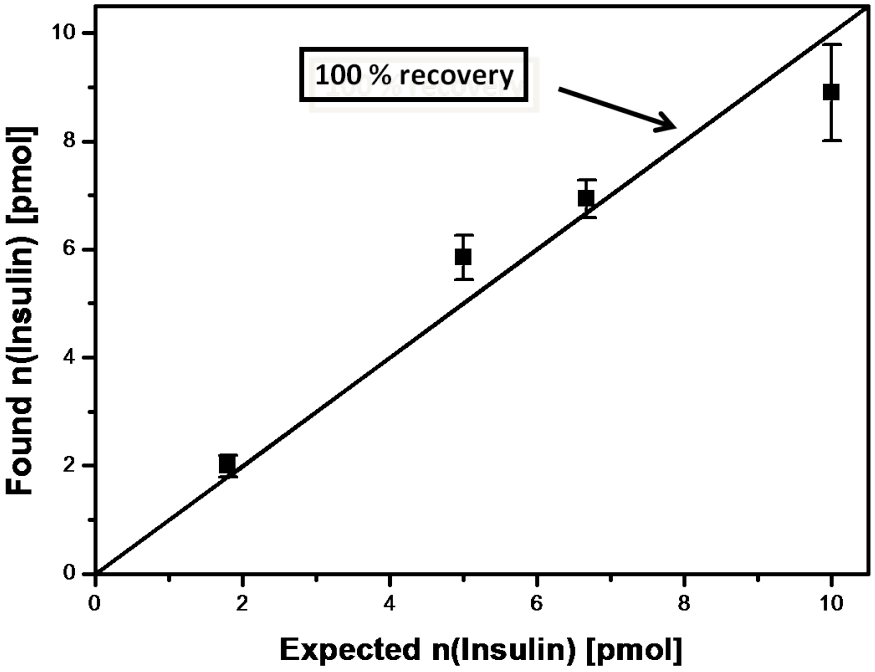


Figure 5.32: Results obtained for absolute quantification of insulin b chain using IPD

5.3.4 Conclusions from this chapter

The results presented in this chapter show the principle applicability of isotope pattern deconvolution as a means for quantification of peptides by molecular mass spectrometry. The complementary use of elemental- and molecular mass spectrometry can help to overcome the potential drawbacks that both techniques present. The lack of quantitative properties of ion sources such as ESI- and MALDI-MS can be reduced by using ICP-MS for absolute quantification of the spike compound which is later on added as an internal standard. On the other hand, the missing structural information which is commonly overcome by comparison of retention times of standards or identification of species in an additional chromatographic run can be obtained in the same run and reduce unexpected contributions of other compounds to the analytical result. Additionally, the possibility of conducting MS/MS analysis can further increase the specificity of the essay for a targeted species. However, care has to be taken for the selection of the ion that is used for quantification, as its size and therefore the effect of the altered isotopic abundance due to the spike to the overall ion cluster can play an important role for the accuracy of the result. The proposed methodology can therefore be an appealing alternative for targeted protein quantification approaches, where peptides appropriate for quantification can be chosen *a priori* and hence peptides can be selected that offer best characteristics for quantification.

5.4 Concluding remarks to protein labelling using pHMB as a labelling reagent

As the last part of this work is dedicated to a fundamentally different topic, some concluding remarks about concerning the previously presented results about protein labelling using pHMB should be made here. First of all, the labelling reagent permitted reduction of the detection limit for the investigated model protein ovalbumin in comparison to the detection of sulphur. Second, all products of the derivatisation could be unambiguously characterised with respect to the number of units of the labelling reagent attached by the derivatisation reaction, so that their accurate quantification is accomplishable via the detector signal of Hg. Furthermore, the resulting bioconjugates have proven their applicability in terms of stability and integrity in combination with various separation techniques commonly used in protein analysis, so that its principal application is possible. In comparison to other proposed methodologies for protein quantification involving elemental labelling, such as antibody labelling with lanthanide complexing agent or direct labelling of proteins using chelating agents, the obtained limits of detection have been in a similar range. However, as shown in table 5.18, not only the contribution of the instrumental configuration contributes to the attainable limit of detection of the method, but also the number of detectable atoms introduced by the label. Generally speaking, although the detection limit obtainable for Hg in ICP-MS is not as good as for example for the detection of lanthanides, similar detection limits can be obtained as the smaller Hg- reagent can access more binding sites than bigger chelating reagents. For example, similar absolute detection limits in the low fmol range were obtained in this study in comparison to [164] using a chelating compound and In detection with ICP-MS. However, if the number of binding units of the labelling reagent can be increased, detection limits clearly inferior to the observed ones are attainable [243]. In contrast to methods involving the use of labelled antibodies, the possibility for conducting isotope dilution analysis is clearly an advantage of the proposed method. Especially in Western-blot procedures proposed for example by [158] or [159], several steps such as electroblotting are probably far from being quantitative, so that potentially occurring analyte losses cannot be compensated. On the other hand, especially using lanthanide labelling, the multielement capabilities of ICP-MS can be used for multiplexed and sensitive analysis of a variety of proteins in one analytical run. In general, the consequent application of label-specific isotope dilution analysis in combination with ICP-MS based labelling approaches is so far not practiced. Besides one other study also using Hg-based labelling [185], other labelling approaches still have to prove their applicability in this context. Other ICP-MS based methods for absolute quantification of peptides and proteins, such as the production of phosphorus-based absolutely quantified standard peptides (PASTA, [97])

or recombinant isotope labelled and selenium quantified proteins (RISQ, [98]) are mainly focussed on the accurate quantification of isotopically labelled standards (as for example for their use in proteomic studies). However, due to the higher number of amino acids that can be phosphorylated, the number of peptides and protein accessible for quantification is higher. However, these methods are therefore not feasible for the direct quantification of biopolymers.

5.5 Investigation on potential binding partners of methylmercury in tuna fish

5.5.1 Scientific interest in the determination of methylmercury

The main reason for the scientific interest in the quantitative determination of methylmercury in biological and environmental samples is its elevated toxicity [244]. For humans, the lowest lethal dosis published for oral admission was 5 mg kg^{-1} for methylmercury, whereas the same value for $HgCl_2$ was 29 mg kg^{-1} [245]. The toxicity of Hg is based in its high affinity towards proteins that can lead to blocking of active centers of enzymes (e. g. acetylcholinesterase [246]) and therefore severely compromise their function. Methylmercury however, is converted into the unpolar methylmercurychloride (MeHgCl), which can be easily distributed over the entire organism via the red blood cells. Furthermore, it can also pass the blood-brain barrier. The main sources for mercury in the environment are for example combustion of fossil fuels such as coal. But also liberation of mercury from sediments due to mining processes is a considerable source for contamination. The release of mercury in different chemical forms can also lead to the formation of $MeHg^+$ due to biogenic methylation. It is long known, that Hg can be converted to methylmercury by bacteria [247], but also other organisms such as algae and fungi [248]. One example for the drastic consequences of uncontrolled mercury release into the environment can be found in the so-called Minamata disease [249]. Here, uncontrolled dumping of residual waters of a chemical fabric into the sea lead to accumulation of methylmercury in fish and seafood, which was the main nutrition source for the people living in Minamata in Japan. During the following years, many victims of chronic methylmercury intoxication were observed in the city.

Analytical methods for the quantitative analysis of methylmercury in tuna fish

Methods for the determination of $MeHg^+$ from tuna fish tissue involve mostly extraction to organic solvents like benzene or toluene after hydrolysis using mineral acids [250] or alkaline solutions [251]. Additional clean-up steps (for example using cysteine acetate as binding partner for $MeHg^+$) and repeated extraction to organic solvents can be conducted before the samples are analyzed. Also other extraction procedures involving for example alkaline-methanol extraction with tetramethylammonium hydroxide (TMAH, [252]) or extraction procedures using ultrasonic probes have been described [253].

From the instrumental point of view most methodologies are based in gas chromatography due to the volatility of alkylated mercury species. But also other techniques, including HPLC methods ([254], [255]), capillary electrophoresis ([256], [257]) and spectrophotometric detection for GC [258] and after chemical isolation of $MeHg^+$

[259] have been applied. For the sensitive and specific detection of separated species, methods based in atomic spectroscopy have been frequently described. Besides atomic absorption (AAS, [260]) and atomic emission (AES, [261]), the coupling of gas chromatography to ICP-MS [262] has been commonly used for quantitative analysis of Hg-species. Here, the combination with species-specific isotope dilution analysis can offer very accurate results [248], as it can correct for species conversion, which can occur during the extraction process. This methodology was also applied for alkylated species of other trace metals, as for example lead and tin [263]. However, the application of IDA is also possible using conventional GC-MS systems equipped with electron impact ionisation [264].

5.5.2 Studies on protein binding of methylmercury

Although there is a considerable interest in determination and speciation analysis of methylmercury, only little is known about its distribution in living beings. Up to now, Hg speciation was mostly concerned about discriminating between Hg^{2+} and methylmercury (or other organic moieties bound to Hg), leaving unaccounted the real counterion or partner (as for example Cl^- in aqueous solutions or sulphur containing biomolecules like cysteine or glutathione). Due to the insights obtained during this study (and other studies aiming at the artificial introduction of mercury to proteins, [175], [179], [182]-[185]) about the binding of mercury compounds to proteins, it is highly probable, that $MeHg^+$ is associated to proteins, and moreover, bound specifically to cysteine residues. This could already be confirmed by X-ray absorption spectroscopy [265]. In this study, the authors compared the spectra from different fish samples to solutions of $MeHg^+$ incubated with different possible binding partners like amino acids. The highest similarity was obtained with a solution of cysteine. In other studies, possible effects on the chemical behaviour (and therefore its toxicity or degradation behaviour) of $MeHg^+$ were investigated [266]. Possible reactions to proteins were also investigated in recent years. In most studies, coupling of liquid chromatography (mostly size-exclusion chromatography) to ICP-MS was applied. In a first study by *Shi et al.* [267], pregnant rats were fed with $MeHg^+$ previously synthesized with Hg enriched in the isotopes $^{196,198}Hg$. Analysis of serum and brain cytosol of the dam and pup rats with SEC-ICP-MS showed three signals in serum ($\geq$ 300 kDa, 300 kDa and 120 kDa), and two signals in brain cytosol (60 kDa and 1.8 kDa). Subsequent quantification with IDA showed that recovery of mercury was quantitative, and that there are differences in its distribution between dam and pup rats. *Li et al.* incubated HSA with different organic mercurials (Hg^{2+}, $MeHg^+$, $EtHg^+$ and $PhHg^+$ and investigated the electrophoretic behaviours of the modified protein, but as well stoichiometry and kinetic of the binding process with a combination of capillary electrophoresis and online electrothermal atomic absorption spectrometry [268]. In contrast, other organomercu-

rial compounds were investigated with regard to their reactivity towards proteins. One example is thimerosal, a compound liberating ethylmercury in aqueous solution, which was used as bactericide and fungicide in vaccines and other drugs [269]. Also here, only incubation experiments with model proteins (β-lactoglobulin A and human serum albumin) were carried out, but the authors were able to identify the tryptic peptide from β-lactoglobulin A containing the only free cysteine residue derivatised with EtHg. *Pedrero et al.* investigated the binding of mercury species in dolphin liver homogenate [270]. After a rough fractionation of the sample (cytosolic fraction and residual fraction), the distribution of Hg species ($MeHg^+$ and inorganic Hg) was determined using gas chromatography coupled to ICP-MS, showing that methylmercury seems to be preferentially (but not uniquely) bound in the cytosol. Further fractionation of this fraction using size-exclusion chromatography revealed the association of Hg species with biomolecules in a wide molecular weight range. Also here, the ratio of $MeHg^+$ and inorganic Hg was determined in all fractions using GC-ICP-MS. In a recent paper, the possibilities for the discovery of mercury containing compounds attached as post-translational modifications were discussed [271]. The authors incubated single protein standards (Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) and extracts of E. Coli cultures with phenyl mercuric acetate to later identify Hg-adducts due to its unique isotope pattern. The use of software-based identification and specific recognition of the isotope pattern decreased the number of false positive detections. However, their study was focussed on the use of isotope pattern as a complementary tool for data evaluation for the discovery and identification of PTM´s. In contrast to protein binding, the binding of mercury to smaller molecules like physiological thiols or phytochelatins was investigated with more effort. *Trümpler et al.* have studied the detoxification mechanism towards methylmercury by incubating whole blood and plasma surrogate samples with Hg^{2+} and $MeHg^+$ followed by addition of commonly used antidotes such as 2,3-dimercaptosuccinic acid (DMSA) and N-acetylcysteine (NAC) [272]. *Krupp et al.* [273] studied the products of low-molecular weight thiols like cysteine and glutathione with methylmercury. After the determination of their structure with molecular mass spectrometry, the compounds were spiked into a plant extract of rice and subjected to reversed phase chromatographic separation and simultaneous ES- and ICP-MS detection. The authors could show, that the plant extract as a matrix did not change the separation and detection capabilities of the proposed methodology. Other studies aimed at the detection and identification of phytochelatins expressed as a reaction of different plants to the exposure to Hg-containing solutions. Also here, binding of metal is realized via cysteine residues [274].

5.5.3 Incubation experiments with human serum albumin (HSA)

To gain first insight into the binding behaviour of $MeHg^+$, incubation experiments with HSA were conducted. Although this protein does not have relevance in the case of tuna fish muscle tissue, it was chosen because it can be involved into the transport of toxic metal compounds due to its high abundance in blood of different types of organisms. To simulate conditions similar to those in the environment, the protein was in excess in comparison to $MeHg^+$. In a first step, the incubated samples were subjected to size-exclusion chromatography with a BioSep S-3000 column. Alternatively, an aliquot of the solution was digested in solution as described in chapter 4.4.1. The digestion was also subjected to size-exclusion chromatography using a Superdex peptide column. Possible mercury containing fractions were collected and concentrated by lyophilisation. After reconstitution, the fractions were injected into the μLC-ICP-MS coupling or subjected to MALDI-MS analysis. The use of multidimensional chromatography was necessary, as the separation power of the size-exclusion column is limited, so that eluting peaks may probably contain more than one mercury containing species. In contrast, reversed-phase chromatography offers better separation methods, but only limited analyte capacity. Figure 5.33 shows the experimental approach.

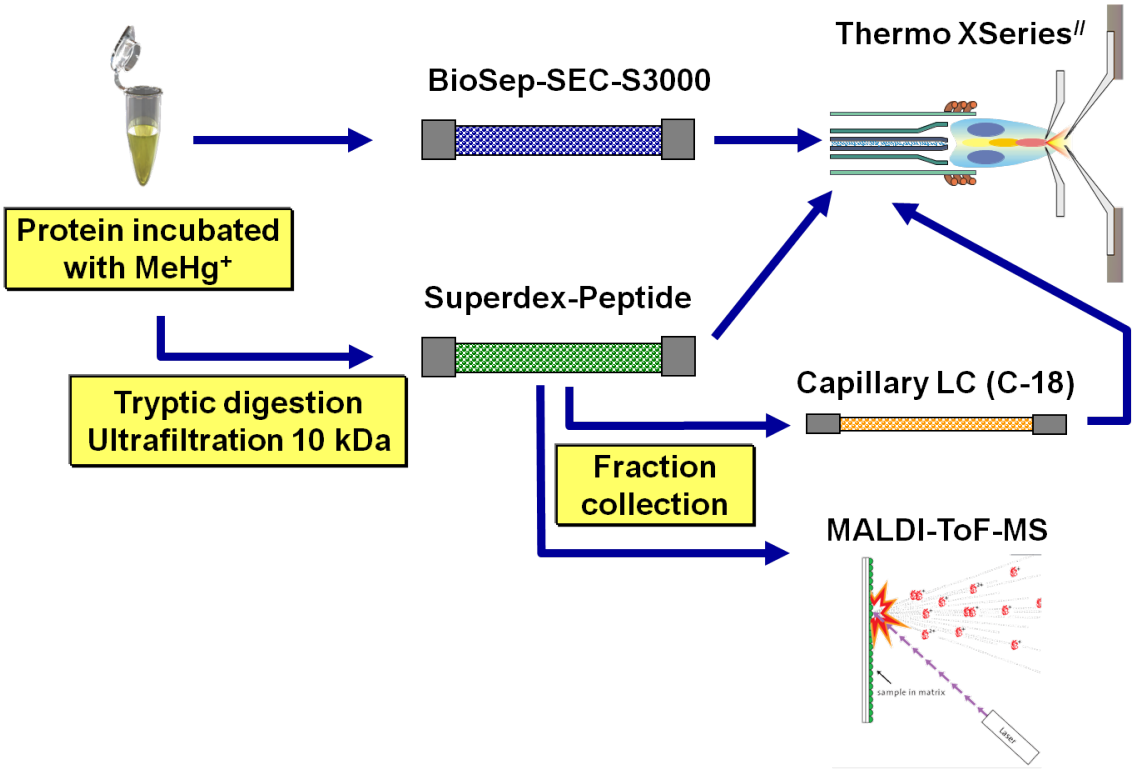


Figure 5.33: Experimental approach for the incubation experiments using HSA

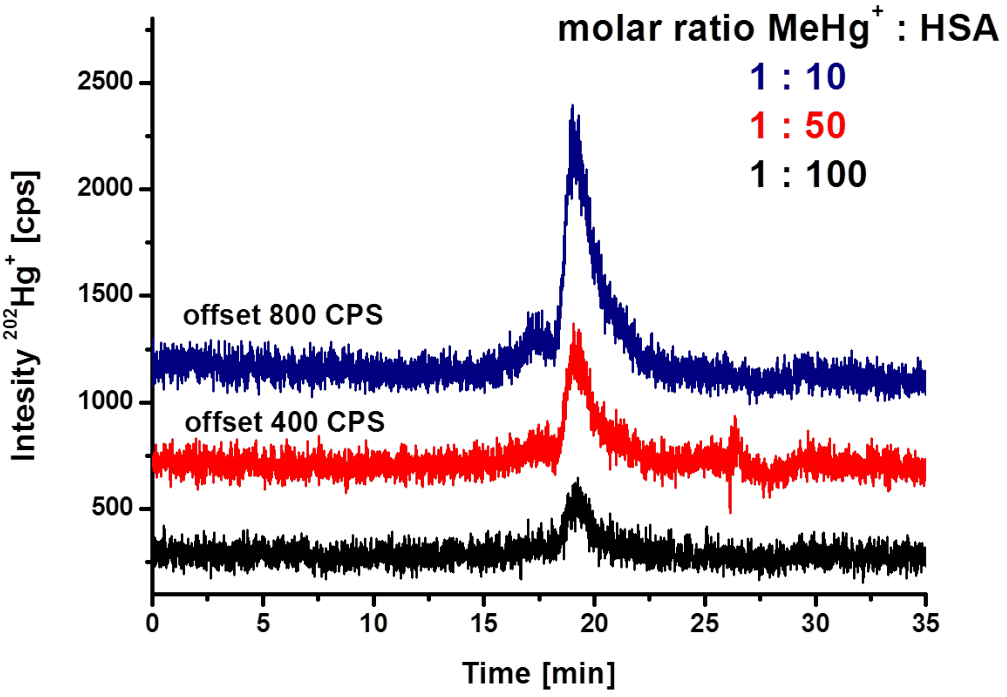


Figure 5.34: SEC of HSA incubated with different relative amounts of $MeHg^+$

SEC of the incubated protein

Figure 5.34 shows the obtained chromatographs from the incubated samples (ca. 90 pmol of HSA were loaded onto the column). As it can be seen, a signal in the $^{202}Hg^+$ trace is appearing for all $MeHg^+$ to protein ratios. The retention time of the signal (approximately 20 minutes) corresponds to the elution volume of HSA. When the amount of $MeHg^+$ is increased relative to the protein amount, also the Hg-signal in the chromatogram increases indicating that all available binding sites in HSA are not yet saturated with $MeHg^+$ and further binding occurs. Also, no Hg signal was observed in the low-molecular mass range corresponding to unbound $MeHg^+$. Similar observations were also made for beta-lactoglobulin (data not shown). In order to further investigate the possible binding sites, a tryptic digestion of the mixture with a 10-fold excess of $MeHg^+$ in comparison to HSA was carried out.

Analysis of the tryptic digest

The digested protein was subjected to size-exclusion chromatography using a Superdex peptide column, which is specially suited for the analysis of peptide mixtures or smallest proteins, as its optimum separation range is between 0.5 to 10 kDa. As it can be seen in figure 5.35, the digestion of the incubated protein leads to the presence of various Hg-containing peptides. There are also two major signal present in the molecular weight range below 1 kDa, which are probably due to the liberation

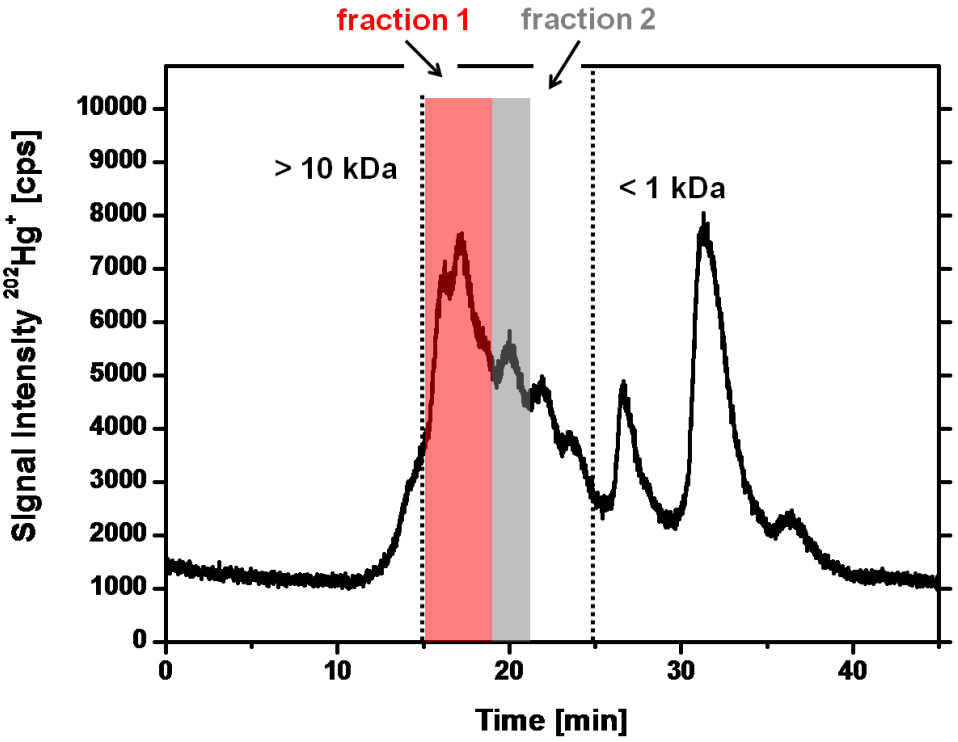


Figure 5.35: SEC of the tryptic digestion of the incubated protein

of $MeHg^{+}$ or Hg^{2+} during the digestion procedure. The fractions corresponding to the dominant signals with retention times 15 - 19 minutes and 19 - 20 minutes were collected and lyophilized to be injected into the μLC-ICP-MS system and to be analyzed with MALDI-MS for the identification of possible Hg-containing peptides. The analysis of the collected fractions with RP-μLC revealed the presence of up to 3 peptides which contain mercury. Although the amino acid sequence of human serum albumin does only contain one free cysteine residue available as a binding site, their existence can be explained by possible missed cleavages of the enzyme during the digestion process, thus forming various larger peptides. The obtained chromatograms are shown in figure 5.36. Whereas fraction one contained more than one peptide derivatised with Hg, fraction two shows only one peptide containing mercury. The retention time of this peptide (ca. 10.5 minutes) corresponds well to a peptide also present in fraction one, so that this peptide may have eluted in between both fractions or was separated during fraction collection.

The results of MALDI-MS analysis of the digested protein are shown in figure 5.37. As it can be seen, there is a variety of peptides present in the fraction, as there are more peptides eluting in the fraction as indicated by the Hg-signal in ICP-MS. No peptides corresponding to self-digestion of trypsin could be observed in the spectrum. Figure 5.37 shows a signal observed at m z^{-1} 2649.24 Da. This signal corresponds to the peptide ^{45}**ALVLIAFAQYLQQCPFEDHVK**^{65}, in which the cysteine residue ^{59}Cys has been derivatised with $MeHg^{+}$. This cysteine residue is the

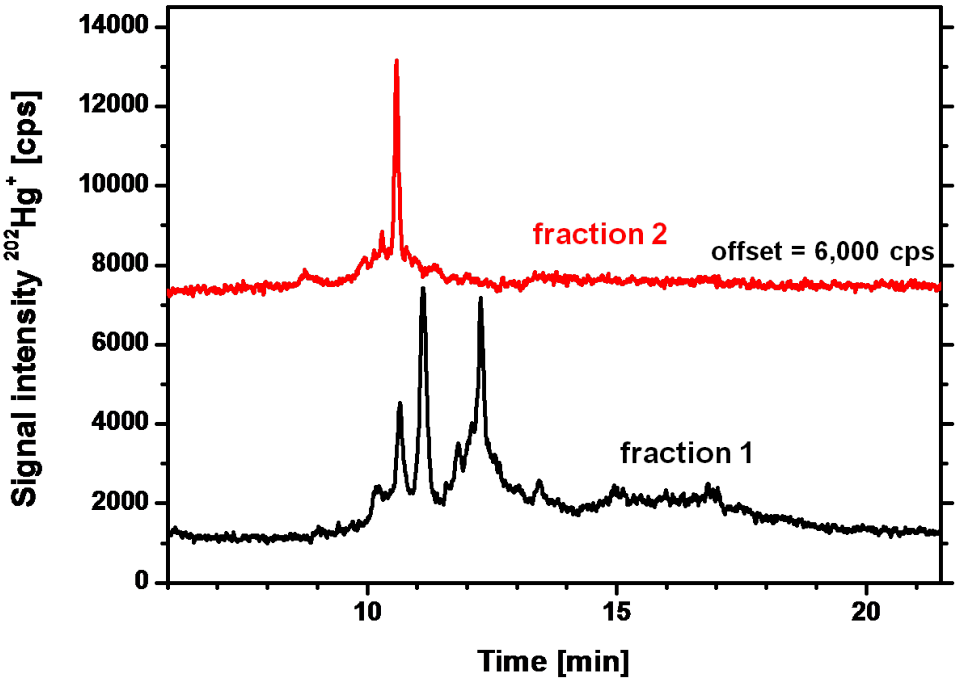


Figure 5.36: Chromatograms obtained with μLC-ICP-MS of both collected fractions

only cysteine residue not involved into a disulfide bridge and therefore a possible binding site for $MeHg^{+}$. A comparison of the isotope pattern to the theoretical pattern for the peptide is shown on the right side in figure 5.37. The measured isotope distribution fits well with to the theoretically predicted distribution, showing the typical broadening of the pattern due to the presence of one Hg-atom. Mass accuracy obtained in the spectrum was 114 ppm which is in the usual range of attainable accuracies.

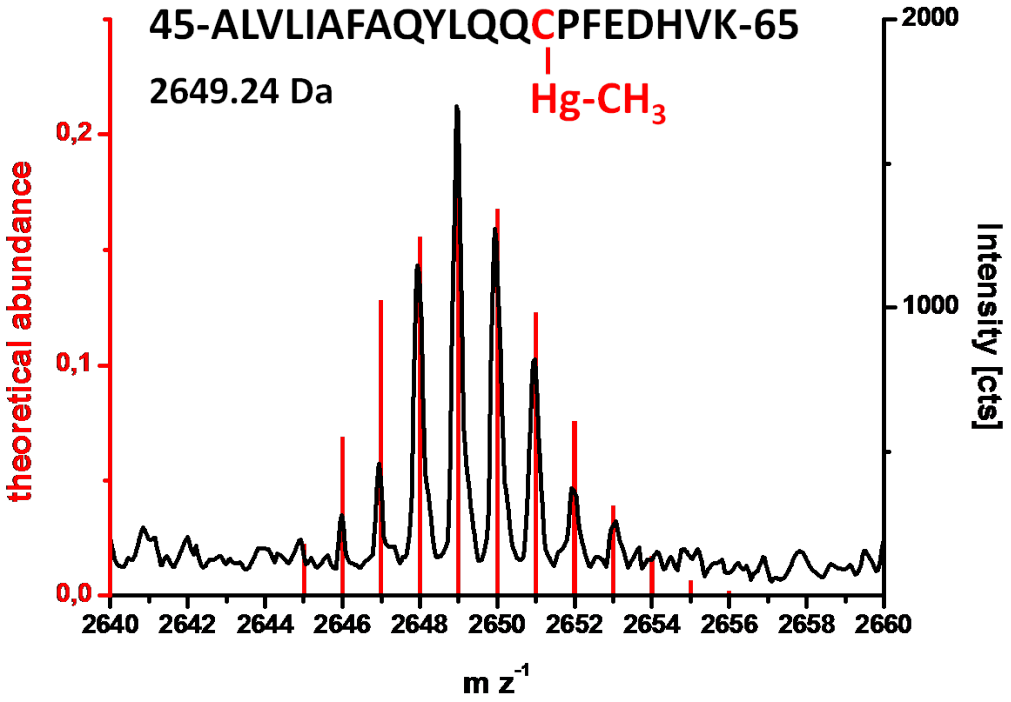


Figure 5.37: Detail of the region between m z^{-1} 2640 and 2660 found in a MALDI-MS spectrum of fraction 1 together with theoretical abundances for the supposed $MeHg^+$-derivatised peptide

Due to the data obtained during the previously presented experiments, it can be concluded, that binding of methylmercury is directed to the free cysteine residue found in human serum albumin. Therefore, attention was focussed on potential Hg-binding proteins in tuna fish muscle tissue.

5.5.4 Experiments with BCR-464 (Tuna fish muscle tissue)

To determine if there are possible proteins which can bind $MeHg^+$ *in vivo*, an extraction procedure had to be chosen, that extracts a sufficient amount of the total Hg content, but at the same time maintains the binding of $MeHg^+$ to the protein. Whereas extraction methods used for the quantitative determination of $MeHg^+$ aim intentionally for the destruction of previous binding partners, in this study extraction methods for proteins are tested for their applicability. As it could be shown in the previous part of this work, the high stability of the S-Hg binding should enable the extraction of the bioadduct even under denaturing conditions.

The certified reference material BCR-464

As a sample for the study of potential binding partners for methylmercury in tuna fish, the certified reference material BCR-464 was chosen. The reason for its selection was that the material corresponds well to the sought matrix, and that the

total mercury concentration is sufficiently high in order to obtain clear indications for the possible presence of mercury containing species. The certified reference materials BCR-463 and -464 have been developed during the beginning 1990s due to the increasing number of methods tackling the determination of methylmercury in tuna fish muscle tissue. Although the previously recognized method for its determination (extraction, clean-up and finally gas liquid chromatography with electron capture detection) was laborious and also suffered from high detection limits and lack of specificity, the new methods evolving could be hardly validated. Therefore, a new certified reference material for high concentrations of $MeHg^+$ was produced by the European Commission and certified by 14 laboratories within the European Union [275]. For the preparation of the reference materials, two catches of bluefin tuna coming from the Adriatic Sea, which were highly contaminated with $MeHg^+$, were withdrawn from the market. The dorsal muscles of the fish were cut, to be afterwards minced and freeze-dried. After homogenization, the material was filled into brown glass bottles. Certification of BCR-464 gave a value of 5.24 ± 0.10 mg kg^{-1} for the total mercury content, and 5.50 ± 0.17 mg kg^{-1} for the $MeHg^+$ content [276]. Therefore, the entire mercury content must be present as methylmercury. A potentially problematic issue concerning this reference material is, that there is only little information available with regard to species conversion effects induced by freeze drying (lyophilisation) of the material. It is clear so far, that this process can lead to denaturation during the freezing process due to the weakening of hydrophobic interactions. These changes in the protein structure [277], that, although reversible, might be associated with loss of biological activity. Also, the formation of adducts has been observed for different kinds of proteins during lyophilisation, so that for example migration distances indicating a higher molecular mass have been observed in SDS-PAGE [278]. However, it was previously reported, that muscle tissue material showed good stability when lyophilised [279]. In a more recent study, an overestimation of methylmercury was observed in freeze dried materials, probably caused by an abiotic methylation transformation reaction during acidic extraction, so that this overestimation cannot be directly related to the process of freeze-drying [280]. In any case, experiments conducted in the former part of this project indicated, that neither denaturating conditions as well as lyophilisation did affect the Hg-binding compound attached to cysteine residues.

Extraction of Hg-containing proteins

Different extraction procedures were evaluated for the extraction of possible Hg-containing proteins. As the concentration of mercury in the tissue material is known, the percentage of extracted mercury could be determined by isotope dilution analysis using $^{200}Hg^{2+}$. Besides the quantitative aspect, also the species of the extracted material was investigated by size-exclusion chromatography using UV-detection. Be-

sides simple extraction using 30 mmol L^{-1} Tris, also denaturating agents as SDS and urea were investigated, following procedures described by *Mounicou et al.* [281]. The extraction procedures tested were the following:

- **A:** 30 mmol L^{-1} Tris, pH 8.0, 4h at 25°C
- **B:** 2% CHAPS ((3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanosulfonate), 4 mol L^{-1} urea, 4h at 25°C
- **C:** 4% SDS in 30 mmol L^{-1} Tris, pH 8.0, 14h at 37°C
- **D:** 4% SDS in 30 mmol L^{-1} Tris, pH 8.0, 4h at 80°C

Extraction was carried out in reaction tubes made of polypropylene. About 100 mg of BCR-464 were weighed and about 4 mL of the respective extraction solution was added, so that the final Hg concentration was in the order of 100 ng g^{-1}. After completion of the extraction procedure, all samples were centrifuged for 20 minutes at 5,000 g in order to separate remaining solids, such as cell debris. Additionally, the extracts were filtered using syringe filters with a pore size of 0.45 μm. The obtained extraction efficiencies (calculated as relative fraction of the expected Hg concentration) is summarized in figure 5.38. As it can be seen, the use of detergents is mandatory for the extraction of significant amounts of mercury. Whereas the exclusive use of Tris does only show an extraction efficiency of 13%, all other extractants show higher efficiencies of at least 75%. The elevated temperature during procedure D was able to compensate for the shorter extraction time in comparison to procedure C and achieved as well an efficiency of close to 100%.

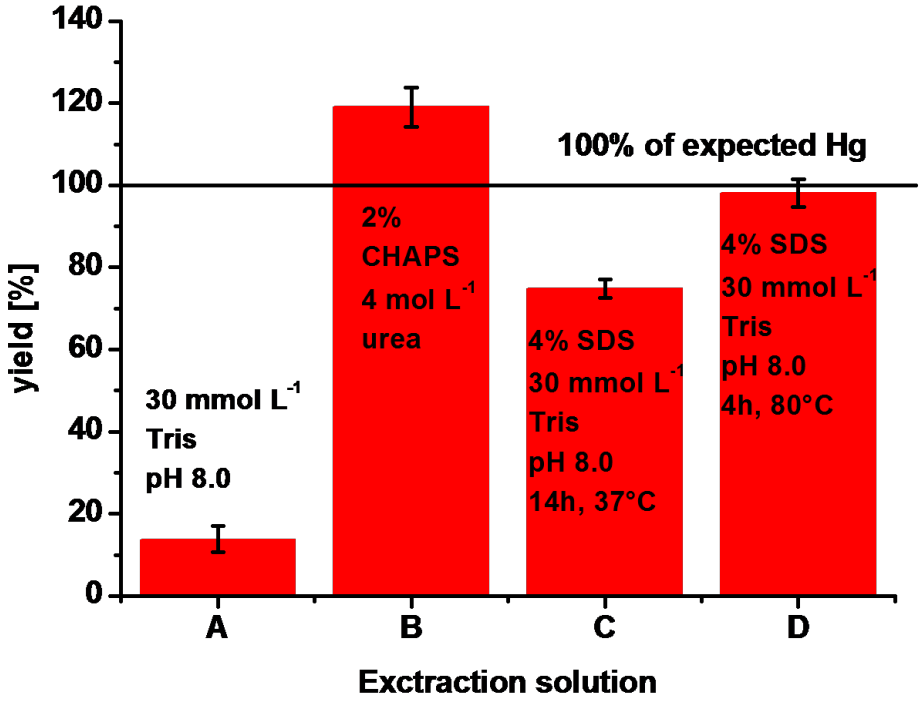


Figure 5.38: Extraction efficiencies for different extraction procedures for $MeHg^+$ containing protein species from the CRM BCR-464

Extraction procedure B gave a nominal extraction efficiency of more than 100 %, which could be related to Hg present in one of its constituting reagents. The molecular weight profiles of the extracted species obtained with SEC with UV detection can be seen in figure 5.39. Before injection, the extracts were diluted 1:5 with mobile phase to reduce the concentration of SDS and urea, resp.. As the use of SDS seems to be mandatory for the extraction of significant amounts of mercury, extraction solution A was already discarded for this analysis. It can be seen, that there is a peak in the high-molecular weight range (retention time 3 minutes) when using the combination of SDS and Tris under both extraction conditions C and D. Both procedures reveal additional species with a molecular weight between ca. 5,000-20,000 Da (retention time 7-10 minutes), and a low molecular weight species with a retention time of 12 minutes. In contrast, the combination of CHAPS and urea does show an additional signal in the low molecular weight range (retention time 13.5 minutes).

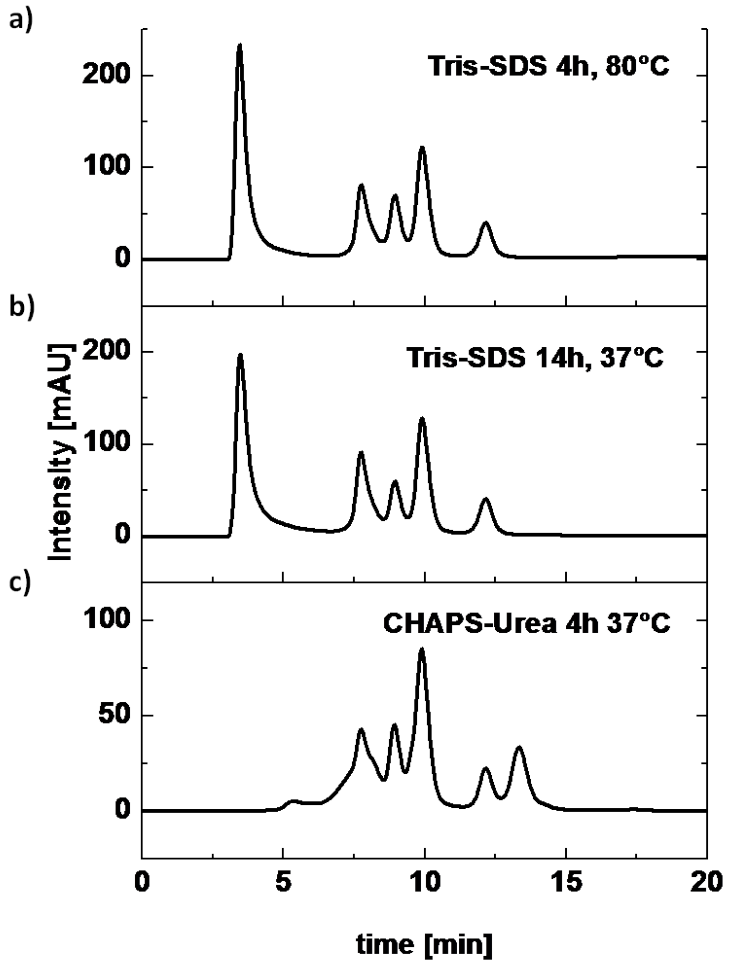


Figure 5.39: SEC chromatograms obtained for the different extraction procedures with UV detection at 254 nm; a: SDS-Tris (80°C, 4 h), b: SDS-Tris (37°C, 14 h, c: CHAPS urea (37°C, 4 h)

Separation of the Hg containing fraction with SEC

In order to investigate which of the previously found species is containing mercury, the size exclusion chromatography was coupled to ICP-MS and the same separation was run under identical conditions. As expected, injection of the extract resulting from procedure B only revealed Hg present as a low molecular weight species. In contrast, the results for extraction procedures C and D indicate, that $MeHg^+$ is attached to a high molecular weight species, as the Hg-containing compound eluted in the void-volume of the column. Figure 5.40 shows the resulting chromatogram for extraction procedure D using UV detection at 254 nm and ICP-MS detection at the $^{202}Hg^+$ trace.

As the separation range of the previously used Superdex 75 column is limited to about 80 kDa, a second size-exclusion column with a higher separation range (10 to 300 kDa) was applied (figure 5.40 b). Also here, co-elution of a high-molecular weight protein with Hg is obvious, and the retention time is similar to the retention

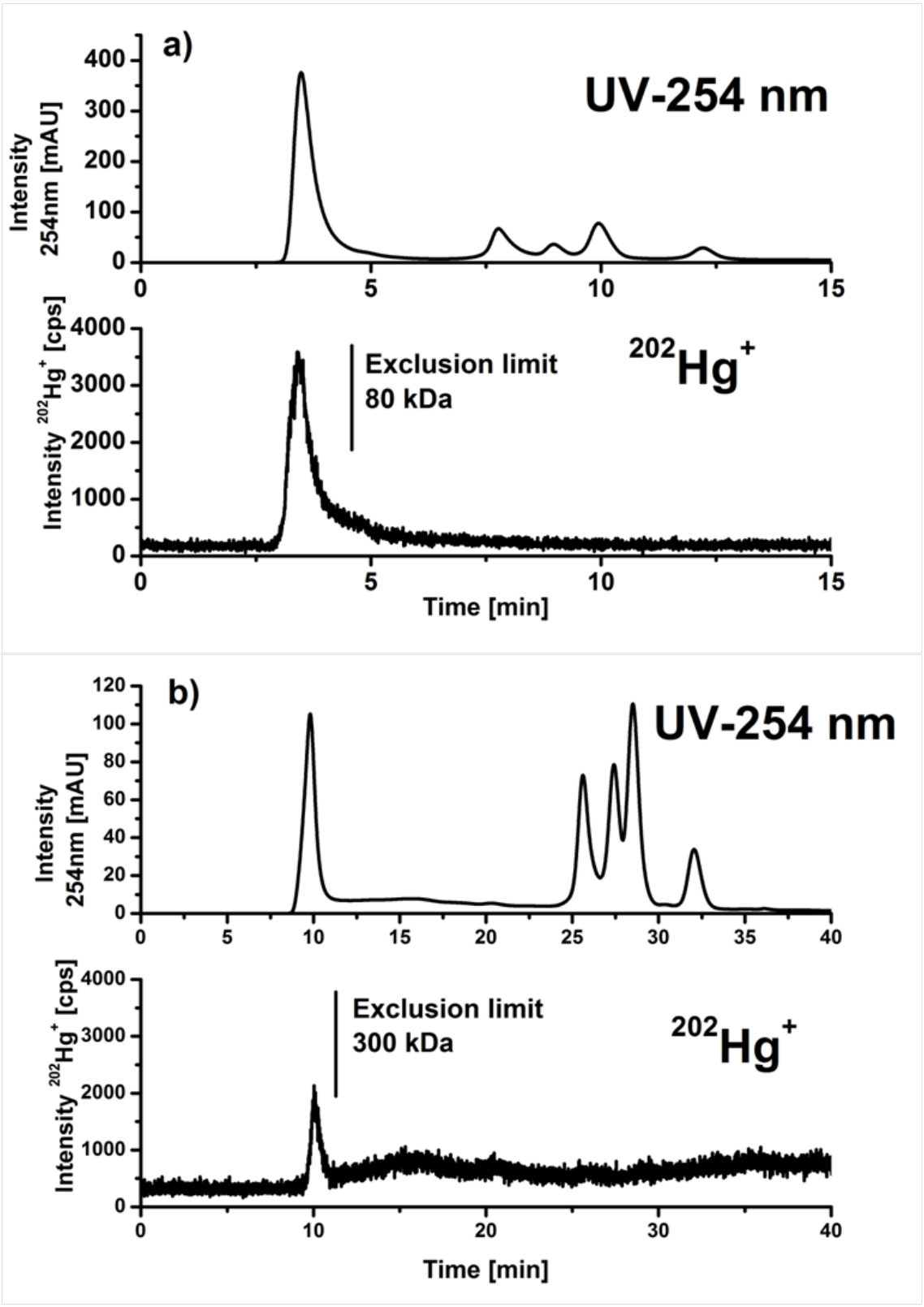


Figure 5.40: SEC chromatograms of the extracted protein species and Hg distribution measured by ICP-MS. a: Superdex 75, b: Superdex 200

time of the biggest protein used for molecular weight calibration of the column. Although one protein potentially able to bind methylmercury, it cannot be excluded, that there are other proteins in muscle tissue that are also available for binding. Further experiments using size-exclusion chromatography were carried out in order to address this point.

Investigation on other potential binding proteins in CRM-464

As previously shown, binding of $MeHg^+$ already occurs under deficit conditions concerning the mercury compound. The reaction conditions for the potential binding of $MeHg^+$ in tuna fish *in vivo* are probably even more deficient regarding the amount of $MeHg^+$ with respect to the protein. These deficit conditions however will probably impede complete saturation of all binding sites in the target proteins, or similarly binding to all proteins present in the organisms that can potentially bind $MeHg^+$. In order to estimate if there are more proteins available for Hg binding, the protein extract was incubated with an excess of ^{199}pHMB to derivatise all cysteine residues possibly remaining. Although the bigger size of pHMB in comparison to $MeHg^+$ may lead to potential differences in the binding behaviour, this has not been observed in any comparative experiments between different protein binding Hg-compounds ([182], [183]). Additionally, one aliquot of the protein extract was reduced using DTT at elevated temperature (5 minutes at 95°C) before incubation with ^{199}pHMB in order to facilitate access to other potential binding sites. The resulting solutions were then analyzed using SEC-ICP-MS using the Superdex 200 column. This column was chosen because it offers the highest separation range, so that eventually new appearing signals can be resolved. The compound enriched in ^{199}Hg was used for incubation, as additional binding of Hg would lead to an altered isotope ratio and thus can be discovered easily.
The results indicate, that as expected the protein fraction carrying Hg was not completely saturated. Whereas incubation without prior reduction does only lead to a slight increase of the Hg signal found in the peak, incubation after reduction leads to an drastic increase in the signal intensity of ^{199}Hg. From the data it can be concluded, that a major part of all cysteine residues in the protein might be part of cystine groups (or otherwise sterically hindered for access), as they are not available for binding under non-reductive conditions, whereas chemical reduction leads to their provision as additional binding sites. Another important conclusion that can be drawn from the results is that there is probably only one protein fraction which is able to bind $MeHg^+$. Neither treatment with DTT and incubation with additional derivatisation agent leads to the appearance of new signals in the chromatogram. However, peak tailing and increased memory effects are observed after injection of the reduced and incubated extract.
In any case, the injected solutions still contained about 1% of SDS, which could

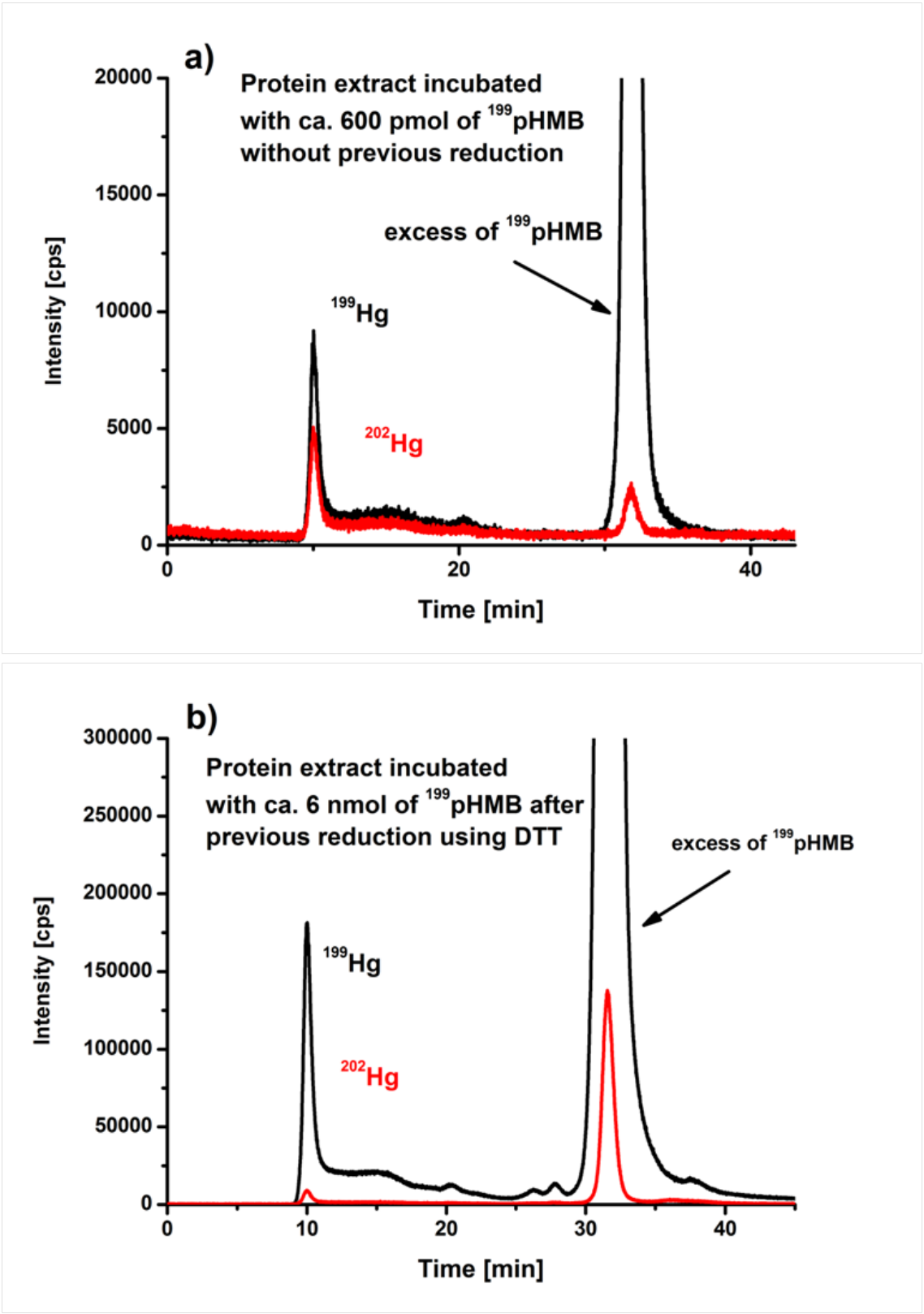


Figure 5.41: SEC-ICP-MS chromatograms obtained after incubation of the protein extract with ^{199}pHMB; (a) without previous reduction and (b) after reduction with DTT

influence the separation in size-exclusion chromatography. Additionally, aggregation of proteins during the freeze drying process could mimick a higher molecular weight observed for the protein. In order to confirm the presence of the supposed protein and the attachment of $MeHg^+$, 1D denaturing gel electrophoresis was chosen as an additional separation technique which is perfectly suited for the separation of protein mixtures from SDS containing samples.

Gel electrophoresis and analysis of the spots

The extraction solution of procedure D was incubated in sample buffer and subjected to slab gel electrophoresis. Figure 5.42 shows an image of the coomassie blue stained gel acquired with a Typhoon scanner. In the lane on the left side of the gel, a molecular weight marker between 20 and 101 kDa was applied. As indicated in the picture, five spots could be identified with different molecular weights. In contrast to the results previously obtained with SEC-ICP-MS, Hg was also detected in the low molecular weight range. This content can also be present in the spots due to contamination with other Hg species probably as a consequence of addition of bromophenolblue. Due to the strong yellow colour of the sample, the amount of bromophenolblue in the sample buffer was increased up to 0.5 %, and was already identified as a potential contamination source in chapter 5.2.4. Similarly to the results obtained using size-exclusion chromatography, a protein spot with a molecular weight of approximately 200 kDa is found (spot no. 5). Additionally, three spots are clearly visible in the low-molecular weight range (below 50 kDa, spots 1-3). Another spot with a molecular weight of about 100 kDa was found (spot no. 4).

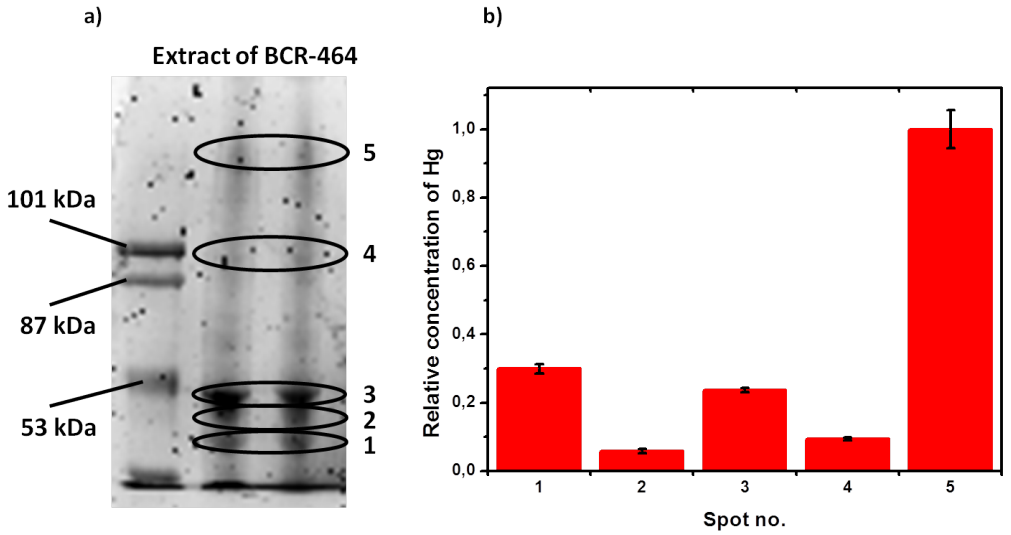


Figure 5.42: Image of a stained gel after separation of tuna fish muscle tissue extract prepared by extraction procedure D (a), Hg-content of the excised protein spots determined using ICP-MS analysis of total Hg after digestion with HNO_3 (b)

The indicated protein spots were excised and afterwards digested in nitric acid. The

presence of Hg in the spots was measured using ICP-MS. The results of the analysis are also shown on the right in figure 5.42. It can be seen, that spot no. 5 shows the highest Hg content, although all other spots also indicate the presence of Hg. This clearly supports the results obtained previously with SEC. Probably, $MeHg^+$ in tuna fish muscle tissue is associated to a protein with a molecular weight of about 200 kDa.

LC-ICP-MS and LC-ESI-MS/MS analysis and database research

After tryptic digestion of the gel spots no. 5 as described in section 4.4.2, the reconstituted peptide mixture was subjected to μLC-ICP-MS analysis in order to find potential Hg-containing peptides. Alternatively, the same sample was subjected to μLC-ESI-MS/MS analysis in order to identify peptides originating from the target protein. Figure 5.43 a shows the results of the ICP-MS based chromatogram and the TIC chromatogram obtained with ESI-MS (figure 5.43 b).

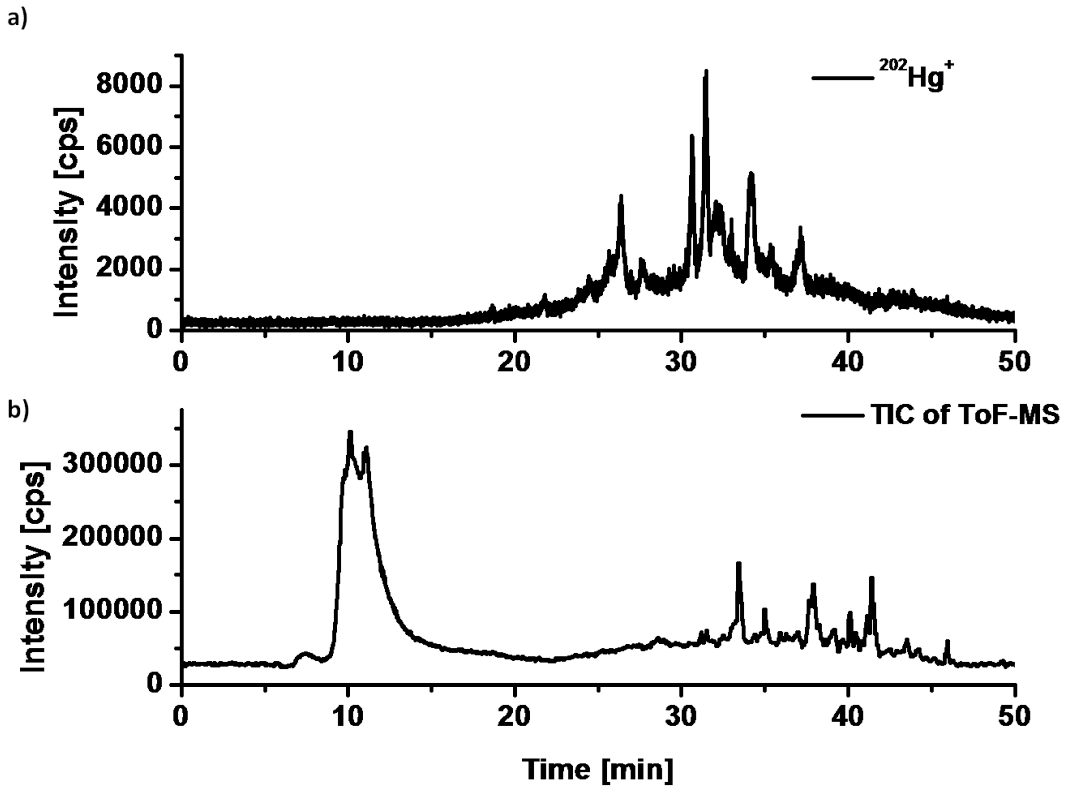


Figure 5.43: LC-ICP-MS chromatogram on $^{202}Hg^+$ (a) and TIC of TOF-MS chromatogram (b) for the tryptic digest of spot no. 5 obtained under identical chromatographic conditions

The ICP-MS based chromatogram on $^{202}Hg^+$ shows the presence of various Hg-containing species, which might be peptides modified with $MeHg^+$. However, the absolute intensity of the signals is quite poor, so that their concentration in the digest is rather low. In contrast, the LC-ESI-MS/MS run generated MS/MS spectra for a number of peptides present in the sample, which were then identified by the Protein Pilot software provided by Applied Biosystems.

Table 5.19: Proteins revealed after database search using Protein Pilot

Nr.	Protein (SwissProt No.)	Organism	Mw (kDa)	Score* (unused)**	Coverage
1	Myosin-1 Q076A6	Canis familiaris	223.1	37.01 (37.01)	27.2
2	Myosin heavy chain P13538	Gallus gallus	223.1	36.54 (17.95)	25.4
3	Myosin 4 Q9Y623	Homo sapiens	223.1	32.21 (5.71)	26.1
4	Myosin-7 Q91Z83	Mus musculus	222.8	18.87 (8.04)	19.7
5	Myosin 13 Q9UKX3	Homo sapiens	223.6	16.26 (9.93)	16.5
6	Myosin heavy chain cardiac muscle isoform P29616	Gallus gallus	128.0	6.33 (2.01)	22.2

* The score value is set by the Protein Pilot software and reflects the number of peptides and the confidence value of their identification contributing to the identification of the protein.
** The unused value reflects the number of peptides not previously used for the identification of another protein. The higher this value, the more peptides contribute uniquely to the identification of this protein.

A complete list of all identified peptides can be found in table A .2 in the appendix. Database search was realized using also the Protein Pilot software. Restrictions concerning the target organism were not included in the search algorithm, as a special option for organisms like or similar to tuna fish was not included in the options. The results of the database search are shown in the table 5.19. A number of proteins from organisms other than tuna fish or other marine species were identified. This result is contradictory on the first view, as tuna fish was the organism from which the sample was taken. On the other hand, the proteins identified (Myosin-type proteins in different forms) do fit quite well to the nature of the sample (muscle tissue). The myosin protein family is a group of motor protein forming part of muscle fibres and thus ahould be present in a considerable amount in the processed sample [283]. Additionally, the molecular weight of the identified proteins corresponds well to the molecular weight observed in the previous experiments involving SEC and SDS-PAGE. Therefore, also possible protein aggregation can be excluded. Unfortunately, the information on tuna fish included in the common databases such as UniProt is rather poor. Searching all entries of this database for Thunnus thynnus (bluefin tuna, the species inhabitated in the mediterranean sea), only 174 entries are found, whereas this number is much higher for other organisms (about 27,000 for

Gallus gallus, about 54,000 for *Danio rerio* (Zebrafish, an important model organism [284])) [285]. As already shown in earlier studies, identification of an unknown protein can also be accomplished if the target protein shows a high similarity to proteins from different organisms (cross species identification, [282]). Taking a closer look to the proteins yet identified for *Thunnus thynnus*, the protein Q91520 (Skeletal muscle myosin, *Thunnus thynnus*) seems to be a potential candidate. However, its entry in the UniProtKB database [286] already shows that it is only partially sequenced, so that only about 800 amino acids are identified up to now. In order to check for similarities between the target protein from tuna fish and the proteins identified via database search, a BLAST (basic local alignment search tool) search against all entries in the UniProtKB database was run. All proteins identified by Protein Pilot were found in the list of proteins similar to Q91520 with identity values between 71 and 80 %. Due to the high degree of similarity, it is highly probable, that the mistakes in protein identification are a result of the missing database information. Therefore, all identified peptides were matched manually against the sequence of Q91520. Tables containing all matched and unmatched peptides can be found in the appendix (table A .2 and A .3, resp.). From the results it can be recognized that most of the identified peptides can be matched against the supposed target protein, as their sequence is identical or only prone to slight deviations, which might also be consequence to errors in the peptide identification algorithm. An example for this is the wrong identification of isoleucine instead of leucine, as both amino acids are not distinguishable using CID as a fragmentation technique. Overall, a sequence coverage of 29 % was achieved, as 226 of 786 amino acids could be identified in Q91520. Additionally, another 149 amino acids were identified in peptides of other myosin proteins, but could not be matched against the known sequence of Q91520. Besides that, the BLAST search indicates that the identified peptides are proteotypic for myosin protein family, thus reinforcing the hypothesis. However, there is also a high number of peptides which could not be matched to the sequence of Q91520. The reason for this is the incomplete content of the database entry for the target protein, as more than 50 % of the protein sequence is unsequenced up to now. The results indicate that the sequenced part of Q91520 corresponds to the final part of the amino acid sequences of the completely identified proteins. For example, the starting sequence (amino acids 1-15) of Q91520 was identified as the peptide containing the amino acids 1154-1168 in P13538 (Myosin heavy chain, *Gallus gallus*). All matched peptides belong to positions above this value in the amino acid chain of the respective protein. Consistently, all peptides that could not be matched belong to the first part of the sequences (below $\approx$ AA 1150). An illustration of these findings is given in figure 5.44.

As it can be seen in the amino acid sequence of Q91520 (see figure A.1 in the appendix), there are three cysteine residues known up to now, which could serve as

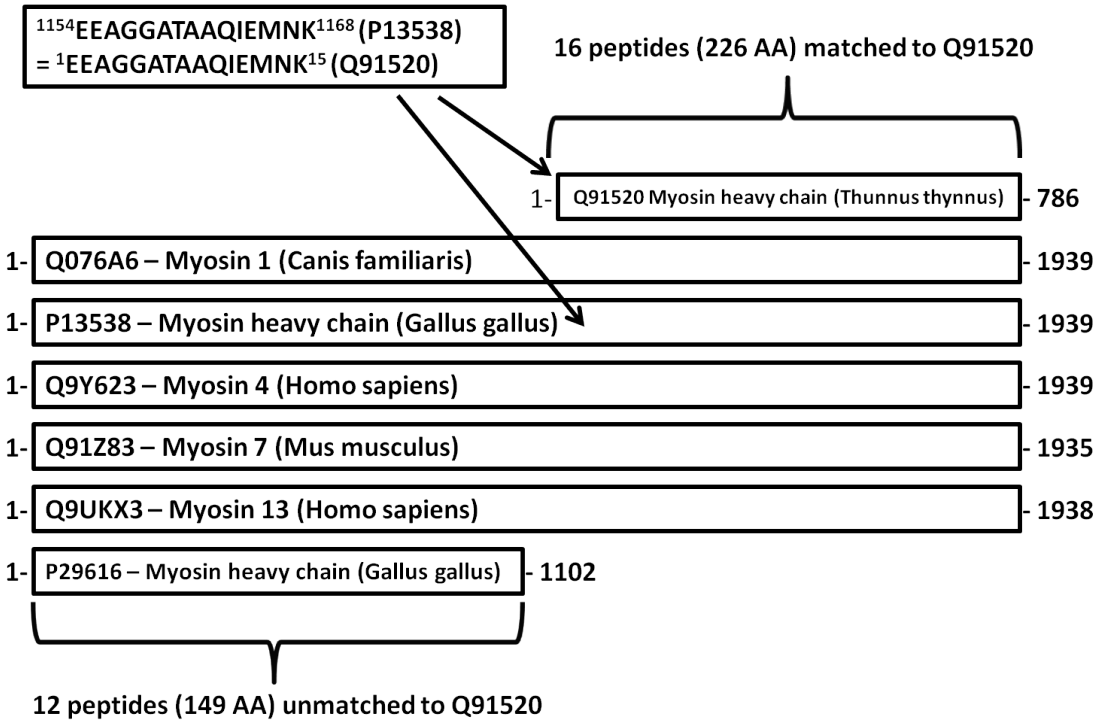


Figure 5.44: Illustration on the positions of the matched and unmatched peptides in the amino acid sequence of Q91520 and in their originating protein

potential binding sites for $MeHg^+$. However, as the sequence information is incomplete, it is highly probable that there are other cysteines present in the unsequenced part of the amino acid chain. In the mistakenly identified proteins, the total number of cysteine residues is considerably higher, varying from 14 in Q91Z83 to 20 in Q9UKX3, so that the total number of cysteine residues in the target protein should be in a similar range. Unfortunately, none of the identified peptides contains cysteine residues, and no isotopic signature of mercury was found in the TOF-MS mass spectra of all eluting compounds before fragmentation, so that there is no evidence for a concrete binding site(s) up to now. One of the reasons for this can be ion suppression caused by other co-eluting peptides. Also, as previously observed in this work, the reduced ionisation rates of Hg containing peptides can be additional issue, so that probably appropriate means of preconcentration of the Hg-containing peptides have to be taken in order to finally identify one or more which may carry the $MeHg^+$ moiety.

5.5.5 Conclusions from this chapter

In this chapter, fundamental investigations on the reaction behaviour of methylmercury towards human serum albumin showed, that $MeHg^+$ can bind to the protein. Additional experiments including tryptic digestion and screening of the peptides for mercury using SEC-ICP-MS and RP-μLC-ICP-MS indicated the presence of at least three Hg-containing peptides of different molecular weight, of which one could be

identified in the MALDI-MS spectrum of the tryptic digest. This peptide contained the only cysteine residue which was available for Hg-binding in human serum albumin under these conditions.

Further investigations on the certified reference material for tuna fish muscle tissue showed, that also here Hg (and hence $MeHg^+$) is bound to a protein, specifically of high molecular mass. This finding could be substantiated by the use of different size-exclusion columns and additional SDS-PAGE, and the molecular weight could be estimated to about 200 kDa. Further incubation with a cysteine derivatising reagent showed, that as expected, only a part of all binding sites available in the protein is really derivatised. Tryptic digestion of the protein spot and mass spectrometric peptide identification followed by database search indicated, that the sought protein could be a myosin protein, namely skeletal muscle myosin heavy chain from *thunnus thynnus*. One reason why $MeHg^+$ binds almost exclusively to this protein is probably its ubiquitous abundance in muscle tissue. Besides the fact, that there is probably only a low number of different proteins present in muscle tissue (especially when compared to other protein rich samples such as protein extracts e. g. from liver or kidney), the found protein is one of the major proteins in this sample. Myosin proteins are structure proteins forming part of muscle fibres, which first of all supports its presence in the sample, and second explains its abundance. Therefore, binding of methylmercury seems to be directed to this group of proteins protein.

6 Conclusions and outlook

Conclusions

The main conclusions that can be drawn from this Ph.D. thesis are summarized in the following:

1. A labelling strategy for the introduction of the reagent p-(hydroxymercuri)-benzoic acid (pHMB) could be successfully developed and proven to enable selective and site-specific derivatisation of free cysteine residues in different kinds of model proteins. It could be shown, that the structure and Hg to protein stoichiometry of the resulting bioconjugate is independent of the reaction conditions in a wide range, concerning e. g. reaction time and excess of reagent used. The structure of the adducts after addition of the labelling reagent could be completely elucidated using molecular MS-techniques.

2. The labelling reagent was synthezised using isotopically enriched mercury in order to perform label-specific isotope dilution analysis. It could be shown, that there is no difference in the formation of the adduct for the commercially available reagent and the synthesised compound, and that there is no alteration of the isotope ratio in the spike due to contamination with natural Hg during synthesis and derivatisation of the proteins.

3. Label-specific isotope dilution analysis was successfully applied to the quantification of labelled proteins. Especially in combination with laser ablation-inductively coupled plasma mass spectrometry, its subsequent use could significantly improve results for protein quantification from polyacrylamide gels, as it compensated for matrix effects and effects of inhomogeneous protein distribution within the gel. Furthermore, the application of an absolutely quantified protein labelled with the enriched compound has been implemented into a typical workflow in proteomics. Also here, the use of label-specific isotope dilution analysis was able to compensate for probably occuring analyte losses during sample procession steps, such as tryptic digestion or incomplete column recovery.

4. Isotope pattern deconvolution (IPD) was successfully applied to the relative and absolute quantification of human insulin using MALDI-MS as a detection system. It could be shown, that IPD can help to achieve a more specific

detection of ion clusters of labelled peptides, thus enabling accurate quantitative results even if co-elution of different mercury-containing species occurs, as these are further separated in the mass spectrum.

5. Investigation on binding of $MeHg^+$ towards proteins showed, that cysteine is its binding partner. Using human serum albumin, the only supposed binding site was proven to be derivatised by $MeHg^+$. Using a certified reference material for tuna fish muscle tissue, it could be shown, that $MeHg^+$ is bound to (a) protein(s) in the high molecular weight range. Using complementary mass spectrometric techniques and typical workflows used in proteomics, a potential target protein could be identified as skeletal muscle myosin heavy chain. Although the protein itself was not identified, its presence was clearly indicated by peptide matching and the molecular weight information distributed by size-exclusion chromatography and gel electrophoresis.

Conclusiones

Las conclusiones derivadas de la presente Tesis Doctoral pueden resumirse en los siguientes puntos, que integran las ideas más importantes recogidas en la discusión de resultados:

1. Se ha desarrollado una estrategia para la introducción del compuesto ácido hidroximercuribenzoico, y se ha demostrado que el proceso de marcaje de proteínas resulta de una derivatización selectiva de las cisteínas libres en diferentes proteínas modelo. Asimismo, se demostró que la estructura y la estequiometría del bioconjugado es independiente de las condiciones de la reacción en un amplio rango. La estructura de los aductos tras la adición del agente derivatizante se esclareció mediante el uso de técnicas basadas en espectrometría de masas molecular.

2. Se ha sintetizado el compuesto de marcaje utilizando mercurio enriquecido en el isótopo 199 para llevar a cabo el análisis por dilución isotópica en forma especifica al compuesto de marcaje. Se demostró que no existen diferencias en la formación de los aductos con el compuesto sintetizado y el compuesto adquirible en el mercado, y además, que no ocurren cambios en la relación isotópica en el trazador debidos a contaminación con mercurio natural durante la sintesis y la derivatisación de las proteínas.

3. Se ha llevado a cabo análisis por dilución isotópica para la cuantificación de proteínas previamente marcadas. Especialmente para la cuantificación de proteínas separadas por electroforesis en gel de poliacrilamida con el uso de ablación laser acoplado a espectrometría de masas con plasma deacoplamiento inductivo podia mejorar significativamente los resultados obtenidos. La gran ventaja de su aplicación es que compensa las efectos de matriz y otros efectos debidos a la distribución no homogenea de la proteína dentro del gel. Además, la aplicación de una proteína marcada con el compuesto de marcaje en su forma enriquecida previamente cuantificada ha sido implementada en protocolos de separación de muestras típicamente utilizados en proteómica. También aqui, el uso del análisis por dilución isotópica específica al compuesto de marcaje ha sido capaz de compensar pérdidas del analito occuridas durante los procesos de tratamiento de la muestra, como por ejemplo, en digestiones enzimáticas o recuperaciones incompletas en columnas cromatográficas.

4. La técnica de deconvolución de perfiles isotópicos (IPD) ha sido aplicada para la cuantificación relativa y absoluta de la insulina humana utilizando el MALDI-MS como sistema de detección. Se demostró, que el IPD es capaz de llevar a cabo una detección más específica de clusters iónicos de péptidos marcados. Asimismo, es posible obtener resultados cuantitativos más fiables,

tambien si ocure co-elución con otras especies que contengan mercurio, como aquellos serán adicionalmente separado en el espectro de masas.

5. Estudios acerca de la unión del $MeHg^+$ a proteínas muestran que el sitio preferido de unión es la cisteína. En la albúmina humana, el único sitio disponible para el enlazamiento ha sido derivatizado tras incubación con $MeHg^+$. Utilizando un material de referencia de tejido muscular de atún se pudo demostrar, que el $MeHg^+$ se enlaza a una fracción de proteínas de alto peso molecular. Con técnicas basadas en espectrometría de masas molecular y procesos de tratamiento de muestras típicos en proteómica, se identificó una proteína que posiblemente sea un sitio para enlazar el metilmercurio, la *skeletal muscle myosin*. Aunque la proteína no ha sido identificada directamente, su presencia está claramente indicada por los péptidos encontrados y la información proporcianoda sobre su peso molecular mediante cromatografía de exclusión por tamaño y electroforesis en gel.

Outlook - future research

Taking into account the results obtained during this Ph.D. thesis, the following suggestions can be made for future research in this field.

1. Although the applicability of labelling with pHMB has proven, that the resulting adduct is stable and can be applied in typical workflows used in protein analysis and proteomics such as denaturing gel electrophoresis and enzymatic digestion, further studies on its general applicability are neccessary. As the analysis of more complex samples normally requires the use of two-dimensional gel electrophoresis (isoelectric focussing followed by SDS-PAGE), it has to be tested if pHMB labelling is also applicable in combination with this technique. Possible points to be investigated in this way might be changes in the isoelectric point (depending on the size and the number of available cysteine residues) of the labelled protein and thus altered migration behaviour in the first dimension, or if there are possible reactions occuring with the buffer strips used for isoelectric focussing which might compromise the stability of the bioconjugate.

2. The use of isotope pattern deconvolution can be extended to ESI-MS/MS applications, as the quantification of species from their fragment ions would further increase the specificity for the targeted compound. However, as pointed out in chapter 5.3, one of the limiting conditions, which has to be taken into account, is the actual size of the peptide to be quantified together with the number of labelling units present, as this certainly affects accuracy of the method. Therefore, it is suggested to prove the applicability of the methodology using a smaller synthesized model peptide (five to eight amino acids), or a tryptic peptide.

3. Concerning the use of labelling with pHMB (or other labelling approaches that permit the use of label-specific IDA), the direct comparison of quantification using ICP-MS based instrumentation and MALDI/ESI instrumentation would be an interesting topic for future research.

4. The application of methodologies based on gel electrophoresis and afterwards tryptic digestion for the identification of special modifications in proteins has proven to be useful alternative to the classical means provided by speciation analysis, such as size-exclusion or ion exchange chromatography followed by fraction collection. However, due to the usually weak binding of metals associated to proteins (Fe, Cu, Zn, ...), there are possibly restrictions in the applicability of such methodology. In the case of identification of selenium containing proteins or phosphorylated proteins, these effects are not to be expected, so that maybe new insights can be achieved.

5. As the final identification of the binding site for $MeHg^+$ in tuna fish muscle tissue was unfortuntely not achieved during this work, further means of preconcentration of the targed peptides have to be taken. Possible means of concentration might be the installation of an enrichment column prior to the μLC set-up used up to now. In this way larger amounts of sample could be loaded onto the column which would finally improve the detectability of the desired compounds.

6. For the same reason it would be interesting to conduct the same workflow that has lead to the discovery of the supposed binding protein (skeletal muscle myosin) with an extract that was previously incubated with an additional quantity of $MeHg^+$ in order to saturate all binding sites and finally obtain a higher concentration of derivatised peptides in order to identify possible binding sites via the unique isotopic pattern of mercury. Due to the strong increase of the Hg signal in SEC after additional reduction, it seems that under these circumstances binding sites may become available, which are not easily accessible, so that maybe a less realistic picture is obtained.

7. The analytical strategies developed during this work, e.g. the combination of ICP-MS based identification of species carrying the element of interest and tryptic digestion followed by molecular mass spectrometry for the identification of distinct proteins can be further extended to other and more complicated sample types, such as liver or kidney extracts.

Sugerencias para Trabajos Futuros

Como resultado de la experiencia conseguida durante la realización de la presente Tesis Doctoral y en vista de los resultados obtenidos, se pueden sugerir los siguientes caminos para continuar en futuros trabajos dentro de esta linea de investigación:

1. Aunque la aplicación del marcaje de proteínas con el compuesto HMB ha demostrado que el aducto resultante es estable y puede ser aplicado en tratamientos de muestras típicas en el análisis de proteínas y proteómica, como por ejemplo, electroforesis en gel bajo condiciones desnaturalizantes y digestión enzimática, serán necesarios estudios más en profundidad. Como el análisis de muestras más complicadas normalmente requiere el uso de electroforesis en gel bidimensional (enfoque isoeléctrico seguido por SDS-PAGE), será necesario comprobar si el proceso de marcaje es compatible con dicha técnica. Posibles puntos a investigar son por ejemplo, si la derivatización puede afectar al punto isoeléctrico de la proteína derivatizada (dependiendo del tamaño y el numero de cisteínas accesibles), y por ello, inducir un cambio en la distancia que migra la proteína en la primera dimensión. También será importante investigar si pueden afectar a la estabilidad del bioconjugado las posibles reacciones con las tiras aplicadas en el enfoque isoeléctrico.

2. El uso de deconvolución de perfiles isotópicos puede ser extendido a aplicaciones del ESI-MS/MS, ya que la cuantificación de especies mediante sus iones fragmentados aumentaría aún más la especifidad para el compuesto seleccionado. En tal caso, como se destacó en el capítulo 5.3, una de las condiciones limitantes que se tiene que tener en cuenta es el tamaño del péptido seleccionado para la cuantificación junto con el número de átomos de mercurio presentes, ya que estos factores afectan a la exactitud del método. Por ello, se recomienda comprobar la aplicabilidad de la metodología con un péptido pequeño (entre cinco y ocho aminoácidos), como por ejemplo, uno sintetizado o tríptico.

3. Relacionado con el uso del marcaje con pHMB e IPD (o con otras estrategias de marcaje que permitan el uso de la dilución isotópica específica al compuesto de marcaje), sería de alto interés un estudio comparativo entre la cuantificación basada en instrumentación de ICP-MS y MALDI/ESI-MS.

4. La aplicación de metodologías basadas en electroforesis en gel seguida de una digestión enzimática para la identificación de modificaciones especiales en proteínas ha demostrado ser una alternativa útil a las metodologías clásicas en especiación, como por ejemplo, cromatografía de exclusión por tamaño o intercambio iónico seguido por recoleción de fracciones. Sin embargo, existen posibles limitaciones al aplicar tal metodología, debido al débil enlace que

suelen presentar algunos metales asociados a proteínas (Fe, Cu, Zn etc.). En el caso de la identificación de proteínas que contienen selenio o de proteínas fosforiladas, en las cuales no se esperan dichos efectos, quizás se pueda obtener más información.

5. Como desafortunadamente no ha sido posible una identifación final del sitio de enlace específico del $MeHg^+$ en tejido muscular de atún, se recomienda llevar a cabo una preconcentración de los péptidos formados. Un método de llevar a cabo esta etapa de preconcentración sería, por ejemplo, el uso de una columna de enriquecimiento de péptidos antes de la instrumentación de μLC que se venía utilizado hasta ahora. De esta forma, sería posible cargar cantidades más grandes en la columna de separación, lo cual finalmente mejoraría la detección de los compuestos seleccionados.

6. Con el mismo objetivo sería interesante llevar a cabo el protocolo de tratamiento de la muestra que resultó en el descubrimiento de la proteína *skeletal muscle myosin* con un extracto previamente incubado con una cantidad adicional de metilmercurio, para saturar todos los posibles sitios de enlace. Asimismo, se obtiene una mayor cantidad de péptidos derivatizados para llevar a cabo la identificación de los sitios de enlace via el perfil isotópico único del mercurio. Debido al aumento de la señal del mercurio observada en cromatografía de exclusión por tamaño parece que, bajo estas circunstancias, podrían aparecer nuevos sitios de unión, de forma que se obtiene una información menos realista sobre la distribución de metilmercurio presente en la proteína.

7. Las estrategias analíticas desarrolladas en la presente Tesis Doctoral, como por ejemplo, la combinación del uso del ICP-MS para la identificación de especies que contienen el elemento de interés con la digestión tríptica seguida por análisis de los péptidos mediante espectrometría de masas molecular para la identificación de proteínas, podrían ser extendidas a otros tipos de muestras aún más complicadas, como por ejemplo, extractos de riñón o hígado.

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7 Appendix

Chemicals

Table .1: List of reagents used throughout this work

Chemicals

Reagent	purity	Manufacturer
Acetic acid (CH_3COOH)	99%	Sigma Aldrich
α-Cyano-4-hydroxycinnamic acid	$\geq$ 99.8%	Bruker
α-Lactalbumin	85 %	Sigma Aldrich
Acetonitrile	HPLC-grade	VWR Prolabo
Albumin, chicken egg	min. 98 %	Sigma Aldrich
Albumin, bovine	for biochemistry	Merck
Albumin, human	96-99 %	Sigma Aldrich
Ammonium bicarbonate (NH_4HCO_3)	99.5%	Sigma Aldrich
Ammonium acetate (NH_4Ac)	99.5 %	Merck
Bromophenolblue	95 %	Sigma Aldrich
4-Carboxyphenylboronic acid	$\geq$ 90 %	Sigma Aldrich
CHAPS [1]	99%	Sigma Aldrich
Coomassie Blue (Bradford Test)	for protein essay	Merck
Coomassie Blue for gel staining		Biorad
Cytochrome C, bovine	$\geq$ 95 %	Sigma Aldrich
Dithiothreitol	99%	Sigma Aldrich
2,5-Dihydroxybenzoic acid	99%	Bruker
Glycerine	pa, 85 %	Merck
Glycine	$\geq$ 99 %	Sigma Aldrich
Formic Acid (HCOOH)	99.5 %	Sigma Aldrich
Hydrochloric acid	pa, 37 %	Sigma Aldrich
Immunoglobulin G, human	$\geq$ 95 %	Sigma Aldrich
Insulin, bovine	$\geq$ 27 units/mg	Sigma Aldrich
Insulin, human	$\geq$ 27 units/mg	Sigma Aldrich
Iodoacetamide	$\geq$ 99 %	Sigma Aldrich

[1] (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanosulfonate)

Lysozyme, chicken	95 %	Sigma Aldrich
2-Mercaptoethanol	99 %+	Sigma Aldrich
Mercury(II)oxide	99 %+	Sigma Aldrich
Methanol	HPLC grade	VWR Prolabo
Nitric acid (NHO_3)	pa, 65%	Fluka
Sinapinic acid	99%	Bruker
Sodium chloride	pa, min. 99.5 %	Merck
Sodium docecyl sulfate	99.5%	Sigma Aldrich
Sodium hydroxide	Suprapur, 30%	Merck
p-(hydroxymercuri)benzoic acid	99.5%	Fluka
Thyroglobulin, bovine	$\geq$ 90 %	Sigma Aldrich
Transferrin, apo-, human	$\geq$ 99.8%	Sigma Aldrich
Tris(hydroxymethyl)aminomethane	$\geq$ 99.8%	Merck
Triflouroacetic acid	$\geq$ 99.8%	Sigma Aldrich
Trihydroxyacetophenone	99%	Sigma Aldrich
Tryptophane	$\geq$ 99 %	Fluka
Trypsin (bovine) TPCK treated	99%	Sigma Aldrich
Trypsin (bovine TPCK treated	sequencing grade	Promega
Urea	99%	Sigma Aldrich
Vitamine B 12	99 %	Sigma Aldrich

Gases

Gas	Purity	Manufacturer
Argon	99.9999%	Air Liquide, Spain
Oxygen	99.9999%	Air Liquide, Spain
Helium	99.9999%	Air Liquide, Spain
Nitrogen	99.9999%	Air Liquide, Spain

Elemental standards

Element	Concentration	Manufacturer
Rhodium	1,000 mg L^{-1}	Merck
Mercury	1,000 mg L^{-1}	Merck

Enriched stable isotopes

Isotope	Chemical form	Source
^{199}Hg	HgO	Chemgas
^{200}Hg	Hg^{2+}, $\approx$ 1,000 mg L^{-1}	ESWE

Identified peptides for the identification of skeletal muscle myosin heavy chain from thunnus thynnus

The following table contains all peptides which were identified with the highest confidence value by Protein Pilot. The sequences were matched manually against the database entry of skeletal muscle myosin heavy chain (*thunnus thynnus*), UniProt Nr. Q91520. Eventually occuring differences in the sequence are indicated as bold letters.

Table .2: Identified peptides by LC-ESI-MS/MS from the tryptic digest of spot no. 5; matched to Q91520

Identified sequence	**Sequence in Q91520**
Q076A6 - Myosin 1 (*Canis familiaris*)	
^{1755}AEEKAKKAI-TDAAMMAEELKK^{1776}	^{601}AE**D**KAKKAI-TDAAMMAEELKK^{622}
^{1580}IAEKDEEIDQLK(R)^{1593}	^{326}**L**AEKDEE**ME**Q**I**K(R)^{339}
^{1705}(K)IAEQELLDASER^{1717}	^{550}(K)**V**AEQEL**V**DASER^{1717}
^{1425}LQNEVEDLMIDVER^{1438}	^{271}LQ**G**EVEDLMIDVER^{284}
^{1490}NAYEESLDQLETLKR^{1504}	^{336}N**S**YEE**A**LD**H**LET**M**KR^{350}
^{1508}NLQQEISDLT-EQIAEGGK^{1525}	^{354}NLQQEISDLT-EQI**G**E**T**GK^{371}
P13538 - Myosin heavy chain (*Gallus gallus*)	
^{1153}LEEAGGATAAQIEMNK^{1168}	^{1}-EEAGGATAAQIEMNK^{15}
^{1867}LQDLVDKLQMK^{1878}	^{714}LQDLVDKLQ**L**K^{725}
^{1229}MEIDDLASNMESVSK^{1243}	^{76}MEIDDL**S**SNME**A**VSK^{1243}
Q9UKX3 - Myosin 13 (*Homo sapiens*)	
^{1200}QADSVAELGEQIDNLQR^{1216}	^{47}QADSVAELGEQIDNLQR^{62}
^{1717}VQLLHSQNTSLINTK^{1731}	^{563}V**G**LLHSQNTSLINTK^{577}
Q91Z83 - Myosin 7 (*Mus musculus*)	
^{1864}LQDLVDKLQLK^{1874}	^{718}LQDLVDKLQLK^{728}
^{1504}NLQEEISDLTEQLGSTGK^{1521}	^{354}NLQ**Q**EISDLTEQLGSTGK^{371}
^{1820}RELENELEAEQK^{1831}	^{670}RELE**S**E**VD**AE**SR**^{681}
Q9Y623 - Myosin 4 (*Homo sapiens*)	

^{1181}DLEESTLQHEATAAALR^{1197}	^{27}DLEESTLQHEAT**S**A**S**LR^{43}
^{1401}LQDAEEHVEAVNSK^{1414}	^{247}LQDAEE**SI**EAVNSK^{260}

Total identified: 226 of 786 AA (29 %)

The following table contains all remaining peptides identified with with the highest confidence value by Protein Pilot. These peptide could not be matched to the sequnece of Q91520, but probably also belong to the same protein.

Table .3: Identified peptides by LC-ESI-MS/MS from the tryptic digest of spot no. 5; unmatched to Q91520

Unmatched peptides

Peptide	**Protein**
^{1002}ALQEAHQQTLDDLQAEEDKVNTLTK^{1026}	Q076A6
^{171}ENQSILITGESGAGK^{185}	
^{417}GQTVQQVYNAVGALAK^{432}	
^{645}GSSFQTVSALFR^{656}	
^{1119}IEELEEEIEAER^{1130}	
^{261}LASADIETYLLEK^{273}	
^{749}LLGSIDVDHTQYK^{761}	
^{586}AGTVDYNISGWLEK^{599}	P13538
^{1028}TKLEQQVDDLEGSLEQEKK^{1046}	
^{216}GTLEDQIIQANPLLEAFGNAK^{236}	Q9UKX3
^{728}ILNASAIPEGQFIDSK^{743}	
^{143}NLTEEMATLDENISK^{157}	P29616

Total identified: 149 AA

Figure .1: Amino acid sequence of Q91520 following the UniProtKB database [286]. All cysteine are marked in red.

Publications

In the following, all scientific of publications derived directly and indirectly from this thesis are summarized.

Publications directly related to this thesis

1. Kutscher, D. J., del Castillo Busto, M. E., Zinn, N., Sanz-Medel, A., Bettmer, J.: Protein labelling with mercury tags: fundamental studies on ovalbumin derivatised with p-hydroxymercuribenzoic acid (pHMB); J. Anal. At. Spectrom. 23 (2008), 1359-1364

2. Kutscher, D. J., Bettmer, J.: Absolute and Relative Protein Quantification with the Use of Isotopically Labeled p-Hydroxymercuribenzoic Acid and Complementary MALDI-MS and ICPMS Detection; Anal. Chem. 81 (2009), 9172-9177

3. Kutscher, D. J., Fricker, M., Hattendorf, B., Bettmer, J., Günther, D.: Systematic Studies on the Determination of Hg-labelled Proteins Using Laser Ablation-ICPMS and Isotope Dilution Analysis; Anal. Bioanal. Chem., DOI 10.1007/s00216-011-5199-5

4. Kutscher, D. J., Sanz-Medel, A., Bettmer, J.: Investigations on Potential Binding Partners for Methylmercury in Tuna Fish Muscle Tissue Using Complemetary Mass Spectrometric Techniques; in preparation

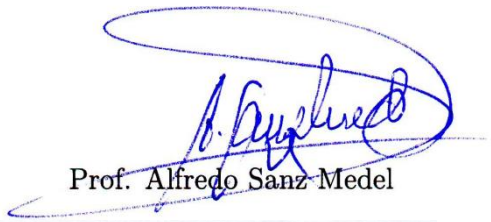
Publications indirectly related to this thesis

1. Fricker, M. B., Kutscher, D., Aeschlimann, B., Frommer, J., Dietiker, R., Günther, D.: High spatial resolution trace element analysis by LA-ICP-MS using a novel ablation cell for multiple or large samples; Int. J. Mass Spectrom. 307 (2011), 39-45

With their signature, the authors additionally contributing to the previously mentioned scientific articles included in this Ph.D thesis agree to their inclusion:

Dr. Maria Estela del Castillo Busto

Dr. Nico Zinn



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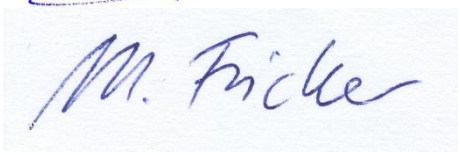

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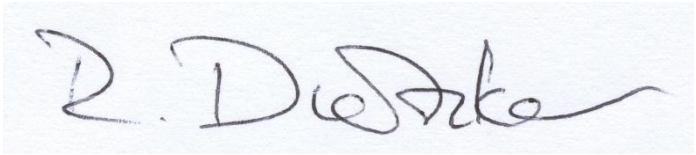
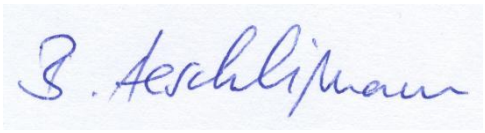


Dr. Rolf Dietiker



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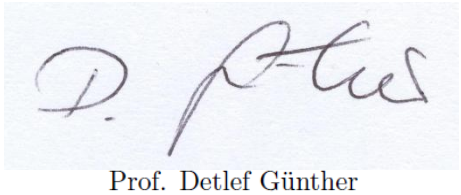

Prof. Detlef Günther

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Protein labelling with mercury tags: fundamental studies on ovalbumin derivatised with *p*-hydroxymercuribenzoic acid (pHMB)

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Protein labelling in combination with mass spectrometry is appointed as a modern approach for quantifying biopolymers, especially proteins. With respect to elemental mass spectrometry, specifically inductively coupled plasma-mass spectrometry (ICP-MS), protein labelling approaches are still scarce, although they offer many advantages, *e.g.* in terms of detection sensitivity. In this fundamental work, we present results on the labelling of ovalbumin with *p*-hydroxymercuribenzoic acid (pHMB). After optimising the derivatisation procedure, the characterisation of the labelled species is necessary, and thus, the use of molecular MS techniques like MALDI-, and ESI-MS is required. Finally, the detection capabilities of ICP-MS are evaluated on the labelled species. Important factors to consider are the reaction yield, the selectivity, and the stoichiometry of the bioconjugate. For instance, the stoichiometry of the bioconjugate is determined by comparative measurements using MALDI-, and ESI-MS. It can be demonstrated that the label/protein ratio is determined to be ~3 : 1 by MALDI-MS, which is lower than the number of expected binding sites (ovalbumin has four free sulfhydryl groups from cysteines). In contrast to these findings, the use of ESI-Q-ToF-MS with its superior mass resolution indicates a stoichiometry of 4 : 1. However, the overall strategy given here on the example of ovalbumin labelling with pHMB might be a promising approach for protein quantification as it provides a significant improvement in terms of detection limits (1 fmol for ovalbumin) in comparison to the use of sulfur as naturally occurring elemental tag.

Introduction

Quantitative proteomics has become an emerging challenge in research and routine work of biochemical and clinical areas. Herein, mass spectrometry has been considered to be an indispensable tool as it can offer both structural and quantitative data.[1,2] The latter aspect has been particularly highlighted by the development of labelling procedures with the use of isotopic codes. Isotope-coded affinity tags (ICAT)[3] provided a starting point for many developments in quantitative protein analysis. The most important and probably most promising advances later in this field are stable isotope labelling by amino acids in cell culture (SILAC)[4] and isotope tags for relative and absolute quantification (iTRAQ).[5] The isotopic labelling was mainly achieved by the use of ^{2}H, ^{13}C, ^{15}N, and ^{18}O. This restricts the application of these strategies to so-called soft ionisation sources like electrospray-ionisation (ESI) and matrix-assisted laser desorption and ionisation (MALDI).

In combination with elemental mass spectrometry, particularly ICP-MS, labelling of peptides and/or proteins aims to introduce an element with low detection limits and high sensitivity.[6] Two different labelling strategies have been explored so far and current research is recently summarised in two reviews.[6,7]

Tanner's group[8] and at the same time Zhang and colleagues[9] have developed an approach using elemental labelling of antibodies. Lanthanides[10] and/or gold nanoparticles[11] were bioconjugated with the antibodies of interests and detected by ICP-MS after sample digestion or laser ablation.[12,13] Particularly, Tanner and colleagues have recently demonstrated the feasibility of ICP-MS in a flow-cytometry based analytical strategy to monitor different biomarkers.[14,15]

The second strategy represents the direct labelling of the protein *via* well-known derivatisation reactions. In this case, the elements, usually lanthanides, were introduced into the protein or peptide in the form of chelates[16] with a reagent directly linked to a reactive group of the analyte. Either covalent binding to sulfhydryl groups[16] or to primary amines[17] have been suggested so far. The first quantitative application of this strategy by flow-injection ICP-MS detection has been recently published by Linscheid and colleagues.[18] In this study, the authors could demonstrate the attractiveness of ICP-MS in terms of low detection limits, *e.g.* 110 amol for bovine serum albumin, and its robustness in the analysis of complex matrices (porcine lens proteins). A new strategy using HPLC-ICP-MS coupling has also been described by Patel *et al.*[19] In this preliminary study, they derivatised peptides with cyclic diethylenetriaminepentaacetic anhydride (cDTPA) and subsequent labelling was carried out with natural and isotopically enriched Eu^{3+}.

However, the direct binding of a metallic tag to a protein has not been explored in detail for its application in elemental mass spectrometry. The main difference to the strategies described above is here the formation of a quasi-covalent bond between the

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metal and the reactive group of the target analyte, *e.g.* the formation of a metal–sulfur bond in the case of derivatisation of free sulfhydryl groups. This protein labelling approach has not been explored very often and it can be prone to several limitations like insufficient labelling yield, ionization suppression effects (especially in soft ionisation sources), and low chromatographic or electrophoretic separation efficiency of labelled and unlabelled compounds. Thus, several preliminary investigations are required to assess the real potential of this procedure to quantitative analyses.

The present work shows fundamental results on protein labelling using *p*-hydroxymercuribenzoic acid (pHMB). This compound has already been used for the derivatisation of metallothioneins[20] and glutathione[21] with detection by ICP-MS and atomic fluorescence spectrometry, respectively. Furthermore, the potential of using pHMB for the characterisation of sulfhydryl groups in native and denaturated proteins has been also suggested using hyphenated techniques based on hydrophobic interaction chromatography.[22,23]

In this work the labelling of ovalbumin as a standard protein will be assayed and the derivatisation procedure itself will be explored: the label/protein stoichiometry, the labelling efficiency, and the selectivity (parameters, which are necessary for further quantitative studies) will be investigated. Complementary techniques, such as MALDI-MS and ESI-MS, will be compared to characterise the bioconjugates obtained.

Experimental

Reagents

All solutions were prepared using ultra pure water (Milli-Q Water Purification System, Millipore, Bedford, MA, USA). The investigated proteins were ovalbumin (Grade V, Sigma-Aldrich, Steinheim, Germany), bovine serum albumin (≥96 %, Sigma-Aldrich), lysozyme from human neutrophils (≥95 %, Sigma-Aldrich), and insulin from bovine pancreas (Sigma-Aldrich). Protein solutions of appropriate concentrations were prepared daily in water for further investigations. The derivatisation reagent, *p*-hydroxymercuribenzoic acid (pHMB, Sigma-Aldrich), was dissolved in diluted NaOH (p.a., >99 %, Merck, Darmstadt, Germany) to a final concentration of 0.18 mg L^{-1} and diluted for the incubation experiments (**Safety note:** pHMB is very toxic!). Elemental standard solutions (1000 mg L^{-1} Hg and Rh, resp.) were purchased from Merck. Mobile phases for μLC experiments consisted of acetonitrile (VWR Prolabo Gradient Grade HiPerSolv, Leuven, Belgium) and trifluoroacetic acid (TFA, 99%+, Sigma-Aldrich). Typically, mobile phase A contained 2% acetonitrile/98% water/0.05% TFA and mobile phase B 98% acetonitrile/2% water/0.05% TFA.

The matrix solution for MALDI-MS was prepared by dissolving 5 mg of sinapinic acid (Sigma-Aldrich) in 1 mL of 30% acetonitrile and 0.1% TFA.

Instrumentation

Thermo XSeries^{II} ICP-MS (Thermo Fisher Scientific, Bremen, Germany) was used under the conditions summarised in Table 1 and was equipped with a DS-5 nebulizer (CETAC, Omaha, USA) and a self-constructed spray chamber.[24] Pt cones were used as oxygen was added into the spray chamber. μLC separations were performed with a dual piston pump with build-in solvent split (Ultimate, LC Packings, Sunnyvale, USA). A typical gradient program was a linear gradient from 20% B to 80% B. A second pump (Ultimate Switchos, LC Packings) delivered an additional flow (50% A, 50% B, 10 μg L^{-1} Rh as internal standard) and mixed with the LC eluent in a Y-connector (Upchurch Scientific, Oak Harbor, USA) in order to meet the total flow rate required for optimum nebulisation. A reversed-phase column (diphenyl, 5 μm particle size, 300 Å pore size, 150 mm × 200 μm inner diameter, GraceVydac, Columbia, USA) was used throughout this work. All connections were made of fused-silica capillaries (inner diameter: 50 μm). In Table 1 the working conditions are summarised for the μLC-ICP-MS system.

Table 1 Operating conditions of the μLC-ICP-MS coupling

ICP-MS system	
Instrument	XSeries^{II} ICP-MS
Plasma Power	1300 W
Auxiliary gas flow	0.7 L min^{-1}
Coolant gas flow	13 L min^{-1}
Sampler cone	Pt, 1.0 mm orifice
Skimmer cone	Pt, 0.7 mm orifice
Dwell time	100 ms
Isotopes monitored	^{103}Rh, ^{199}Hg, ^{200}Hg, ^{202}Hg
Nebulizer	DS-5
Spray chamber[a]	Laboratory-made
Nebulizer gas flow	0.95 L min^{-1}
Oxygen gas flow	40 mL min^{-1}
Capillary LC	
Instrument	Ultimate μLC
Chromatographic column	Diphenyl, 5 μm particle size, 300 Å pore size, 150 mm × 200 μm ID[b]
Flow rate	3–4 μL min^{-1}
Injection volume	2 μL
Additional flow	8 μL min^{-1}

[a] See ref. 24.[b] ID: inner diameter.

In order to investigate the derivatisation procedure more in detail, molecular mass spectrometric techniques needed to be applied.

MALDI-ToF measurements were carried out with a Voyager-DE-STR Workstation (Applied Biosystems, Langen, Germany) equipped with a N_2 laser (337 nm, 3 ns pulse, 1600 μJ). The observed mass range was between 5000 and 100 000 Da, further parameters were as follows: accelerating voltage 20 kV, grid 90%, and delay time 35 ns. Typically mass spectra were acquired by averaging 10 accumulated spectra of 100 single laser shots. External calibration was performed for molecular assignments using a standard of bovine serum albumin. Sample preparation for MALDI-MS measurements was performed on a stainless steel hydrophobic target (Voyager 96_2 Sample Plate P/N V700813) using the dried-droplet technique. For that, an aliquot (0.5 μL) of the sample solution and an equal aliquot of the matrix solution were mixed on the target in the given order and dried at room temperature.

The ESI instrument used for this study was a QStar XL model (Applied Biosystems) equipped with the ion spray source (voltage 5.5 kV) and using nitrogen as nebulisation gas. Mass calibration was performed daily using a standard solution of poly(propylene glycol). All proteins (labelled and unlabelled)

were reconstituted in 80% acetonitrile/0.1% TFA to an appropriate concentration and injected at a flow of 5 μL min^{-1}. The scanned mass range was chosen from *m*/*z* 500 to 4000. A Bayesian deconvolution algorithm available in the Analyst software (Applied Biosystems) was applied.

Labelling procedure

If not otherwise stated in the text, the labelling procedure was carried out as follows: freshly prepared aqueous solutions of ovalbumin were diluted to the appropriate concentration range in water. The applied molar excess of pHMB was calculated based on the number of free cysteine residues in ovalbumin (four sulfhydryl groups per protein), and the pHMB solution prepared as described above was added at room temperature. If not stated otherwise, a ten-fold excess of pHMB was used throughout the experiments (MALDI-MS or ESI-MS analysis) and the reaction time was 1 h. The excess of pHMB (500 μL sample volume) was then removed by ultrafiltration (Ultracel YM-10 centrifugal filter device, Millipore) in a MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) for 30 min at 13 500 rpm. In the case of testing the labelling efficiency in dependency of the reagent's excess (see Fig. 1b), this ultracentrifugation step was repeated after re-suspending the retentate in 300 μL water. Finally, the protein-containing residue was re-diluted in water (for MALDI-MS or ICP-MS analysis) or acetonitrile/TFA (ESI-MS analysis) for further experiments. The recovery of the bioconjugate after ultrafiltration was checked for two different protein amounts (200 fmol and 1 pmol) and was determined to be higher than 95%.

Results and discussion

The herein presented strategy using a mercury compound follows the potential labelling of free sulfhydryl groups derived from cysteines ubiquitously present in proteins. The reagent used throughout the work was *p*-hydroxymercuribenzoic acid (pHMB) and is well-known to form covalent bonds to SH groups of peptide or proteins. In the 1950s its potential of quantifying free sulfhydryl groups was already demonstrated[25] and is tested here for reproducible labelling of ovalbumin with subsequent detection and quantification by μLC-ICP-MS.

Ovalbumin (Swiss-Prot P01012) consists of 386 amino acids including six cysteines, of which two are bridged as disulfide (cys74–cys121). Furthermore, it has 16 methionines as potential reaction sites, so that overall 22 sulfur atoms are present in ovalbumin.

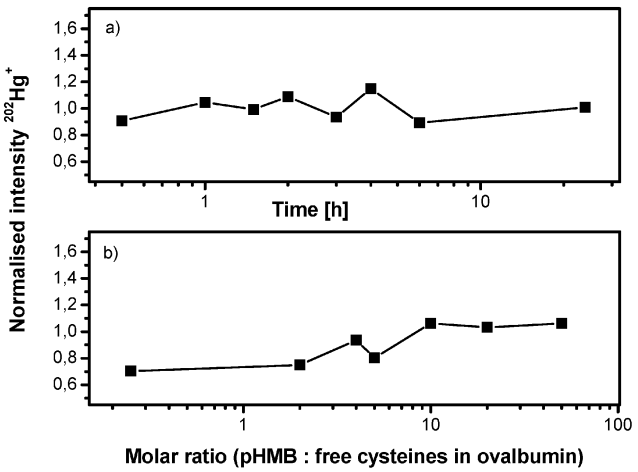

Fig. 1 Dependency of (a) the reaction time and (b) the molar excess of pHMB on the signal intensity of $^{202}Hg^+$.

Optimisation of the labelling procedure

The initial experiments of optimisation of our labelling methodology were directed to the reaction time and the stoichiometric excess of pHMB. The labelling procedure was carried out, including the ultrafiltration step, as described above. These reactions were monitored in general by total mercury analysis using ICP-MS and moreover by μLC-ICP-MS (Hg detection) in order to prove the purity of the labelled protein.

Fig. 1 shows the effect of the labelling time (Fig. 1a) and the reagent's excess (Fig. 1b) on the $^{202}Hg^+$ signal intensity obtained for the modified ovalbumin. As can be seen, the labelling time between 0.5 and 24 h (with 10-fold molar excess with respect to the number of cysteines per ovalbumin) does not influence the mercury signals significantly. The determined concentration of mercury (normalised to the amount of ovalbumin, 1 pmol) showed a variation of ±8%. From these results, it can be concluded that once the reaction has taken place on the specific sites, no further side reactions or adsorption processes seem to occur, which could then cause an uncontrollable change of the stoichiometry of the bioconjugate. Furthermore, the bioconjugate has proven to be stable for several days under storage at 4 °C. Typically, 1 h incubation reaction time was applied for further investigations.

Then, the effect of the stoichiometric excess of pHMB was investigated: at molar ratios lower than ten (referring to the free cysteine residues) the derivatisation has been determined to be incomplete, which could be observed due to the slight increase of the mercury signal (Fig. 1b), but higher ratios (here ratios up to 50 were investigated) did not influence the mercury signal significantly as the signal remained stable. In all case, μLC-ICP-MS proved that the detected mercury was exclusively bound to ovalbumin, thus, the excess of pHMB was removed quantitatively.

A typical chromatogram obtained is given in Fig. 2b using the optimised conditions for labelling. The derivatised ovalbumin elutes as a narrow peak (full width at half maximum, FWHM: 0.22 min) and only slight memory effects could be observed. As ultrafiltration was used after the labelling process, the excess of pHMB could be successfully removed. This is somehow necessary as the reagent itself showed remarkable memory effects due to its high concentration resulting in an elevated background (Fig. 2a). Furthermore, it is noteworthy that the obtained S/N ratio of the peak turned out to be 2.5 times higher after ultrafiltration, although the peak height diminished.

Stoichiometry of the bioconjugate

The artificial introduction of an elemental tag into a protein is done for two main reasons: (i) a significant improvement of the detection limits for the protein, and (ii) its application to

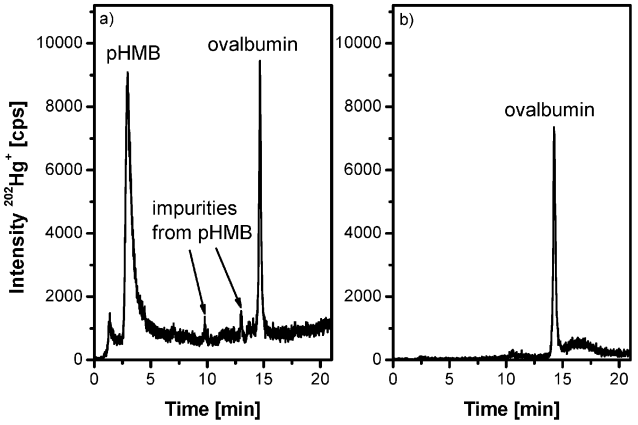

Fig. 2 Chromatograms of 200 fmol ovalbumin injected after labelling with pHMB, (a) without ultrafiltration, and (b) with ultrafiltration.

quantitative analyses. The latter requires knowledge about the stoichiometry of the bioconjugate.

In order to determine the stoichiometry, MALDI-MS was compared with ESI-MS measurements of the entire and the labelled protein. The graphs in Fig. 3 represent the MALDI-MS spectra of ovalbumin before and after labelling. The overall working conditions were kept constant for better comparability. For both, the protein and the bioconjugate, a typical distribution of the different charged species like $[M + H]^{2+}$, $[M + H]^{+}$ and $[2M + H]^{+}$ could be observed with the highest abundance of $[M + H]^{+}$. Furthermore, all observed ions showed a mass shift between the entire and derivatised protein. The mass difference was determined to be (940 ± 40) Da for the signals derived from the molecular ions ($[M + H]^{+}$ and $[M^{*}+H]^{+}$, resp.). Provided that the bond formation of a sulfhydryl group and pHMB releases one water molecule, a mass shift of 321 Da should be observed for each label introduced into the protein. Consequently, from these MALDI-MS experiments an average bioconjugate stoichiometry could be determined to be (2.9 ± 0.2) : 1 (pHMB : ovalbumin). However, it is worthwhile to mention that the peak width (FWHM) for $[M + H]^{+}$ was found to be about 350 Da for the

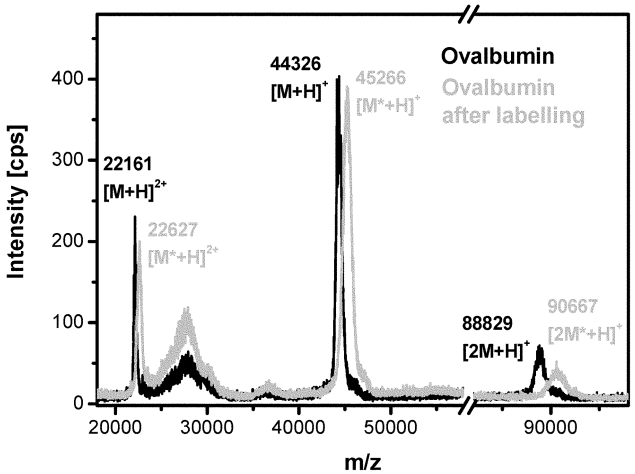

Fig. 3 MALDI-MS spectra of ovalbumin before and after labelling with pHMB.

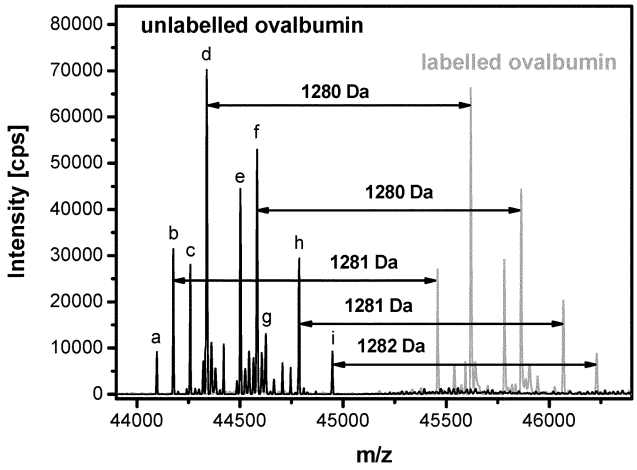

Fig. 4 ESI-MS spectra of ovalbumin before and after labelling with pHMB.

unlabelled and about 950 Da for the labelled ovalbumin. This observation might indicate that besides the micro-heterogeneities in terms of different glycoforms for ovalbumin, the labelled form showed an additional mass distribution due to the mercury tag. As the MALDI process itself can be responsible for structural artefacts, ESI-Q-ToF-MS measurements were carried out as an alternative method. Solutions of both the entire and the labelled protein were prepared in the same way (see Experimental section) and were introduced into the ESI-MS at the same concentration (~25 μmol L^{-1}) at 5 μL min^{-1}.

Fig. 4 compares the deconvoluted mass spectra obtained for the unlabelled and the labelled ovalbumin. As ESI-MS serves as an excellent tool for studies of protein glycosylation,[26,27] the structural heterogeneity of ovalbumin could be observed originated from its different glycoforms (mass range from 44 000 to 45 000 Da). A minimum of 21 peaks were detectable out of which nine could be identified by comparison with data from current literature[28] (see Table 2). The mass accuracy achieved for these glycoforms was better than 100 ppm (typically better than 50 ppm) and distinctive peak widths (FWHM) were about 5 Da, which was a significant improvement in comparison to the MALDI-MS analyses. Moreover, a similar signal pattern with comparable signal intensities at a mass shift of 1280 ± 2 Da was obtained for ovalbumin after labelling with pHMB. The difference correlates very well to a total number of four labels (1284 Da, 4 × 321 Da). This indicates that all free cysteine residues were derivatised with pHMB, which was in contrast to the results obtained by MALDI-MS. This reflects that both ionisation sources could lead to different conclusions with respect to the investigations on labelled compounds. Obviously, ESI-MS as the softer ionisation source preserves the intact bioconjugate structure,[29] while a partial degradation could be observed in MALDI-MS. Moreover, complementary experiments by μLC-ESI-MS confirmed that the bioconjugate remains stable during the chromatographic separation.

Reaction yield of the labelling procedure

Another important parameter for the application of labelling procedures in quantitative studies is the reaction yield. First

Table 2 Average masses (M_r) of observed ovalbumin glycoforms before and after labelling with pHMB

M_r/Da (ovalbumin) [a]	Calculated M_r/Da [b tab2fnc]	Glycoform [d]	M_r/Da (labelled ovalbumin) [e]	Mass difference/Da [f]
44097 (a)	44098	$Hex_5HexNAc_2$	45378	1281
44177 (b)	44180	$Hex_3HexNAc_4$	45458	1281
44259 (c)	44260	$Hex_6HexNAc_2$	45539	1280
44339 (d)	44342	$Hex_4HexNAc_4$	45619	1280
44502 (e)	44504	$Hex_5HexNAc_4$	45782	1280
44583 (f)	44587	$Hex_3HexNAc_6$	45863	1280
44665 (g)	44667	$Hex_6HexNAc_4$	45943	1278
44787 (h)	44790	$Hex_3HexNAc_7$	46068	1281
44948 (i)	44952	$Hex_4HexNAc_7$	46230	1282

[a] Characters in brackets correspond to the peaks in Fig. 4a.[b] Calculated M_r of the oligosaccharide added to M_r of ovalbumin (42881 Da) minus 18 Da (elimination of H_2O) (from ref. 28).[c] It has be to mentioned that ovalbumin is phosphorylated, so that assigned masses may also refer to phosphorylated glycoforms.[d] Hex: hexose, HexNAc: *N*-acetylhexosamine (from ref. 28).[e] M_r of ovalbumin glycoforms after labelling with pHMB.[f] Mass difference by subtracting M_r (labelled ovalbumin glycoform) from M_r (unlabelled ovalbumin glycoform).

experiments were therefore conducted by MALDI-MS, and a mass shift between the unlabelled and labelled protein could be observed as explained in the previous section. However, these MALDI-MS data (Fig. 3) could only serve for a rough estimation due to the limited mass resolution, so that a complementary strategy needed to be applied.

ESI-Q-ToF-MS was applied to follow the reaction yield for the derivatisation under optimised reaction conditions. Fig. 5 represents the deconvoluted mass spectrum of the labelled ovalbumin. In the mass range 45 300 to 46 300 Da several intense signals were obtained, which could be referred to different glycoforms of ovalbumin (see Table 2). However, there were no detectable signals corresponding to those species in the mass range between 43 900 and 45 100 Da (see enlarged mass spectrum in Fig. 5), in which the native (and/or partially labelled) ovalbumin would be expected. The signals visible in the enlarged mass spectrum, *e.g.* the most abundant one at *m*/*z* 44 548 Da, did not overlap with any of the previously detected ovalbumin glycoforms, even under consideration of the worst mass accuracy (100 ppm). These findings indicate that the proposed labelling procedure itself did not only provide a high selectivity in terms of bioconjugate stoichiometry as mentioned before, but also a high reaction yield.

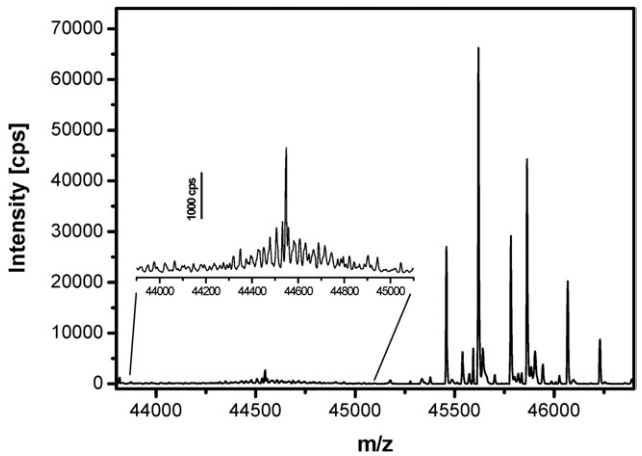

Fig. 5 ESI-MS spectrum of ovalbumin after labelling with pHMB with the expanded mass region between 43 900 and 45 100 Da.

However, it has to be kept in mind that different ionisation efficiencies in electrospray ionisation can be expected for the entire and derivatised protein, so that quantitative conclusions are difficult to derive exclusively from the ESI-MS signal intensities. As any signal at all was absent in the expected mass range (Fig. 5) and as the ESI-MS signal intensities for both the entire and the labelled ovalbumin were comparable at equivalent concentrations (Fig. 4), we assumed in this case an excellent labelling efficiency (here estimated to be higher than 95%). This might be very sufficient for a sensitive determination of ovalbumin by μLC-ICP-MS.

Selectivity of the labelling procedure

As ovalbumin represents a protein with different glycoforms, a first indication could be given that the labelling procedure (in terms of stoichiometry) did not depend on their occurrence. Clearly, the binding sites in ovalbumin for pHMB are the free sulfhydryl groups of cysteines.[25] In order to substantiate this statement we incubated further proteins, which do not contain reactive groups for pHMB. On the example of human lysozyme (Swiss-Prot P61626) and insulin from bovine pancreas (Swiss-Prot P01317) (both proteins do not contain any free sulfhydryl groups), ESI-MS was applied to study the reaction of the protein and the labelling reagent. In both cases, it could be proven that no reaction took place under the optimised derivatisation conditions for ovalbumin. This finding was confirmed by μLC-ICP-MS measurements, in which consequently no signal in the mercury trace could be detected for these proteins.

Once the bioconjugate has been fully characterised, the labelling procedure and its subsequent analysis by μLC-ICP-MS showed satisfactory results in terms of repeatability (200 fmol ovalbumin, $N = 6$, 3.3% peak height, 4.2% peak area). So far, the detection limit (D.L.) for ovalbumin was determined to be 1 fmol abs. (3σ criterion, relative D.L.: 0.5 nmol L^{-1} based on 2 μL injection volume) and presents a significant improvement in comparison to other ICP-MS based methods. As an example, post-column isotope dilution of sulfur by μLC-ICP-SFMS was applied to the determination of ovalbumin, and reported detection limits were 500 fmol.[24] Nevertheless, extensive research work is required in order to apply the proposed labelling procedure to the quantitative analysis of proteins in real and more complex samples.

Overall, these investigations are a striking example of the potential of the use of complementary mass spectrometric techniques on the characterisation of this type of bioconjugate.

Conclusions

Fundamental studies were carried out on the labelling procedure of ovalbumin with *p*-hydroxymercuribenzoic acid (pHMB). It could be demonstrated that pHMB represents a feasible labelling reagent for ovalbumin in terms of labelling efficiency (reaction yield), stoichiometry, selectivity, and repeatability.

The main benefit achieved was the tremendous decrease of the detection limits (1 fmol abs. for ovalbumin, this work) in comparison to ICP-MS detection of sulfur (500 fmol abs. for ovalbumin).[24] In combination with further pre-concentration steps, these detection limits might allow in future the detection of low-abundant proteins in the attomol range, a concentration range of high interest in quantitative proteomic studies.[30]

We suggest the complementary use of elemental (ICP-MS) and molecular mass spectrometric techniques (especially ESI-MS) for the detailed evaluation of any labelling procedure intended to be used in quantitative protein analysis.

In general, fundamental studies as presented within this work are likely to be absolutely mandatory for any labelling procedure applied to quantitative protein analysis. The detailed knowledge about the bioconjugate stoichiometry and the labelling efficiency forms the basis for any further quantitative studies. However, these parameters have to be checked very carefully in the case of the analysis of complex biological samples, as the presence of interfering biomolecules, *e.g.* other sulfhydryl-containing proteins, might influence the labelling reaction itself.

It should be mentioned here that labelling strategies (following fundamental investigations as suggested here) might open the door for ICP-MS based hyphenated techniques in absolute quantitative proteomics in the low concentration range. Nevertheless, they do not automatically provide accurate results, unless the whole analytical process (including sample preparation, pre-concentration, and chromatographic steps) has been evaluated for the recovery of each individual protein. Thus, further studies should reveal whether the presented labelling strategy can be implemented into a general quantitative proteomic approach including multi-dimensional separation techniques.

Acknowledgements

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Absolute and Relative Protein Quantification with the Use of Isotopically Labeled *p*-Hydroxymercuribenzoic Acid and Complementary MALDI-MS and ICPMS Detection

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Chemical labeling with subsequent mass spectrometric detection represents a common approach for protein quantification. Whereas most methods make use of stable isotope labels from natural elements such as ^{2}D, ^{13}C, ^{15}N, or ^{18}O, artificially introduced metals have gained interest as alternative markers. In this work we present the application of *p*-hydroxymercuribenzoic acid (pHMB) as a labeling reagent for cysteine-containing proteins. As a proof of concept, insulin was chosen as the model protein, and two different workflows were developed to its absolute and relative quantification with the use of complementary MALDI-MS and ICPMS. On the basis of the synthesis of isotopically labeled [^{199}Hg]pHMB, and thus on the basis of the label-specific isotope dilution concept, a differential labeling procedure can be applied either to the comparative study of two different samples (relative quantification) or to the absolute quantification of insulin. In both cases, final detection by MALDI-MS followed by isotope pattern deconvolution was applied to extract the quantitative data from the mass spectra. Good agreement with the expected values was obtained for the relative insulin quantification, and the recovery for insulin applying the absolute quantification workflow was between 90% and 110% with an RSD of better than 5% in the low picomole range.

Current quantitative approaches based on mass spectrometric detection predominantly make use of different labeling strategies, in which internal standardization of amino acids is achieved by chemical, enzymatic, or metabolic labeling.[1,2] SILAC (stable isotope labeling in cell culture)[3] and enzyme-catalyzed $^{18}O_2$-labeling[4] are two well-established concepts representing the two latter ways of labeling, whereas chemical labeling concepts are exceedingly manifold due to the huge variety of labeling reagents developed.[5] Principally derived from ICAT (isotope-coded affinity tags),[6] several methodologies have been developed for relative and absolute quantification purposes, in which the concept of iTRAQ (isobaric tag for relative and absolute quantitation) plays nowadays a major role.[7,8] Recently, Brun et al. suggested the application of in vitro-synthesized isotopically labeled full-length proteins, so-called protein standard absolute quantification (PSAQ).[9] The authors pointed out that this type of labeling provides accurate absolute protein quantification as the used protein standards perfectly match the biochemical behavior of the target analytes. In general, all these methods have in common that the earlier the isotope dilution step takes part in the workflow, the better the accuracy which can be obtained for the analytical results.[2,10] Referring to chemical labeling procedures, labeling at the protein level before subsequent enzymatic digestions might be therefore superior.

Although the relative aspects of protein analysis have been tackled with analytical strategies such as ICAT, absolute protein quantification remains rather difficult. Trying to solve such a fundamental challenge, new isotope/elemental labeling methods in conjunction with mass spectrometric tools have been explored; among them the potential of metal tags has been recognized.[11−13] The common way here is mainly represented by the use of lanthanide ions chemically introduced into the protein or peptide of interest[14−16] or into a corresponding antibody in the form of

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complexes of DOTA, DPTA, etc.[17−21] Metal tags offer the added value that they are detectable not only by ESI- and MALDI-MS, but moreover by complementary elemental MS techniques such as inductively coupled plasma mass spectrometry (ICPMS).

In this paper we present as a proof of concept an alternative route for relative and absolute protein quantification based on protein labeling with metal tags, namely, mercury-containing tags. Proteins are specifically labeled at available sulfhydryl groups of cysteine (present in about 70% of all proteins in a certain proteome) with *p*-hydroxymercuribenzoic acid (pHMB), a compound which was already used more than 50 years ago[22] for the determination of "free" sulfhydryl groups in proteins, and which has been recently recovered for analytical purposes.[23−25] On the basis of the synthesis of ^{199}Hg-enriched pHMB, the current approach is divided into two different steps. The first follows the concept of differential isotope labeling for comparing two different samples (relative quantification) and is here demonstrated on insulin as a model protein by the use of MALDI-MS and following isotope pattern deconvolution. To extend the approach to absolute protein quantification, isotopically labeled insulin was characterized quantitatively by ICPMS and added to the sample as an internal standard throughout the proteomic workflow. The main advantage over other approaches based on isotopic labeling can be seen in the fact that the mercury tag contains the necessary information for consequent absolute quantifications in terms of isotopic composition and due to a previously determined mercury−protein stoichiometry the absolute protein concentration added as an internal standard.

EXPERIMENTAL SECTION

Reagents. All solutions were prepared using ultrapure water (Milli-Q Water Purification System, Millipore, Bedford, MA). Insulin from bovine pancreas (UniProtKB/Swiss-Prot P01317) and human insulin (UniProtKB/Swiss-Prot P01308, recombinant, expressed in yeast) (both obtained from Sigma-Aldrich, Steinheim, Germany) were used as standard proteins to prove the described concept. Protein solutions of appropriate concentrations were daily prepared in 6 mol L^{-1} urea (Acros Organics, Geel, Belgium). The derivatization reagent pHMB (Sigma-Aldrich) was dissolved in diluted NaOH (p.a., >99%; Merck, Darmstadt, Germany) and 6 mol L^{-1} urea to a final concentration of 25 mg mL^{-1} and used without further dilution before the incubation experiments. *Safety note: pHMB is very toxic!* 4-Carboxyphenylboronic acid (Strem Chemicals, Kehl, Germany) was used for the synthesis of the isotopically labeled mercury tag. ^{199}HgO was obtained from Chemgas (Boulogne, France) and had the following isotopic composition: ^{196}Hg, 0.11%; ^{198}Hg, 1.58%; ^{199}Hg, 91.71%; ^{200}Hg, 4.87%; ^{201}Hg, 0.73%; ^{202}Hg, 0.87%; ^{204}Hg, 0.13%. For reverse isotope dilution analysis of the bioconjugate, aqueous mercury standard solution (2% nitric acid) was used after appropriate dilution (1000 mg L^{-1} Hg; Merck, Darmstadt, Germany).

Mobile phases for μLC experiments consisted of acetonitrile (VWR Prolabo Gradient grade HiPerSolv, Leuven, Belgium) and trifluoroacetic acid (TFA; 99+%, Sigma-Aldrich). 1,4-Dithiothreitol (DTT; >99%) as a reducing agent was obtained from Sigma-Aldrich. *Safety note: DTT is toxic!*

The matrix solution for MALDI-MS was prepared by dissolving 5 mg of α-cyano-4-hydroxycinnamic acid (CHCA; Bruker Daltonics, Bremen, Germany) in 1 mL of 50% acetonitrile and 0.1% TFA.

Instrumentation. *MALDI-MS Experiments.* The MALDI-ToF instrument was a Voyager-DE-STR workstation (Applied Biosystems, Langen, Germany) equipped with a N_2 laser (337 nm, 3 ns pulse). For the analysis of labeled insulin the mass range was set between 700 and 6000 Da. Other parameters were as follows: accelerating voltage 20 kV, grid 90%, and delay time 35 ns. Typically mass spectra were acquired by averaging the spectra of 250 single laser shots. Sample preparation for MALDI-MS measurements was performed on a stainless steel hydrophobic target (Voyager 96_2 sample plate P/N V700813) using the dried-droplet technique. For that, equal aliquots of the sample solution and matrix solution were mixed and pipetted (1 μL) onto the target and dried at room temperature. Mass calibration was performed using a mixture of standard peptides (angiotensin I, adrenocorticotropic hormone fragments 1−17, 18−39, and 7−38). Mass resolution was found to be in the range of 8000−13000 (fwhm), and the mass accuracy was better than 90 ppm.

Quantitative information was generated out of the mass spectra by the use of a least-squares algorithm analyzing the superimposed isotope pattern. For this, the observed signal pattern was baseline corrected and the peak area corresponding to every signal was integrated and normalized to 1 to determine the relative abundances of the signals.[26,27] Typically, at least four independent spectra from different spots were analyzed.

ESI-MS. A QStar XL model (Applied Biosystems) was equipped with the ion spray source (voltage 5.5 kV) and using nitrogen as the nebulization gas. Mass calibration was performed daily using a standard solution of poly(propylene glycol). Insulin samples were reconstituted in 72% acetonitrile/0.5% acetic acid/0.1% TFA to an appropriate concentration (typically nmol L^{-1} to μmol L^{-1}) and injected at a flow rate of 5 μL min^{-1}. The scanned mass range was chosen from m/z 500 to m/z 4000. A Bayesian deconvolution algorithm available in the Analyst software (Applied Biosystems) was applied.

ICPMS Analyses. A Thermo XSeries^{II} ICPMS instrument (Thermo Fisher Scientific, Bremen, Germany) was used under the conditions summarized in a previous paper[25] and was equipped with a DS-5 nebulizer (CETAC, Omaha, NE) and a

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self-constructed spray chamber.[28] Pt cones were used as oxygen was added into the spray chamber in the case of μLC separation. For SEC separations (BioSep-SEC-S3000, 300 × 7.8 mm, 5 μm particle size, 290 Å pore size; Phenomenex, Aschaffenburg, Germany), regular Ni cones and no oxygen addition were applied.

Isotope analysis of Hg (all Hg isotopes were monitored in this case) was performed with a Meinhard nebulizer and a conical glass spray chamber containing an impact bead (Thermo Fisher Scientific). All solutions were prepared in 2% nitric acid. ICPMS parameters: forward power 1400 W, nebulizer gas flow 0.96 L min^{-1}, dwell time per isotope 100 ms. Typically 3 main runs with 100 repetitions per run were acquired for statistical reasons.

For μLC−ICPMS analysis (diphenyl, 5 μm particle size, 300 Å pore size, 150 mm × 300 μm inner diameter; GraceVydac, Columbia, MD), typical ICPMS parameters were forward power 1400 W, nebulizer gas flow 0.8 L min^{-1} argon, 40 mL min^{-1} oxygen, and dwell time per isotope 100 ms.

μLC separations were performed with a dual piston pump with build-in solvent split (Ultimate, LC Packings, Sunnyvale, CA). Typically, mobile phase A contained 2% acetonitrile/98% water/0.05% TFA and mobile phase B 98% acetonitrile/2% water/0.05% TFA. The gradient program was a linear gradient from 0% B to 80% B. A second pump (Ultimate Switchos, LC Packings) delivered an additional flow (50% A, 50% B, 20 μg L^{-1} Rh as the internal standard) and mixed with the LC eluent in a Y-connector (Upchurch Scientific, Oak Harbor, WA) to meet a total flow rate required for optimum nebulization.[28] Flow rates of the μLC system were 3.5 μL min^{-1} from the column and 8 μL min^{-1} for the makeup flow.

RESULTS AND DISCUSSION

The application of chemical labeling processes for quantitative purposes requires, in general, the evaluation of the chemical process in terms of reaction yield and identification of the reaction products. As already shown on the example of different standard proteins,[24,25] the reaction efficiency of pHMB is very close to quantitative for the derivatization of cysteine-containing analytes. In this work, bovine and human insulin were used for further investigations, and the first step was again the identification of the bioconjugate. The derivatization was carried out as described before.[25] Briefly, the protein was reduced with use of DTT, and a 10-fold molar excess (with respect to the free cysteine groups) of pHMB was added and incubated for 1 h at room temperature. The excess of the reagent was finally removed by 2-fold ultrafiltration (3 kDa). The purified adducts were analyzed by both ESI-MS and MALDI-MS, illustrated in Figure 1. The ESI-MS spectrum of labeled bovine insulin shows a series of multiply charged (3+ to 5+) ions of the fully labeled insulin A chain (4-fold derivatized by pHMB) and the labeled insulin B chain (2-fold labeled with pHMB). Sodium adducts can be observed as well (especially for triply charged species), whereas the abundance of unlabeled or partially labeled species is very low, so that a reaction yield can be derived from these data to be >97%. However, in the MALDI-MS spectrum (Figure 1b) only signals from the insulin B chain could be found under the chosen experimental conditions (positive ion detection),

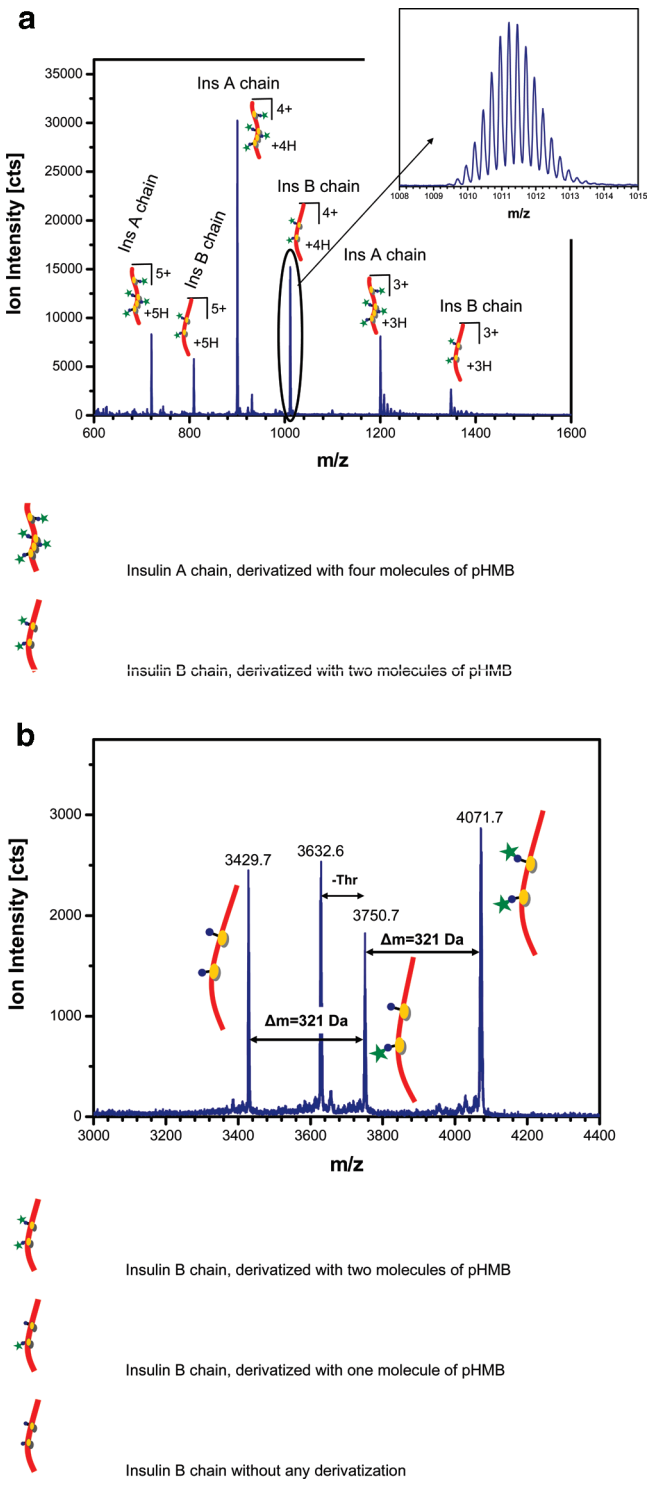

Figure 1. (a) ESI-qToF-MS spectrum of labeled bovine insulin (P01317) and (b) MALDI-ToF-MS of labeled human insulin (P01308).

which is in agreement with earlier studies.[29] Furthermore, the MALDI ionization process can be regarded as more destructive for these labeled species. In contrast to ESI, the insulin B chain was found in its unlabeled and singly labeled forms, indicating cleavages of the mercury−sulfur bond at an elevated level. However, with regard to the planned application of isotope dilution analysis for quantitative studies the observed cleavage should not

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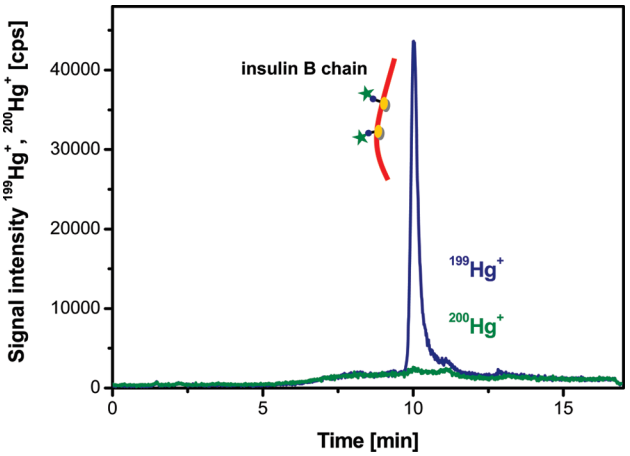

Figure 2. μLC−ICPMS chromatogram of purified [^{199}Hg]pHMB−insulin B chain.

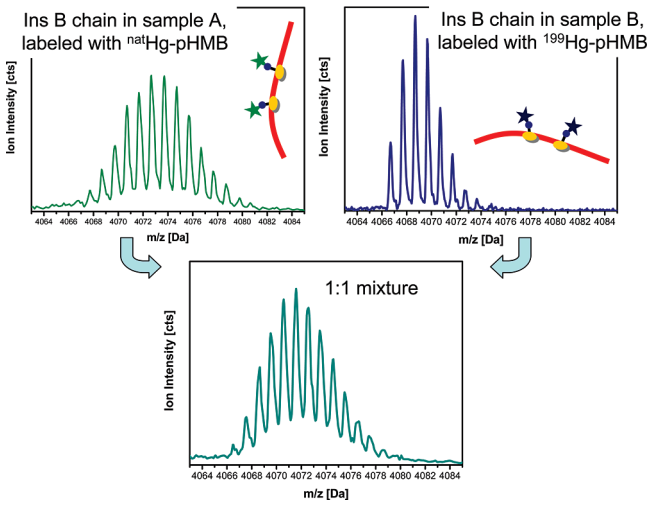

Figure 3. Isotope patterns of the quasi-molecular ions of fully labeled human insulin B chain: top left, mass spectrum of insulin B chain labeled with [^{nat}Hg]pHMB; top right, mass spectrum of insulin B chain labeled with [^{199}Hg]pHMB; bottom, mass spectrum of a 1:1 mixture.

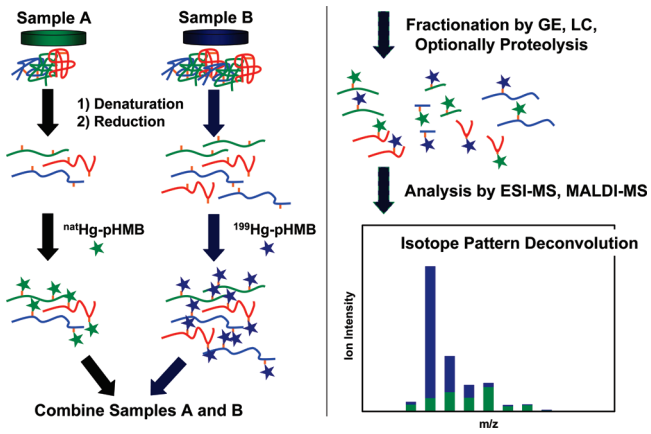

Figure 4. Workflow for relative quantification purposes using label-specific isotope dilution and MALDI-MS detection.

influence the accuracy of the results. Once the isotope dilution step occurs, the isotopically labeled species are affected to the same extent as the protein derivatives with the mercury tag with natural isotopic composition. Thus, any cleavage induced by the ionization process will not influence the isotope ratios monitored, but will influence the absolute signal intensity. As a consequence, further studies within this work were carried out with the aid of MALDI-MS, although it has to be mentioned that ESI-MS can principally be very useful for the same approach.

A major step for a subsequent label-specific isotope dilution approach was the synthesis of the isotopically enriched label. For that, the two-step synthesis (see the Supporting Information) was carried out with enriched ^{199}Hg. In a preliminary experiment, the labeling efficiency of [^{199}Hg]pHMB was tested with human insulin after reduction with DTT and the bioconjugate (in this case labeled insulin B chain) was purified by ultrafiltration and SEC. A typical chromatogram obtained by μLC−ICPMS is shown in Figure 2, in which only a signal for the derivatized polypeptide appeared as expected. The isotope ratio (^{199}Hg:^{200}Hg) of the detected peak was determined to be 18.1, which was in good agreement with the expected one (R_{exp} = 18.8). Furthermore, MALDI-MS spectra of the derivatized human insulin B chain were recorded (Figure 3, top right). The observed isotope pattern dominated by the presence of ^{199}Hg was in excellent agreement with the expected one and could be easily distinguished from the polypeptide derivatized with ^{nat}Hg (Figure 3, top left). As observed in Figure 1b, cleavage products could also be detected in the case of the isotopically enriched label (spectrum not shown here).

These basic results already proved the principal applicability in further studies using ICPMS and label-specific isotope dilution. However, it has to be kept in mind that any quantitative study in complex samples based on this type of isotope dilution analysis requires chromatographic peak purity. Any coeluting mercury-containing compound, which cannot be discovered solely by ICPMS, potentially originating from other derivatized proteins with free sulfhydryl groups will influence the isotope ratio and, thus, significantly the accuracy of the analytical result. As a consequence, a complementary technique, preferably molecular mass spectrometry, needs to be employed for further quantitative studies based on label-specific isotope dilution as they can provide the detection and identification of the coeluting species. Moreover, the isotope pattern, e.g., observed in MALDI-MS, should provide quantitative information by the use of isotope pattern deconvolution.[26,27]

The general concept for relative quantification studies[6] involves principally the comparative analysis of two different and independent samples (e.g., cell tissues, body fluids) with respect to the abundance of one or more proteins. The workflow using the labeling with pHMB is represented in Figure 4. Two samples (for instance, sample A and sample B) were treated under exactly the same conditions with respect to protein denaturation and reduction as described in the Experimental Section. The following derivatization step involved the protein labeling in sample A with [^{nat}Hg]pHMB, whereas sample B was incubated with [^{199}Hg]-pHMB. Afterward, the excess labeling reagent was removed by ultrafiltration in each sample. The two purified samples were then combined, resulting in the isotope dilution step. Once a complete mixture was achieved, any analyte loss being very probable during the subsequent analytical steps[28,30] (e.g., multidimensional chromatography) would not affect the accuracy of the analytical result, but would affect the detection limit.

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Table 1. Results for the Relative Quantification of Human Insulin Using MALDI-MS with Subsequent Isotope Pattern Deconvolution

ratio (sample A:sample B)	determined ratio ($n = 3$)	SD (%)
1:1	0.89	10
1:2	0.52	5
1:3	0.39	7
1:10	0.11	10

However, the presented workflow was applied to the relative quantification of a standard solution containing different concentrations of human insulin. A typical isotope pattern is shown in Figure 3 (bottom spectrum), which was obtained from two samples with the same concentration. The spectrum indicates clearly that the isotope pattern was a composite of the two differentially labeled samples (with [^{nat}Hg]pHMB and [^{199}Hg]-pHMB, respectively). With the application of isotope pattern deconvolution (which represents a mathematical tool for calculating the molar fractions of the contributing isotope patterns[26,27]) a calculated ratio (sample A:sample B) of 0.89 was obtained. Further results are summarized in Table 1 and showed good agreement with the expected values. The relative standard deviations were found to be below 10% and are mainly affected by the manual evaluation of the peak areas. Although the MALDI process induced cleavages of the mercury−sulfur bonds within the labeled insulin B chain, they should not influence the quality of the results as the isotope dilution step occurred before. Thus, isotope pattern deconvolution could be applied not only to the quasi-molecular peak (at m/z 4071) for the quantitative elucidation, but also to the singly labeled species at m/z 3750 (Figure 1b). In our experiments, the obtained results for both different isotope patterns revealed consistency. However, if any disagreement is observed, it will be a clear indication that the labeling procedure in the two different samples had varying efficiencies. In the case of insulin and other proteins, which contain more than one available sulfhydryl group, the revision of the isotope patterns in two differently labeled species can give an added value on the labeling procedure and, consequently, on the accuracy of the obtained results. In any case, these initial results demonstrate that the suggested label-specific isotope dilution might serve as an attractive alternative for relative protein quantifications.

Besides the manifold application of relative quantification approaches, there is an increasing need for reliable absolute quantification methods. This work depicts the seminal usefulness of combining label-specific isotope dilution analysis using metal labels (in this case pHMB) and the complementary use of elemental and molecular mass spectrometry. The suggested workflow for implementing this approach into a scheme for absolute protein quantification is shown in Figure 5. Following denaturation and reduction of the protein mixture, a standard protein, which was labeled with [^{199}Hg]pHMB, was added to the sample. This represents the decisive step within the whole workflow as the added bioconjugate could be quantified individually and independently by reverse isotope dilution analysis as described in the following.

The ^{199}Hg-labeled standard protein, here human insulin B chain, was first purified with repeated ultrafiltration and size-exclusion chromatography and characterized in terms of its

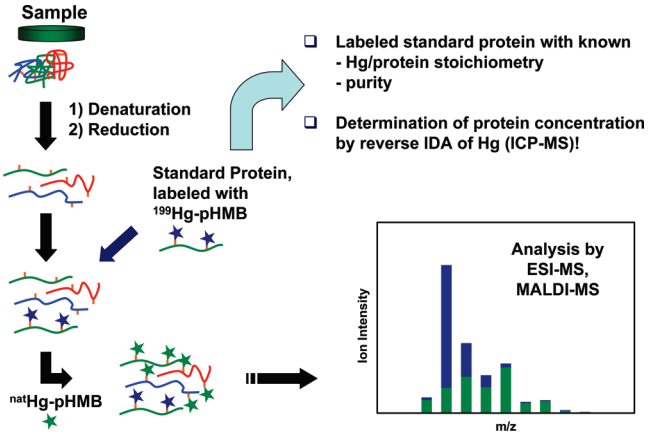

Figure 5. Proposed workflow for absolute quantification purposes using label-specific isotope dilution and MALDI-MS detection.

labeling stoichiometry by ESI-MS (two pHMB molecules per insulin B chain), its purity by μLC−ICPMS, and its mercury isotopic composition (see the Experimental Section). The missing and decisive parameter, the concentration of the added protein, could now be determined by reverse isotope dilution analysis of the purified bioconjugate, a common and highly accurate quantification method for isotopically labeled species.[31,32] Briefly, an aliquot of the purified bioconjugate was digested with concentrated HNO_3, and after addition of a mercury standard with natural isotopic composition and known concentration, the unknown concentration of the isotopically labeled mercury originating from the bioconjugate could be determined (Figure 5). As a result the added protein concentration could be determined with the knowledge of the bioncojugate stoichiometry and a typical precision of better than 5%.

After addition of the ^{199}Hg-labeled standard protein the proteins present in the sample were labeled with [^{nat}Hg]pHMB (Figure 5). Further processing on the protein level involved the purification of the sample including multidimensional chromatography (SEC and RP-LC), but also gel electrophoresis in combination with enzymatic digestion might serve as a suitable technique for future investigations. Final detection was again achieved by MALDI-MS of the quasi-molecular ion of the 2-fold-labeled insulin B chain with subsequent isotope pattern deconvolution. The results obtained are summarized in Figure 6. In the low picomole range a good agreement with expected values could be obtained, which emphasized that any losses during the analytical process can be compensated with label-specific isotope dilution. The achievable detection limits were in the subpicomole range for MALDI-MS and in the subfemtomole range for ICPMS detection, which were of the same order of magnitude as reported for other ICPMS-based approaches.[15,16] These initial results already demonstrate the future potential of the complementary mass spectrometry in protein quantification studies.

CONCLUSIONS

Complementary mass spectrometric techniques, namely, MALDI-MS and ICPMS, were used for protein quantification based on isotopically enriched mercury labels. The labeling strategy was

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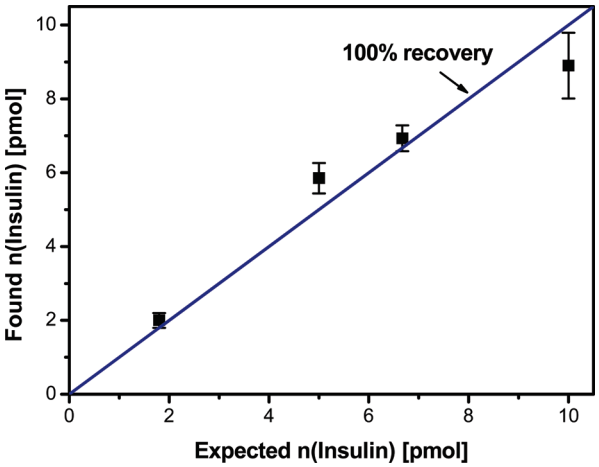

Figure 6. Results for recovery experiments for the absolute quantification of human insulin.

tested for its use in relative quantification approaches as it is often applied in protein analysis and, furthermore, for absolute protein quantification. The latter concept involved the development of label-specific isotope dilution analysis, which was realized by the synthesis of ^{199}Hg-enriched pHMB. Proteins, here demonstrated with the example of human insulin, labeled with either [^{199}Hg]pHMB or [^{nat}Hg]pHMB, could easily be distinguished by the observed isotope pattern provided by MALDI-MS. After deconvolution of the isotope pattern quantitative results were obtained for the analysis of the standard sample. An important benefit from this work can be seen in that the isotopically labeled protein used as the internal standard can be independently quantified by ICPMS on the basis of reverse isotope dilution analysis, whereas molecular mass spectrometry can serve for final detection and quantification purposes.

However, the presented approach is currently restricted to the analysis of proteins, which contain available sulfhydryl groups, and which are available as standards for subsequent derivatization, and future investigations need to demonstrate its full potential for the analysis of complex biological samples.

Generally speaking, as demonstrated here with the example of labeling with pHMB the application of metal-containing labels can principally serve as an attractive approach for the relative, and moreover the absolute, quantification.[13,33−35] Many metals, except for monoisotopic ones, fulfill all requirements necessary for chemical isotopic labeling using mass spectrometric approaches. Thus, future label-specific isotope dilution analysis might also cover the application of metal-containing tags besides the common use of ^{2}D, ^{13}C, ^{15}N, or ^{18}O. The main advantage of this general strategy can be seen in the use of ICPMS detection—as a tool complementary to MALDI- and ESI-MS—for quantitative purposes[36] and in that ICPMS allows a continuous monitoring and thus control of the isotopically enriched label during all analytical steps.

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SUPPORTING INFORMATION AVAILABLE

Description of the synthesis of ^{199}Hg-enriched pHMB and characterization of pHMB. This material is available free of charge via the Internet at http://pubs.acs.org.

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ORIGINAL PAPER

Systematic studies on the determination of Hg-labelled proteins using laser ablation-ICPMS and isotope dilution analysis

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Abstract A method was developed for the precise and accurate determination of ovalbumin labelled with *p*-hydroxymercuribenzoic acid (pHMB) using polyacrylamide gel electrophoresis with ns-laser ablation–inductively coupled plasma mass spectrometry. Following systematic optimisation of the ablation process in terms of detection sensitivity, two different quantification strategies were applied: external calibration using standards of the derivatized protein after $^{13}C^{+}$ normalization and, as a proof of concept, label-specific isotope dilution analysis (IDA) using pHMB enriched in the isotope ^{199}Hg. Due to the inhomogeneous distribution of the protein within the gel bands, it could be demonstrated that the IDA approach was superior in terms of precision and accuracy. Furthermore, it permits a reliable quantification, if more complex separation protocols are applied, as typically occurring analyte loss and degradation can be compensated for as soon as complete mixture of spike and sample is achieved. The estimated limit of detection was 160 fmol in the case of ovalbumin. In contrast to earlier studies using metals naturally present in proteins, no loss of mercury was observed during separation under denaturing conditions and other sample preparation steps. Using label-specific IDA, the measured isotope ratios in the gel corresponded to recoveries between 95% and 103%.

Keywords ICPMS · Laser ablation · Gel electrophoresis · Protein quantification · Isotope dilution analysis · Protein labelling

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Introduction

Gel electrophoresis is one of the most applied separation techniques for protein samples in biochemistry and molecular biology. It is widely employed as a first-dimension separation for the investigation of protein expression in biological systems. Besides the classical detection methods such as gel staining with dyes and fluorophores, mass spectrometry (MS)-based methods have gained interest [1], since different ways of labelling (for example metabolic or chemical labelling) were described that allow unambiguous identification and mostly relative quantification of the proteins at the same time [2]. These approaches were usually developed for so-called soft ionisation sources such as electrospray ionisation (ESI)-MS or matrix-assisted laser desorption ionisation (MALDI)-MS.

An appealing alternative for the quantification of proteins is the use of elemental MS, especially inductively coupled plasma (ICP)-MS [3]. In recent years, its potential for protein quantification has attracted a lot of attention [4]. Besides heteroatoms like phosphorous and sulfur, metals were frequently detected and quantified in different types of proteins [5].

Laser ablation (LA) is the method of choice for solid sampling in ICPMS. Since its introduction for the ablation of metal-containing proteins separated in polyacrylamide

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gel electrophoresis (PAGE) [6], the combination of PAGE and LA-ICPMS was widely applied for the detection of different elements present in proteins, either heteroelements like phosphorous [7–9] and selenium [10] or metals such as Cu, Zn and Cd [11, 12]. However, LA is prone to elemental fractionation and other matrix effects so that accurate quantification still remains difficult. Several approaches for quantification have been published in recent years. Already the first publication on this technique suggested the use of element doped gels as standards for external calibration [6]. Although the authors achieved a good calibration precision of 6% RSD, this calibration approach does not take into account the possibility of inhomogeneous distribution of the protein within the gel, and therefore, may lead to greater uncertainty and inaccuracy of the quantification. Also, the use of liquid standards has been suggested [8]. This however, besides the aforementioned problems of the heterogeneous distribution, the different characteristics of a nebulised solution and the laser-generated aerosol in an ICP, leads to another significant source of uncertainty. Another approach frequently used for LA is to use the ion signal of a matrix element as internal standard, in the case of PAGE $^{13}C^{+}$ [10]. However, the internal standard and the analyte have to enter the ICP in the same form, which might not always be given in the case of carbon and other analytes (Frick, unpublished results). Therefore, different results using C as internal standard have been observed.

On the other hand, isotope dilution analysis (IDA) was demonstrated to be a useful tool for accurate quantification with LA-ICPMS sampling [13–15], and a strong impact of IDA can be expected in the field of absolute protein quantification [16, 17]. Recently, the use of species-specific IDA was described for the quantification of superoxide dismutase (SOD) using non-denaturing GE-LA-ICPMS [18]. As the isotopically enriched spike, in this case SOD enriched in ^{65}Cu and ^{68}Zn, was chemically identical, instrumental drifts, e.g. during the ablation process and the aerosol transport, could be compensated. Furthermore, this type of internal standardisation allowed correcting for any loss during the separation process and especially for inhomogeneous protein distribution within the gel spots or bands. In general, this approach is limited to the analysis of thermodynamically and kinetically stable metalloproteins, so that its application might be restricted to a relatively small number of analytes.

In order to broaden the applicability of ICPMS-based methods in quantitative protein studies, concepts were developed for their sensitive detection after chemical labelling with an ICPMS detectable element [19, 20], Several methods were suggested, either using the labelling of antibodies [21] or the direct labelling of proteins or peptides [22]. In contrast to metals naturally incorporated in a protein, these labels can exhibit a higher stability under typically applied separation conditions, as for example, denaturing SDS-PAGE. Among these labelling approaches, mercury compounds have been investigated extensively [22–25]. One of the most widely used compounds is *p*-hydroxymercuribenzoic acid (pHMB). Due to the highly covalent binding of mercury to free sulfhydryl groups in proteins and the relatively small size of the derivatisation agent, complete derivatisation is achieved under mild reaction conditions. Characterisation of the resulting bioconjugates is possible using molecular MS-techniques such as electrospray- or MALDI-MS.

In this study, a method was developed for the sensitive detection of ovalbumin from PAGE gels after labelling with pHMB. Ovalbumin was chosen as a model protein, as earlier studies already showed that treatment with pHMB resulted in specific and selective derivatisation of the four sulfhydryl groups theoretically available [23]. The LA process was first optimized in order to achieve high sensitivity and spatial resolution of separated compounds. In order to explore the potential of label-specific IDA for accurate quantification, it was compared to external calibration using standard solutions of the labelled protein. Overall, this study represents a proof of concept of the potential of labelling with pHMB and label-specific IDA for quantitative protein analysis.

Experimental

Sample preparation and gel electrophoresis

All solutions were prepared from reagents with the highest purity available. Ovalbumin (SwissProt Nr. P01012, Sigma Aldrich, Madrid, Spain) was dissolved in 50 mmol L^{-1} ammonium bicarbonate (Sigma Aldrich). After appropriate dilution, pHMB (dissolved in 0.05 mol L^{-1} NaOH, Suprapur, Merck, Darmstadt, Germany) was added in tenfold molar excess per cysteine either with natural isotopic composition (Sigma Aldrich) or enriched in the isotope ^{199}Hg (synthesized as described elsewhere [26]; safety note: pHMB is highly toxic!). Reaction conditions for the derivatisation were 1 h at room temperature. After twofold ultrafiltration using a 10-kDa cut-off membrane (Amicon Ultra, Millipore, Bedford, MA, USA), the purified protein was diluted in 50 mmol L^{-1} NH_4HCO_3. Before electrophoresis, aliquots corresponding to the desired protein amount were further diluted if necessary and incubated in sample buffer. For IDA, different mixtures between analyte (ovalbumin derivatized with pHMB in natural isotopic composition) and spike (ovalbumin derivatised with ^{199}Hg-pHMB) were prepared and also incubated in sample buffer. The sample buffer contained 62.5 mmol L^{-1} tris(hydroxymethyl)aminomethane (Tris; Merck), 2% sodium dodecylsulphate (SDS, Sigma

Aldrich), 25% glycerol (Merck) and 0.1% bromophenol blue (Sigma Aldrich) at pH of 6.8. The addition of dithiothreitol (DTT) or other reducing agents was avoided due to possible side reactions with the derivatized protein. Gel electrophoresis was carried out using the PhastGel System (GE Healthcare, Otelfingen, Switzerland). Precast gels on a plastic support were used (dimensions 50×43×0.45 mm, GE Healthcare) with acrylamide concentrations in the separation gel of 12.5% if not otherwise stated. Running buffer for denaturing electrophoresis was provided by buffer strips supplied from the same manufacturer. Electrophoresis was carried out at 15 °C with 250 V applied for 30 min. After electrophoresis, the gels for LA were kept in 30% MeOH (Merck), 10% acetic acid (Carlo Erba Reagents, Rodano, Italy) to precipitate the proteins and thus stop their continuing migration due to diffusion. Before drying for 2 h at 70 °C, the gels were shaken in glycerol for exchange with remaining water to prevent deformation and cracking of the gels during drying [27]. Some gels were also stained using Coomassie blue for visual comparison. Although no protein loss or contamination has been observed due to the staining process and because the migration distance was reproducible within <2%, gels for LA were generally not stained. For LA, the gels were cut in the middle and attached to a glass slide using double-sided tape to mount them inside the ablation cell. In order to determine the spatial distribution of mercury within the gel, line scans were acquired following the migration direction of the protein.

Laser ablation ICPMS

An ArF excimer laser operated at a wavelength of 193 nm (GeoLasC, Lambda Physik, Göttingen, Germany), with a computer-controlled *xyz*-stage was used during this study. Observation of the sample surface was possible through an Olympus BX 51 microscope. Helium was used as carrier gas at a flow rate of 1.0 Lmin^{-1}. Table 1 shows a summary of the operating conditions.

The ablation cell used in this study was custom made at the mechanical workshop of ETH Zurich. The effective gas volume and gas dynamics in the ablation region are optimized in order to minimize the aerosol distribution across the cell. This reduces the aerosol washout times significantly and thus improves the attainable spatial resolution for scanning mode or depth profile ablation. Additionally, the design of this cell allows to directly insert samples of larger size (maximum dimensions, 230×34×16 mm (L×W×D)), which are placed on an internal sled that allows a fast change of the respective sampling position without the need to open the cell. A more detailed description can be found elsewhere [28]. The cell outlet was connected to the torch of the ICPMS via a laminar flow adapter where argon gas was added as make-up gas for stable operation of the ICP.

Table 1 Instrumental parameters of the LA-ICPMS system

Agilent 7500cs ICPMS	
Forward power	1,600 W
Carrier gas (He)	1.0 Lmin^{-1}
Nebulizer gas (Ar)	0.75 Lmin^{-1}
Plasma gas	1.0 Lmin^{-1}
Coolant gas	12.0 Lmin^{-1}
Ion optics setting	Maximized intensity for $^{202}Hg^{+}$
Collision cell	Evacuated
Dwell time per isotope	100 ms
GeoLas C	
Energy density	14.5 J cm^{-2}
Repetition rate	20 Hz (5–20 Hz)[a]
Spot size	160 μm (44–160 μm)[a]
Scan velocity	50 μm s^{-1} (25–100 μm s^{-1})[a]

[a] Values in brackets refer to the range in which the parameters were optimised

All measurements were carried out using an Agilent 7500cs ICPMS instrument under dry plasma conditions. A more detailed summary of instrumental parameters can be found in Table 1. The use of a collision or reaction gas in the octopole ion guide was unnecessary as potential spectral interferences are not to be expected in the mass range of mercury from the sample matrix in this study.

The instrument was optimized daily for high sensitivity using a NIST 610 glass standard. Besides sensitivity, also the ratios $^{238}U^{+}/^{232}Th^{+}$ and $^{248}ThO^{+}/^{232}Th^{+}$ were monitored to check for plasma temperature and oxide formation. $^{238}U^{+}/^{232}Th^{+}$ was optimized by adjusting the make up gas flow to a value near 1, while $^{248}ThO^{+}/^{232}Th^{+}$ under these conditions was typically below 0.3%.

For LA, the following isotopes were measured: $^{13}C^{+}$ as matrix element and $^{34}S^{+}$ as indicator for the buffer front. During method optimisation, only $^{199,\ 200,\ 202}Hg^{+}$ were monitored, whereas all mercury isotopes were monitored for IDA.

For liquid analysis (characterization of the spike and determination of the initial isotope ratio of isotope diluted samples), the Agilent 7500cs ICPMS was used with a self-aspirating pneumatic nebulizer (PFA 100, ESI Omaha, USA) made from perfluoroalkane (PFA) with a nominal flow rate of 100 μL min^{-1}. An aliquot of the protein solution after labelling with pHMB was digested with nitric acid, and the concentration of mercury was determined using inverse IDA against a standard solution with certified concentration (Merck, Darmstadt, Germany). All isotopes of mercury (^{196}Hg, ^{198}Hg, ^{199}Hg, ^{200}Hg, ^{201}Hg, ^{202}Hg and ^{204}Hg) were monitored with a dwell time of 100 ms, and 50

repetitions were acquired to improve counting statistics. The initial isotope ratio of the samples was determined after dilution of the protein solution in 2% nitric acid to a final Hg concentration of about 10 ng g^{-1}.

Results and discussion

Optimization of the LA-ICPMS system

For method optimisation, a gel containing approximately 40 pmol of ovalbumin, labelled with pHMB containing Hg with natural isotope composition (corresponding to 32 ng of Hg), in each of the total eight lanes of the gel was used. The obtained sensitivity in LA-ICPMS is dependent on the amount of ablated material containing the analyte of interest. Therefore, the most influential parameters such as laser repetition rate, spot size and scan velocity were carefully optimised. This optimisation aimed to find conditions that enable a complete in-depth ablation of the entire protein amount present in one line scan with a sufficient spatial resolution to separate proteins and short analysis time. Figure 1 shows the fractions of the Hg ion signals acquired in a sequence of line scans across the protein band in the same lane using different laser repetition rates with identical scan speed. A higher repetition rate corresponds to a proportionally greater number of laser pulses on the gel surface, thus creating deeper ablation lanes. After each line scan, the surface of the ablation crater was refocused, and another line scan was acquired. This procedure was repeated until no further Hg signal was detectable above the instrumental background. After peak integration and normalization to the sum of all signals, the fraction of the respective ion signal for each line scan was determined. Using a repetition rate of 5 Hz (Fig. 1a), four consecutive line scans were necessary to remove the majority of the Hg-containing protein. With a repetition rate of 10 Hz (Fig. 1b), the same amount was removed in two line scans, whereas consistently a repetition rate of 20 Hz (Fig. 1c) was able to remove the same amount in one single line scan. For this experiment, also $^{29}Si^{+}$ was monitored as an indicator whether the laser beam had removed the entire gel matrix and thus started to ablate on the microscope slide. However, complete ablation of the gel was not observed. The maximum depth penetration necessary for complete removal of the protein from the gel was estimated using light microscopy to be 170± 5 μm, whereas the gel matrix had a thickness of 450 μm. It is also important to note that the highest concentration of the protein is found close to but not directly at the surface of the gel, which would be removed when using lower frequency or higher scan rates.

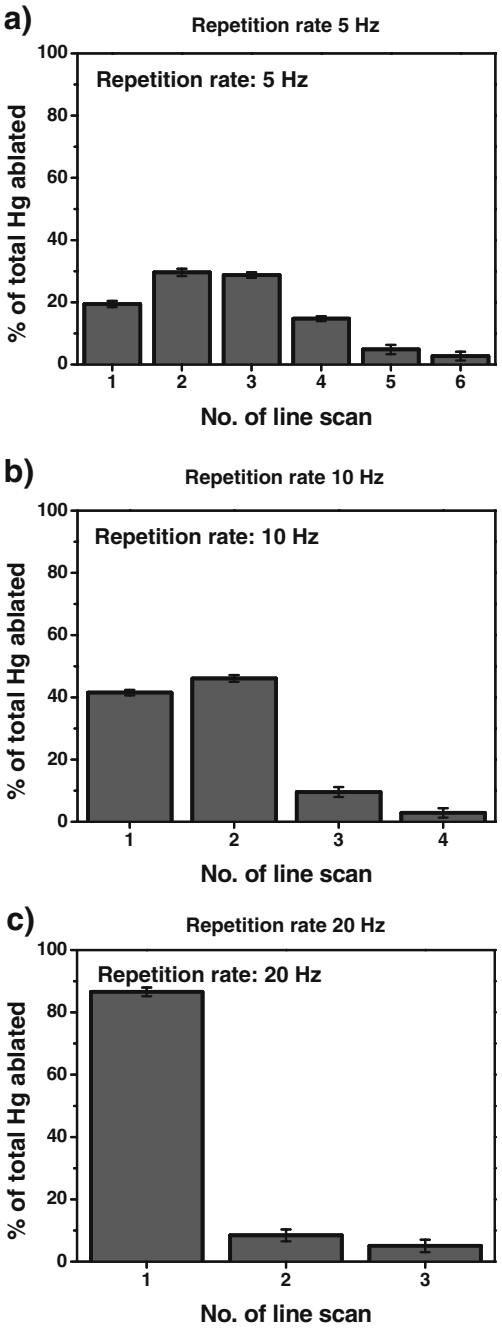

Fig. 1 Depth profile of the Hg content in the band using different laser repetition rates (spot size, 160 μm; scan rate, 50 $\mu m s^{-1}$). **a** Repetition rate 5 Hz. **b** Repetition rate 10 Hz. **c** Repetition rate 20 Hz

The signal intensity obtained is furthermore directly correlated with the spot size of the laser beam. A larger spot size leads to an increase in signal intensity with approximately quadratic dependency, but might compromise lateral resolution attainable. Figure 2b shows that the resolution of the protein bands in the transient signals was not improved with a smaller beam diameter, so the largest spot size available on the laser system (160 μm) was used throughout this work. The protein amount per band was 15 pmol in

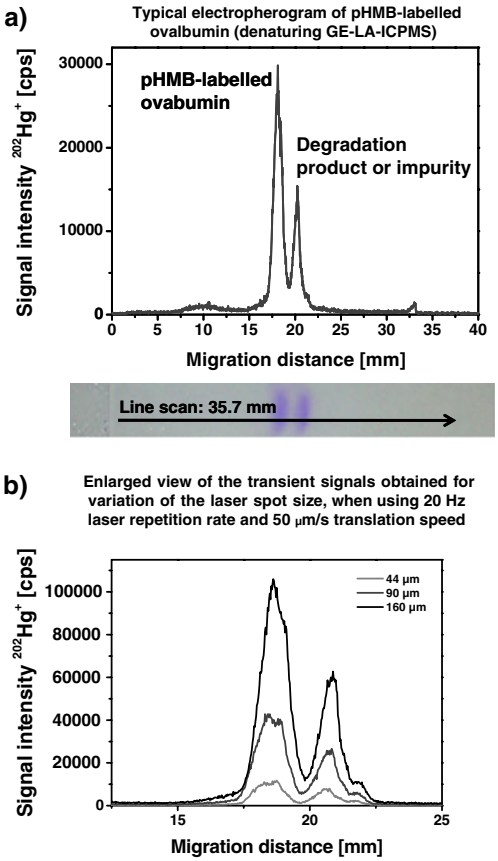

Fig. 2 **a** Typical electropherogram of pHMB-labelled ovalbumin (denaturing GE-LA-ICPMS). **b** Enlarged view of the transient signals obtained for variation of the laser spot size, when using 20 Hz laser repetition rate and 50 μm/s translation speed

this case. It needs to be noted, however, that the actual width of the scan was significantly wider than the beam diameter employed. Typical ablation lines showed a lane width of 225±5 μm, which is most likely due to additional thermal desorption of material during the ablation event.

Together with the laser repetition rate, the scan velocity determines the number of laser pulses that will hit a certain area of the gel surface. Therefore, it affects sensitivity, but also the time needed to conduct a line scan and may additionally influence the spatial resolution attainable. A scan velocity of 50 μm s^{-1} was finally chosen for further measurements because it represents a reasonable compromise between analysis time and sensitivity. Even shorter analysis times or higher sensitivity might be attainable by using lasers with higher repetition rates. Using this velocity, the time necessary for one line scan was around 700 s. Under optimised conditions (20 Hz repetition rate, 160 μm spot size, 50 μm s^{-1} scan velocity), ablation of the ovalbumin standard resulted in a mercury (and thus protein) distribution along the migration direction in the gel as shown in Fig. 2a. Although only ovalbumin was presumed to be present in the sample, two distinct peaks are clearly separated and can be observed in both the ICPMS-based data and also the Coomassie blue stained gel. As both peaks were also observed in untreated ovalbumin after electrophoresis (data not shown), we attribute the second signal to impurities or degradation products present in the ovalbumin standard. Analysis of the protein standard with MALDI-MS indicated the presence of a second protein species with a molecular weight of 40 kDa, which fits well to the molecular weight of the second peak, when considering the respective migration distances. In any case, the migration distance and width of the protein bands showed good agreement between the stained gel and the transient signal obtained by ICPMS. Reproducibility of the migration distance was in all cases better than 2%, also between different gels.

Calibration using external standards

Under optimized ablation conditions, a linear calibration was carried out using external standardization with different amounts of derivatized ovalbumin in order to determine the figures of merit and for comparison with IDA analysis for quantification. For each electrophoresis lane, five individual line scans were acquired. Figure 3 shows the calibration curves obtained using the signal intensity of mercury

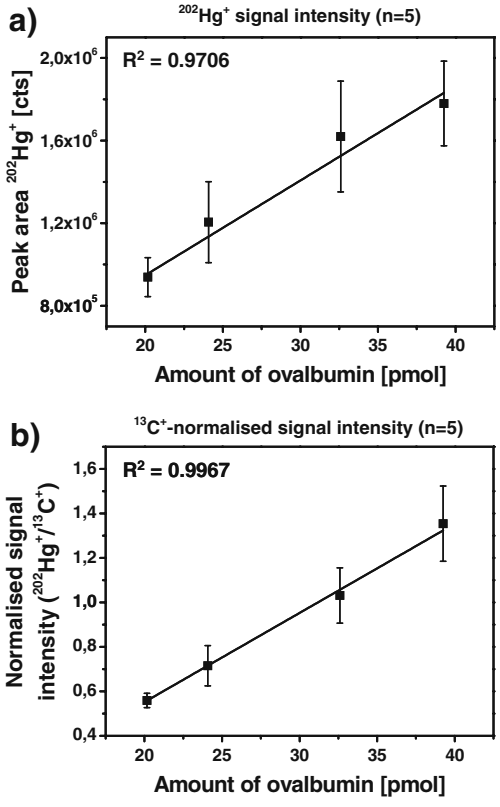

Fig. 3 Comparison of external calibration using the (**a**) $^{202}Hg^{+}$ signal intensity (n=5) and the (**b**) $^{13}C^{+}$-normalised signal intensity (n=5)

(Fig. 3a), and the signal intensity normalised to the ion signals of $^{13}C^+$ (Fig. 3b). It can be seen that normalization to the matrix element carbon does significantly improve the correlation and precision [10]. As the surface of the gel is not perfectly uniform after drying, slight changes in its height lead to changes of the surface relative to in the focus position of the laser beam. This results in varying ablation rates and thus varying signal intensities. Normalization to $^{13}C^+$ can to some extent compensate for this. Hg and C are both predominantly transported in a gaseous form (presumably as elemental Hg and CO_2), which was verified using a gas exchange device [29]. This most likely alleviates the potential problem of different transport efficiencies for particle and vapour, which could otherwise lead to problems in the quantification. However, this must not necessarily be true for other metals attached to proteins.

Taking into account the 3σ criterion (standard deviation of the mean signal during ablation of a point where no protein is found), the limit of detection was estimated to be 8 pg of mercury (corresponding to 160 fmol of ovalbumin). The signals for the Hg-isotopes recorded at a protein-free position however did not show significantly higher intensities than the gas blank recorded without ablation of the gel. The variability of the intensity ratios from individual line scans in a single lane showed relative standard deviations between 15% and 20%, which would ultimately limit any precise quantification of the labelled proteins. This is mostly a result of the non-uniform distribution of the protein in the gel. A line scan perpendicular to the migration direction (Fig. 4) shows that the protein is enriched in the centre of the lane. From these data, the width of the entire band can be estimated to be 3.3 mm. Furthermore, it was also observed that the signal intensity between two lanes did not completely reach the background level, so that slight diffusion effects cannot be excluded. Taking into account a distance of about 2.25 mm (the width necessary to ablate up to five individual line scans), the standard deviation obtained for the normalised signal intensities ($^{202}Hg^+/^{13}C^+$) is about 15%, corresponding well with the value observed for the individual line scans conducted for the linear calibration. In contrast, the isotope ratio (here the natural isotope ratio for $^{202}Hg/^{199}Hg$, R_{nat}= 1.77) remains constant over the entire width of the protein containing band (see also Fig. 4). The standard deviation was determined to be lower than 4% for the same distance as mentioned above. Obviously, the determination of the isotope ratio can be carried out more precisely for quantification than the determination of the peak area and comparison to external standards. Whereas for the use of external calibration slight deviation from the centre of the gel lane may lead to an erroneous determination of the protein amount, the isotope ratio remained constant (Fig. 4). Furthermore, the absolute amount of ablated material is becoming only relevant for the sensitivity but not for quantification anymore. For external calibration, it has to be assured that the same amount of material is ablated for all standards and samples to give similar signal intensities, whereas the isotope ratio measurement is independent of the absolute amount ablated. Also, the distribution of the analyte, e.g. due to diffusion effects, played a critical role for the accurate determination.

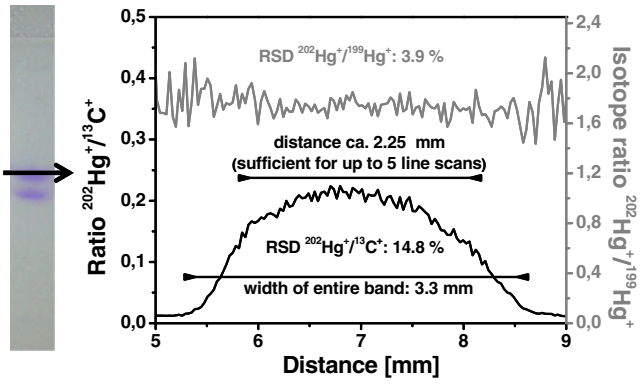

Fig. 4 Line scan perpendicular to the protein band showing the $^{13}C^+$-normalised signal intensity and the isotope ratio $^{202}Hg^+/^{199}Hg^+$

Isotope dilution analysis

In order to verify the better precision offered by IDA for the determination of proteins from PAGE gels, ovalbumin derivatised with pHMB with natural isotopic composition was quantified with label-specific IDA after derivatisation with ^{199}Hg-pHMB [26]. As there is a lack of appropriate certified reference materials, the methodology proposed only represents a proof of concept. Mixtures with different quantities (from 3 to 21 pmol) and spike-to-sample ratios between 0.4 and 2.5 were prepared and subjected to gel electrophoresis. Each sample was analysed in duplicate with one empty lane (filled with sample buffer only) in between [18]. Additionally, after digestion in nitric acid and appropriate dilution, the initial Hg isotope ratio in the samples was determined using ICPMS with pneumatic nebulisation. The concentration of the spike was determined by inverse IDA. Mass bias was determined by analyzing a standard with natural isotopic composition of Hg. For LA-ICP-MS, this was done using ^{nat}pHMB-derivatised ovalbumin. Isotope ratios were corrected using the exponential function (currently more prominent as "exponential law") [30]. The gels were treated as usual and ablated using optimized conditions as mentioned above. After evaluation of the isotope ratio in the electropherograms, the quantity of ovalbumin was calculated. Table 2 summarises the obtained results. As it can be seen, the determined amounts of ovalbumin correspond very well to the expected values. The analysis was quantitative with

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Table 2 Results obtained for the determination of labelled ovalbumin using label-specific isotope dilution analysis

Sample	R ($^{202}Hg^{+}/^{199}Hg^{+}$) (PN-ICP-MS)	R ($^{202}Hg^{+}/^{199}Hg^{+}$) after GE (LA-ICP-MS)	n expected [pmol]	n found [pmol]	Recovery [%]
1	0.2858±0.0083	0.2818±0.0088	3.04	3.03±0.17	99.9±5.6
		0.2815±0.0166		3.03±0.21	99.8±8.9
2	0.3761±0.010	0.3780±0.0243	3.24	3.32±0.17	102.7±5.2
		0.3770±0.0153		3.31±0.22	102.3±6.6
3	0.2819±0.0070	0.2767±0.0097	10.06	9.72±0.66	96.7±6.8
		0.2771±0.0018		9.74±0.62	96.9±6.4
4	0.2173±0.0063	0.2165±0.0052	13.02	13.25±1.05	101.8±7.9
		0.2154±0.0042		13.17±0.68	101.1±5.2
5	0.5642±0.0186	0.5610±0.0093	21.47	20.65±1.25	96.5±6.1
		0.5545±0.0073		20.29±1.19	94.5±5.9

accuracy between 94.5% and 102.7%, whereas the precision was between 5% and 9%. The isotope ratio was similar for both protein bands observed in the electropherograms, which indicates that the unknown protein was also successfully derivatised by ^{nat}pHMB and the isotopically enriched reagent. However, the precision obtained here takes into account not only the isotope ratio measurement but also possible errors occurring during sample preparation. Therefore, it is slightly higher than the values obtained for the RSD of the isotope ratio measurements (about 4%). In comparison to external calibration, the use of IDA was not only superior in terms of precision but also less prone to instrumental drifts or matrix effects.

The limit of detection (160 fmol for ovalbumin) was identical to those obtained using the external calibration. In comparison to other GE-LA-ICPMS approaches for the detection of proteins (Table 3), it is obvious that the artificial labelling with metals significantly improves the achievable detection limits [17, 19]. Therefore, the proposed methodology (GE-LA-ICPMS in combination with label-specific IDA) can help to combine the principle features for the precise and accurate absolute quantification of proteins.

Conclusion

The combination of gel electrophoresis and LA-ICPMS was demonstrated for the determination of ovalbumin labelled with pHMB. The comparison of two different quantification strategies showed that external calibration required signal normalization to $^{13}C^{+}$ in order to improve correlation and precision. Label-specific IDA (using ^{199}Hg-enriched pHMB) on the other hand improves precision and accuracy of the results. It was found that the analytical results were independent from the analyte distribution within the bands. As the quantitative information is encoded in the isotope ratio, which is constant over the entire width of the spot, the effective amount of ablated sample in one line scan does not affect the analytical result. This might be of special importance when spots from two-dimensional gels are investigated. Here, the small size of the spots will impede uniform distribution in any direction; therefore, external calibration might be probably prone to errors. For the analysis of real samples in a proteomic study, more sophisticated separation protocols are mandatory which may require additional sample fractionation, e.g.

Table 3 Comparison of laser ablation conditions and detection limits for the determination of proteins using GE-LA-ICPMS

Protein [reference]	Element	Laser conditions	Observations	Detection limits
SOD [18]	Cu, Zn	ns 266 nm [3.5 J cm^{-2}, 20 Hz, 250 μm spot]	Protein-specific IDA	16 pmol
β-Casein [7]	P	ns 213 nm [55%, 20 Hz, 120 μm]	Blotting onto PVDF membranes	16 pmol
α-Casein [9]	P	ns 266 nm [0.7 mJ, 8 Hz, 100 μm]	Blotting onto NC membranes	5 pmol
BSA [31]	I	ns 266 nm [3 mJ, 15 Hz, 500 μm]	Blotting onto NC membranes after iodination	150 fmol
CYP1A1 [32]	Eu	ns 266 nm [3 mJ, 15 Hz, 500 μm]	Blotting onto NC membranes, incubated with Eu-labelled Ab	140 fmol
GSHPx [33]	Se	fs 1,030 nm [13 J cm^{-2}, 40 μm]	Ablation of 2 mm wide lane	Low fmol levels
Mre 11 [34]	Au	ns 213 nm [4.2 J cm^{-2}, 10 Hz, 100 μm]	Incubated with AuNP-labelled Ab, blotting onto membranes	0.2 amol
Ovalbumin [this study]	Hg	ns 193 nm [14 J cm^{-2}, 20 Hz, 160 μm]	Labelling with pHMB and subsequent label-specific IDA	160 fmol

PVDF polyvinylidene fluoride, *NC* nitrocellulose, *Ab* antibody, *AuNP* gold nanoparticle

2D gel electrophoresis. Due to the high stability of the labelled protein, the isotope dilution step can already be accomplished in an early point of sample preparation, which can help to improve accuracy of the quantitative data obtainable.

With respect to achievable sensitivity and accuracy in protein determinations, it is possible to conclude that the combination of protein labelling with metal-containing compounds, label-specific IDA and gel electrophoretic separations with subsequent laser-ablation ICPMS might serve as a powerful tool in absolute protein quantification.

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