Chemical labels and natural element tags for the quantitative analysis of bio-molecules

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Besides the qualitative analysis of a bio-molecule or even a whole proteome, their quantitative determination is also a topic of growing interest, since only the quantity of proteins or changes in their abundance really reflects the status and changes of a biological system. Various approaches have recently been suggested for the comparative investigation of different sample states resulting in different proteomes (comparative proteomics) on the basis of either relative or absolute protein quantification. These use molecule specific mass spectrometry and stable isotope labels with different chemical properties, which are covalently bound to, or incorporated *in vivo* into the various protein species within the sample entities compared. Various groups in the field of analytical atomic spectrochemistry have recently been developing new strategies and analytical tools for the complementary implementation of elemental mass spectrometry (*e.g.* inductively coupled plasma mass spectrometry (ICP-MS)), which is still generally known as only a "metal" detector, in new, "non-traditional" application areas targeting bio-molecular chemistry or even the fast growing field of proteomics related research, which is currently dominated by ESI or MALDI based MS techniques. At this time, three main strategies are emerging for the complementary application of ICP-MS to bio-molecule quantification in life-sciences orientated research. These include (i) the utilisation of natural (hetero) element tags (covalently bound elements *e.g.* sulfur and phosphorus), which are present in nearly all protein bio-molecules, (ii) the controlled labelling of bio-molecules such as proteins, peptides or antibodys with ICP-MS detectable elements using bi-functional chelating agents (by means of a chemical reaction, which forms a covalent bond between the chelating agent and a specific functional site of the bio-molecule) and (iii) the chemical labelling of bio-molecules with nano-particles which contain elements detectable by ICP-MS. The idea behind all three strategies is to

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make bio-molecules detectable by ICP-MS and thus benefit from its high sensitivity, linearity and matrix robustness. This would allow the qualitative detection and quantitative determination of nearly all bio-molecules provided the stoichiometry of either the natural tag or the chemical label is known. This article will give a critical review of the latest developments in the field of quantitative proteomics and will highlight key applications of ICP-MS as a complementary tool to ESI or MALDI MS techniques for selected proteomics or biochemistry related applications. Special emphasis is placed on the quantification of bio-molecules using elements that are *covalently* bound to proteins or peptides and which show wide distributions in these bio-molecules as tags.

1 Introduction

With the ongoing accumulation of tremendous numbers of DNA sequences, ending up in the recent deciphering of the genome of different species, it has been realised that simply gaining information on a fully-sequenced, static genome is in many cases not sufficient to clarify biological functions. Living cells rely on complex interactions between thousands of different bio-molecules that maintain cellular integrity and morphology and execute many biological functions.

There is no strict linear relationship between genes and the encoded proteins, since living systems are normally dependent upon a multitude of metabolic and regulatory pathways, which often involve the formation of protein isoforms or their post-translational modification products.[1,2]

The application of genomics techniques, such as measuring the mRNA level for the determination of the expression level of selected proteins has a long tradition. However, the measurement of the mRNA transcripts does not necessarily allow for description of the resulting entity of proteins, their amounts, their localisation or possible post-translational modifications at any point in time.[3,4]

In order to overcome these limitation of genomics based approaches, the complementary large scale analysis of the encoded protein products and their post-translational modified active forms has become the focus of several life science related research disciplines.[1]

As defined by Wilkins *et al.* and more recently by Pandey and Mann, proteomics represents the study of the entire proteins (the proteome) of a cell or an organism, how they are modified, when and where they are expressed, how they are involved in metabolic pathways and how they interact with each other.[1,5]

Besides the qualitative analysis of a proteome, which includes the analysis of all different protein species expressed at a given time inside a defined biological entity, their quantitative determination is also of growing interest, since only the quantity of proteins or changes in their abundance can reflect the status and changes of a biological system or of different biological states.[6–8]

Quantitative proteome profiling, defined as the systematic identification of proteins in complex samples and the determination of their quantity or quantitative change, is an essential sub-discipline in the emerging field of systems biology. It is expected to provide new functional insights into biological processes, in particular with regard to the identification of new diagnostic or prognostic disease markers and the finding of new therapeutic targets at the protein level.[9]

Fig. 1 shows the number of peer-reviewed papers in the ISI database published between 1998 and 2007 that deal with quantitative proteomics (search terms: quantitative and proteomics). The near exponential expansion in this field over an exceptionally short period of time is clear and various strategies for either the relative or the absolute protein quantification have been developed. Some of them are already commercially available. In the following sections the term *relative quantification* will be used for quantification approaches which compare the intensities of corresponding mass spectrometric signal pairs separated by a defined mass difference which provide a measure of the relative expression level of the related peptides or proteins within the sample entities compared. The term *absolute quantification* will be used for those approaches which give real quantitative data allowing the direct conversion of signal intensity into a corresponding concentration level.

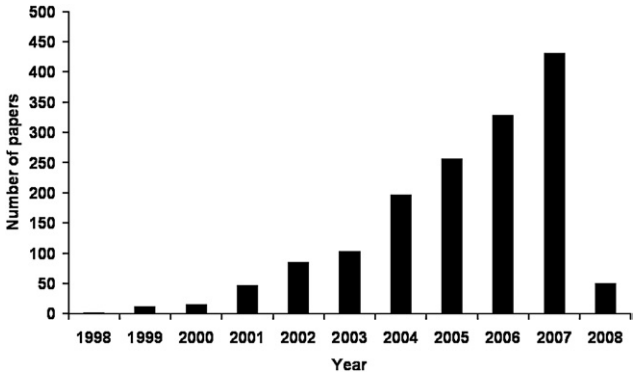


Fig. 1 Number of peer reviewed papers listed in the ISI database over the last ten years on quantitative proteomics (search terms in Title, Abstract, Keywords: quantitative and proteomics).

Despite this impressive evolution, the accurate quantification of large sets of proteins is still one of the ongoing challenges in life-science related research disciplines such as proteomics.

The application of stable isotope (^{2}H, ^{13}C, ^{15}N, ^{18}O) labelled analogues of the compounds under investigation as internal standards has a long tradition in mass spectrometry, especially for the accurate quantification of small organic molecules. In this context, reports on the synthesis of labelled peptides for the quantification of selected proteins can be found in the literature.[10–12]

Since it is impractical to synthesise thousands of species and proteome specific labelled protein or peptide standards in the field of proteomics, new techniques are necessary to allow the investigation of a large set of proteins or peptides at the same time without the need for highly specific standards.[8]

A number of strategies have recently been suggested for the comparative investigation of different proteomes on the basis

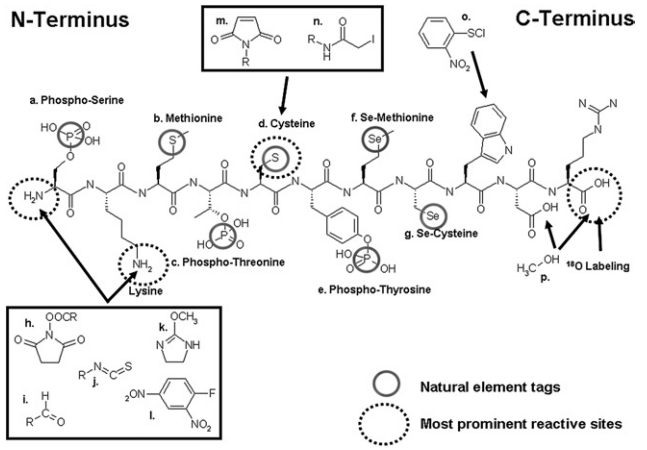


Fig. 2 Overview of the most prominent reactive sites and suitable chemicals allowing the covalent modification of proteins and peptides and possible natural element tags, which can occur in an amino acid chain: Natural (hetero) element tags (a) phospho serin, (b) methionine, (c) phospho threonine, (d) cysteine, (e) phospho thyrosin, (f) Se methionine, (g) Se cysteine, amine reactive groups (h) *N*-acyloxysuccinimide esters of acids, (i) aldehydes, (j) isothiocyanates, (k) imidazole based reagents, (l) 2,4 dinitro-1-fluorobenzene, cysteine reactive groups (m) maleimide based reagents, (n) iodoacetamide, miscellaneous reagents, (o) 2-nitrobenzenessulfenyl chloride, (p) esterification.

of either relative or absolute protein quantification. Most of them use stable isotope containing labels with different chemical properties, which are covalently bound to the various protein species within the sample entities compared.

As shown in Fig. 2, the amino acid chain of all proteins or peptides features a number of reactive centres which can covalently bind to isotopically labelled reagents due to specific reaction chemistry.

The basic idea behind most isotopic labelling approaches is to modify a sample before or after enzymatic digestion with a reagent containing only naturally abundant (light) stable isotopes and to modify a second sample with an identical reagent in which several atoms have been exchanged with their stable heavy counterparts. The samples are then mixed, fractionated, usually with reversed phase liquid chromatography (RP-HPLC), and then analysed by mass spectrometry. Since the light and the heavy reagents normally show identical chemical properties, the differently labelled peptides will ideally co-elute from the RP column into the mass spectrometer generating a peak doublet in the mass spectrum separated by a significant mass shift defined by the number of heavy isotopes introduced. The abundance ratio of each individual peptide can be measured, thus allowing the relative determination of the amounts of individual proteins originating from the two different samples.

Since their introduction, electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) MS techniques have become the gold standards for the large scale identification of proteins, and in particular for their relative quantification using stable isotope labelling techniques.

An ideal labelling approach should fulfil a number of criteria:

– All proteins/peptides in one sample should be labelled.
– Labelling should generate a predictable mass window between differently labelled peptides.
– Labelling chemistry should be quantitative.
– Minimal sample treatment should be required.
– Spectral interpretation should not be impacted by the presence of the labelling reagent.
– Labelling reagents should not cause detrimental effects on the ionisation efficiency.
– Labelling should not cause chromatographic resolution of peptide isoforms.
– Multiplexing should be possible (comparison of more than two samples).
– Methods should be applicable to all types of organisms including mammals.

However, currently available approaches to stable isotope labelling show certain strengths, but also a number of limitations; these will be highlighted in section 2.

As an alternative, a huge number of proteins contain trace metals naturally as functional or structural groups. These can be utilised as tags, both for qualitative detection and for quantification without the need for chemical derivatization procedures provided their stoichiometry within the compounds targeted is thermodynamically stable and known.

Various groups in the field of analytical atomic spectrochemistry have been developing new ideas for the implementation of elemental mass spectrometry, namely inductively coupled plasma mass spectrometry (ICP-MS), which is still widely known as only a "*metal*" detector into a number of "non-traditional" new applications targeting bio-molecular chemistry or even the rapidly emerging field of proteomics related research.

In recent years, the ICP-MS technique has matured from being a metal detector to become one of the most versatile and sensitive complementary tools in bio-inorganic analytical chemistry. Together with ESI or MALDI MS techniques, it is capable of detecting most of the elements present in the periodic table, allowing their application as tags for the determination of various compounds. This trend has been especially boosted by the availability of collision and reaction cells and more robust high resolution ICP-MS instrumentation, as well as the development of suitable interface technologies for the coupling of chromatographic or electrophoretic separation techniques to ICP-MS.

In analogy to other "*omics*" approaches, *e.g. proteomics* or *genomics*, this recent development has led to the proposal of a new research discipline termed *metallomics*, which is defined as the global study of the so called *metallome*. This concept includes the global investigation and characterisation of the entirety of metal and metalloid species such as metalloproteins or other metal-containing bio-molecules (the metallome) within a defined biological entity.[13–17]

The advantages of existing "*omics*" approaches, such as *proteomics* are based on the availability of techniques such as 2D gel electrophoresis which allow the simultaneous handling of nearly whole, albeit somewhat biased, proteomes that provide a global view of a large section of the expressed protein species inside a defined sample at a given point in time. Unfortunately, there is currently no comparable technology available which allows the simultaneous investigation of the whole *metallome* within a defined biological entity at a given point in time. This should be one of the pre-requisites for an "*omics*" approach, since normally these concepts try to give a global, holistic view

on *e.g.* all proteins and how they interact rather than focus on the global role of a compound, in this case metals, whose importance in biological systems is without question.

Up to now the essential differences between *metallomics* and the well known field of *bio-inorganic chemistry* have not been described sufficiently. As pointed out by Koppenaal and Hieftje it might help to further promote the field of bio-inorganic chemistry and to bring atomic spectrometry into more contact with the biological sciences in a fully meaningful way.[18] Most *metallomics* papers still deal predominantly with the utilisation of selected trace metals as tags for the detection of bio-molecules using ICP-MS, rather than with the investigation of the global role of these elements or their ligands in a larger biological context.

Even though trace metals play a vitally important role in living systems and their application as tags for selected bio-molecules has been demonstrated in a number of papers, they are of limited use as tags in a wider, more general context, since they are often only weakly associated with their ligands. This makes them susceptible to changes in the tag stoichiometry especially during complex sample preparation procedures which may result in inaccurate quantitative results.

As an alternative, most proteins, and therefore also the resulting peptides, naturally contain *covalently* bound tags, such as phosphorus, sulfur and selenium (see Fig. 2), which show more general distributions in living systems and whose utilisation will be highlighted in this review. These elements can be used for both the qualitative detection and the quantification of proteins or peptides without the need for chemical derivatisation procedures, provided their stoichiometry within the compounds targeted is known.

In this context Sanz Medel and more recently Szpunar *et al.* have coined the concept of hetero (element) tagged proteomics, which includes the study of a proteome in which analytical information is acquired by complementary application of elemental mass spectrometry utilising the presence of a heteroatom (S, P, Se, I, lanthanide, element coded nano-particles) in a protein or introduced *via* controlled chemical labelling, especially for fast screening and quantification purposes.[15,19,20]

In general, ICP-MS cannot contribute much information to the structural characterisation and final identification of a bio-molecule containing an ICP-MS detectable element, due to the fact that all structural information is lost as a result of the 7000 K plasma conditions. At the same time, this drawback represents the main strength of ICP-MS since the result is a drastic reduction in sample complexity, as indicated in Fig. 3.

The use of ICP-MS therefore has much to gain from the complementary application of molecule specific detection techniques based on electrospray ionisation (ESI) or matrix assisted laser desorption/ionisation (MALDI). Fig. 4 indicates the typical proteomic workflow from the sample to the final protein identification and already existing application fields, where the complementary application of ICP-MS has been realised successfully.

This strategy allows the utilisation of selected (hetero) elements which are naturally present in nearly all protein bio-molecules such as phosphorus and sulfur or more recently the application of labels containing ICP-MS detectable elements, which have been attached to proteins *via* controlled chemical derivatisation of the protein species under investigation. The result is sensitive qualitative detection and quantitative determination provided the label stoichiometry is known.

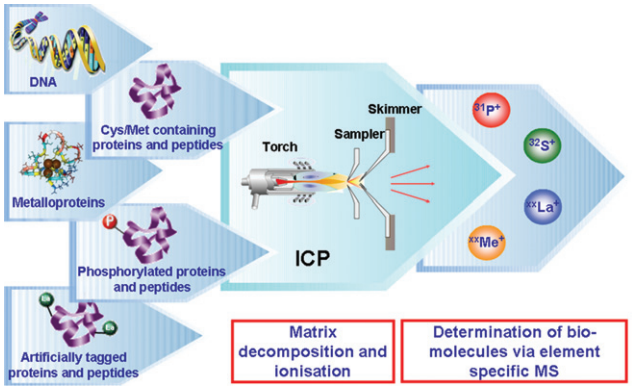


Fig. 3 Reduction of sample complexity using a 7000 K inductively coupled argon plasma (ICP) as ion source. The ICP allows matrix decomposition and shows a compound independent response, especially when using low flow sample introduction systems allowing the qualitative and quantitative determination of selected bio-molecules *via* either natural or artificial (hetero) element labels.

As a detector ICP-MS features some unique properties with respect to calibration and quantification:

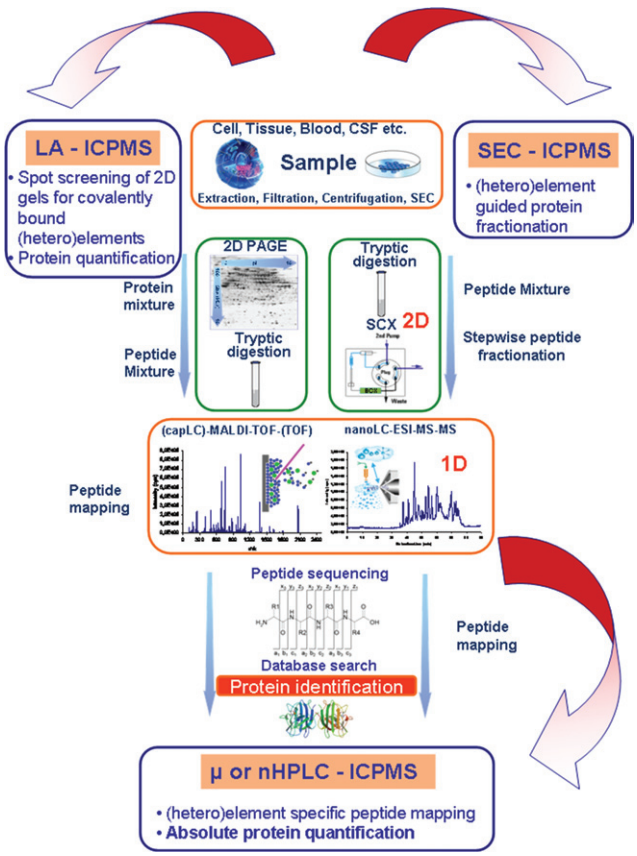


Fig. 4 Most prominent fields of application for the complementary use of ICP-MS within a typical proteomic workflow (either gel-based or gel-free). It includes a multidimensional sample fractionation, (hetero-) element specific screening of 2D gel separations using LA-ICP-MS, (hetero-) element specific peptide mapping using capillary- or nano-LC hyphenated to ICP-MS. In particular the latter allows also absolute protein quantification.

In comparison to organic MS techniques ICP-MS shows a much larger linear dynamic detection range (up to 9 orders of magnitude), nearly no matrix effects, especially when working with low flow sample introduction techniques such as capillary HPLC or nano HPLC, and a high sensitivity for the detection of elements. Since the instrumental response of ICP-MS is only defined by the amount of an element present in a molecule and not by its chemical form, basically two straightforward strategies can be used for quantification:

a. Application of a *compound independent calibration* using *sample-unspecific*, defined standards (*e.g.* inorganic salts, small organic molecules) which contain the target element at a known concentration.

b. *Isotope dilution analysis* whenever the tag element used features at least one further stable isotope (*e.g.* sulfur, selenium, selected lanthanides).

The advantages and limitations of this straightforward approach will be highlighted in this review. In addition the focus will lie on the latest developments in the use of inductively coupled plasma mass spectrometry (ICP-MS) as a complementary tool alongside the molecule specific MS techniques for selected proteomics or protein biochemistry related applications. This will open up new possibilities, especially within the field of protein or peptide quantification as well as in the development of multiplexed assay setups which extend the possibilities of current approaches.

The following sections will first give a critical overview on the most prominent techniques currently used for the global, either relative or absolute, quantification of large protein sets using electrospray ionisation (ESI) or matrix assisted laser desorption/ionisation (MALDI) based molecule specific MS techniques. This is important for the understanding of the basic idea behind all label-based techniques recently developed for the comparative large scale analysis of differently expressed proteomes, as well as their advantages and limitations. We will also show how elements, besides the main constituents of bio-molecules (C, H, O, N), can contribute to simple and sensitive, especially absolute, bio-molecule quantification.

2 Quantitative proteome analysis *via* stable isotope labels using organic mass spectrometry

Up to now various approaches have been suggested for either the relative and absolute quantification of proteins and peptides as already addressed in various reviews.[8,21–26]

The basic idea behind most isotopic labelling techniques is to modify a sample before or after enzymatic digestion with a reagent containing only natural abundant (light) stable isotopes and to modify a second sample with an identical reagent in which several atoms have been exchanged for their stable heavy counterparts such as ^{2}H, ^{13}C, ^{15}N or ^{18}O. Both samples are mixed and finally fractionated mostly by using reversed-phase liquid chromatography (RP-HPLC). This is followed by mass spectrometric detection.

Since the isotopes have identical physico-chemical properties, the lightly labelled compounds elute simultaneously with their heavy counterparts after the separation step into the MS, generating a peak doublet in the mass spectrum with a significant mass shift defined by the number and type of heavy isotopes that have been introduced. The abundance ratio of each individual peptide can be measured, allowing the relative determination of the amounts of individual proteins originating from the two different samples.

A number of different techniques have been developed for the controlled labelling of proteins and peptides. Labelling can be performed either *metabolically*, *enzymatically* or *via chemical reactions* with reagents targeting selected functional sites of the different protein species (see Fig. 2).

The most prominent functional sites are the primary amine groups which can be found at the N terminus and within the lysine residues of all proteins or peptides. Some reagents also specifically target cysteine residues.

In the following section we will highlight the most prominent approaches currently used for the relative and absolute quantification of large sets of proteins and peptides in the field of comparative proteomics. Most of them have been developed to utilise molecule specific MS techniques for either the identification or in most cases the relative quantification of the compound under investigation.

However, some of the latest methods are readily suited to the complementary use of elemental ICP-MS and organic MS even though they were originally designed for organic MS applications.

It should be kept in mind that besides the techniques for quantitative, comparative proteomics described here, various other strategies with additional advantages as well as limitations have been already published. Details of these other possible approaches have been covered in a number of published reviews.[21–25,27]

2.1 Metabolic labelling

In contrast to the *in vitro* labelling technique, which will be highlighted in subsequent sections, *metabolic labelling* takes place *in vivo* and has been widely used for the relative quantification of protein expression in differently treated cell culture systems. Recently metabolic labelling has been used for the whole organism labelling of model species such as *Caenorhabditis elegans* and *Drosophila melanogaster* or even small mammalian species such as *Rattus norvegicus*.[28,29]

Two alternative methods are mainly used in metabolic labelling:

Ong and co-workers introduced an approach that has been termed *stable isotope labelling by amino acids in cell culture* (SILAC). This technique involves the addition of selected isotopically-modified amino acids to amino acid deficient cell culture media. These amino acids are then incorporated metabolically into all proteins as they are synthesised by the organism kept in culture.[30]

Since the labelling takes place at the protein level, full protein information especially on post-translational modifications or protein degradation is available and the sample complexity may be reduced by different pre-fractionation steps. Depending on the particular labelled amino acid used and its statistical natural abundance, many peptides will be labelled and this improves protein identification and sequence coverage.

In comparison to other labelling techniques, the SILAC approach does not change the isoelectric point (p*I*) of the proteins and therefore their migration properties during 2D gel separations. Unfortunately, from a practical point of view, this

approach is not applicable to all organisms for both ethical and cost related reasons.

An alternative metabolic labelling strategy is to grow the test organism (mainly cell cultures of bacteria or yeasts) in media enriched in ^{15}N (>96 %) as the only nitrogen source. In contrast to other labelling schemes, where a defined modification is made and a defined mass shift is introduced into every peptide, the number of labelled atoms incorporated into peptides with this approach will vary from peptide to peptide resulting in the need to determine the number of labels present before the ratio between the labelled and unlabelled peptides can be calculated.[28]

Metabolic labelling meets most of the criteria of an ideal labelling approach, however, it should be kept in mind that some cell types will not grow in isotopically enriched or depleted media and differential transport of the labelling agent between different cellular compartments and organs can often be observed.[31]

2.2 Enzymatic labelling

A simple way to introduce stable isotopes into peptides is to carry out enzymatic digestion of the sample in $H_2{}^{18}O$ while the control sample is digested in normal $H_2{}^{16}O$. This will result in the introduction of up to two ^{18}O into the C-terminal carboxylic group formed during the digestion procedure.

In principle, all serine proteases such as trypsin, chymotrypsin and GluC are amenable to ^{18}O introduction. Fig. 5a illustrates the reaction scheme of this labelling approach.

Overall this strategy is limited due to the high cost of ^{18}O labelled water, the often incomplete incorporation of ^{18}O and

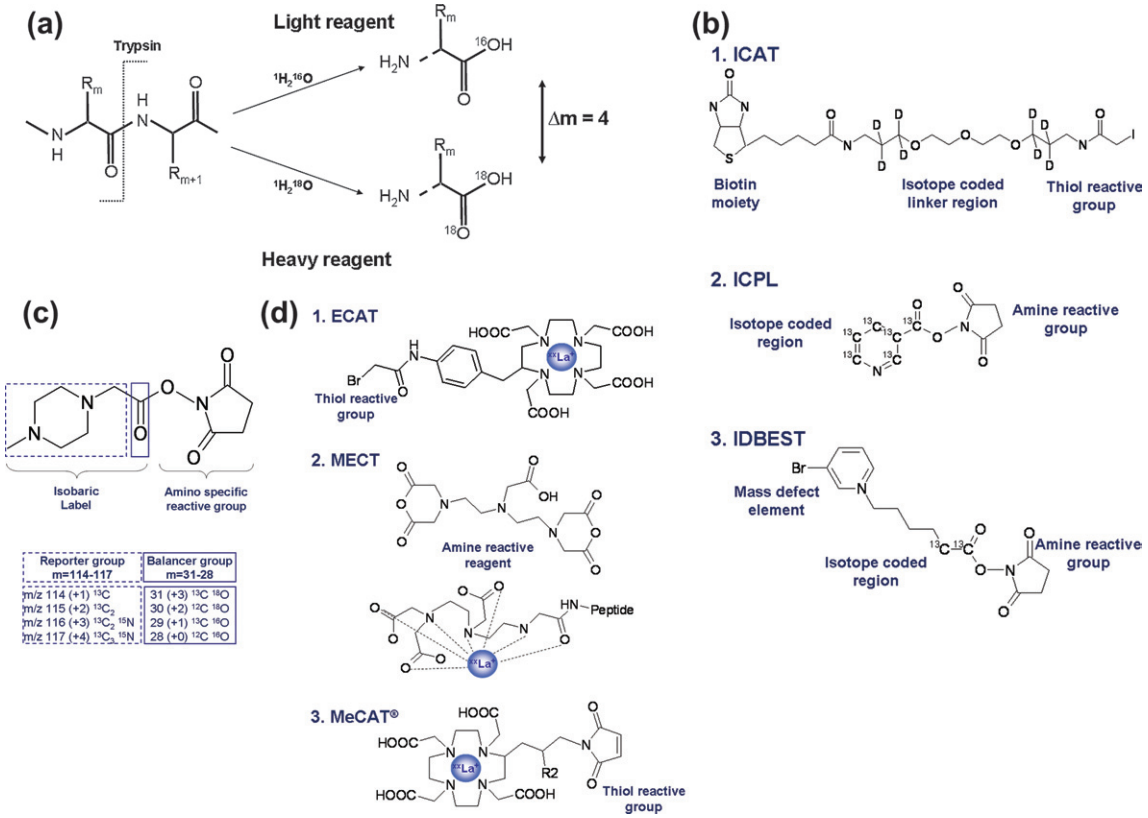


Fig. 5 (a) Reaction scheme of the ^{18}O water labelling approach based on the application of serine proteases such as trypsin, chymotrypsin and GluC, allowing the introduction of up to two ^{18}O atoms into the C terminus of every peptide generated during the proteolytic cleavage of the peptide bound resulting in a mass difference of 4 Da between the differently labelled peptide pairs. (b) (1) Schematic structure of the original ICAT® reagent, which consists of an isotope coded liker region (containing 8 ^1H (light version) or 8 ^2D (heavy version) atoms), a thiol-reactive iodoacetamide group that allows the selective alkylation of cysteine residues and a biotin moiety that allows enrichment of the cysteine containing labelled peptides *via* biotin-avidin affinity chromatography. (2) Schematic structure of the ICPL™ reagent. The reagent consists of ^{12}C or ^{13}C containing *N*-nicotinoyloxy-succinimide and allows the labelling of primary amine groups, which can be found at the N-terminus and within lysine residues. (3) Structure of the IDBEST™ reagent, which features a aminoreactive group (*N*-hydroxysuccinimidyl ester), two carbons, which can be selectively labelled with ^{12}C or ^{13}C to create a light and a heavy version of the reagent, the mass defect element (bromine) and a positive charge for high mass spectral ionisation efficiency. (c) The four chemically identical iTRAQ™ labels consist of a reporter group (*N*-methylpiperazine moiety), a balancer group (C═O moiety) and a reactive group targeting all primary amines (*N*-hydroxysuccinimide). The labelling process of the four samples, which takes place at the peptide level after the enzymatic digestion yields labelled peptides identical in mass and hence also identical in the single MS mode, but which produce strong diagnostic, low mass signature ions at masses 114, 115, 116 and 117 in the MS/MS mode. (d) (1) Structure of the element-coded affinity tag (ECAT), which consists of a derivative of the bi-functional chelating agent 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA), which contains a bromoacetamido reactive group to specifically target cysteine residues. (2) Structure of the metal element chelate tag (MECT) based on the bicyclic anhydride diethylentriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA). (3) Structure of the DOTA based MeCAT® reagent, which features a cysteine reactive malemide group and a further position which can be modified with other functional groups facilitating affinity purification.

the comparatively small mass shift (2 or 4 Da respectively) obtained between the two samples which results in significant overlap of the isotopic patterns of the light and the heavy digestion products.[32–35]

2.3 Chemical labelling due to derivatisation of selected amino acid residues using isotopically (^{2}H, ^{13}C, ^{15}N, ^{18}O) modified reagents

Proteins and peptides feature different reactive centres depending on their amino acid composition, which can be derivatised.

The derivatisation of the primary amino groups found at the *N-termini* of proteins and peptides and in *lysine* residues using *succinimide* or *isothiocyanate* based reaction chemistry is a frequently used approach.

Other concepts focus on the derivatisation of the *SH group* found in *cysteine* residues using *maleimides* or *iodoacetamides* as reactive centres to form covalent bonds.

Even though different techniques are available which allow the large scale comparative, relative quantification of whole proteomes, absolute protein quantification has still not been fully realised.

However the development of several reagents for the stable isotope labelling of proteins and peptides has been reported and several products are commercially available.

The following section will give an overview on selected reagents currently available for comparative and quantitative proteomics *via* stable isotope labelling together with their advantages and limitations. In general labelling can take place before (*protein level*) or after the enzymatic digestion procedure (*peptide level*).

2.3.1 ICAT® (isotope-coded affinity tag). The ICAT® technology (*isotope-coded affinity tag*) introduced by Gygi and co-workers represents the first commercially available reagent for the determination of the relative expression level of individual proteins originating from two different samples of the same biological system.[36]

Therefore, the control and the pertubated samples were individually labelled using a light and a heavy version of the ICAT® reagent. The differently labelled samples were then mixed and enzymatically digested. As shown in Fig. 5b, the original ICAT® reagent consists of an isotope coded linker region, containing 8 ^{1}H (light version) or 8 ^{2}D (heavy version) atoms, a thiol-reactive iodoacetamide group that allows the selective alkylation of cysteine residues and a biotin moiety that allows enrichment of the cysteine containing labelled peptides *via biotin–avidin affinity chromatography*.

Meanwhile various examples for the application of the ICAT® approach for the global relative quantification of protein expression levels in complex samples can be found in the literature, especially as a result of the commercial availability of the reagent and the implementation of this strategy into different database search engines and instrument software.[37–41]

The most critical point of the ICAT® approach and other amino acid specific labelling reagents is the potential underrepresentation of proteins and loss of peptide redundancy for a given protein species, since proteins in a mixture would be represented by at least one *e.g.* cysteine or histidine containing peptide. Clinically relevant features, such as different protein isoforms or post-translational modifications, might be completely missed during MS analysis unless these features are located in the enriched peptide species.[26]

Avidin affinity chromatography also often shows a significant unspecific binding of unlabelled cysteine free peptides or irreversible adsorption effects of some cysteine containing labelled peptides.

Chromatographic co-elution of the differently labelled species into the mass spectrometric detector is an essential prerequisite for the accurate measurement of their ratios and therefore their relative quantification.

A general, critical issue of the ICAT® reagent has been highlighted by Zhang and co-workers who demonstrated the influence of the heavy label on the liquid chromatographic separation since the significant substitution of ^{1}H for ^{2}D changes the retention time of the labelled species in RP-HPLC. In some experiments they even obtained a baseline separation of the light and heavy labelled counterparts.[42,43] However, this problem has also been observed with other ^{2}D based labels. In addition, the rather hydrophobic biotin moiety, which is a part of the reagent, causes peptides to elute in a relatively narrow time window. To overcome the various problems related to the original ICAT® reagent, the so called *cleaveable ICAT®* (cICAT) has been introduced recently.[44]

Instead of using ^{1}H or ^{2}D the new reagent uses ^{12}C and ^{13}C to obtain a significant mass shift between the differently labelled sample species and to eliminate the known chromatographic effects. Additionally, the reagent contains a cleavable biotin moiety which can be removed after the affinity purification process resulting in reduced hydrophobicity of the labelled peptides, which now elute in a much wider time window.

Different applications of the cICAT can be found already in the literature indicating a significant improvement in this labelling strategy in comparison to the original reagent.[45–49] However, the high cost of both reagents might still be a reason for limiting a more widespread application of the ICAT® approach.

2.3.2 ICPL™ (isotope-coded protein label). The ICPL™ strategy (*isotope-coded protein label*) was introduced by Schmidt and co-workers in 2005.[50] The reagent consists of ^{12}C or ^{13}C containing *N*-nicotinoyloxy-succinimide (see Fig. 5b) and allows the labelling of primary amine groups found at the N-termini and within lysine residues. The labelling step is performed before the proteolytic digestion step at the protein level and results in a mass shift of $\Delta m = 6.0204$ Da. This also allows a reduction in sample complexity due to the possibility to perform a pre-fractionation at the protein level. Since the labelling takes place at the protein level, information on post translational modifications or protein degradation can also be obtained. Due to the amine specific labelling, a much better sequence coverage can be obtained in comparison to thiol specific labelling. Furthermore, an up to 100 fold enhancement in MS sensitivity has been obtained due to the derivatisation of the samples with nicotinic acid.

The influence of the ICPL™ label on the p*I* of the protein species and the current limitation on the comparison of two samples represent the main disadvantages of this approach. However a number of publications have already shown successful applications of this labelling technique.[51–54] Also, reagents for a triplex assay are currently under development.

2.3.3 IDBEST™ (isotope-differentiated binding energy shift tag). Hall and Schneider introduced the *isotope-differentiated binding energy shift tag* (IDBEST™) technique which incorporates elements with a substantially different nuclear binding energy (mass defect) in comparison to the elements common in bio-molecules. The mass defect is related to the nuclear binding energy liberated during the formation and stabilisation of the nucleus of a given isotope. The most abundant elements in proteins such as C, H, N and O have negligible mass defects in comparison to ^{12}C, which by convention is assigned a mass defect of zero. Especially after the incorporation of high mass defect elements (elements with atomic numbers between 35 (Br) and 63 (Eu)), the mass spectral peaks that correspond to peptides carrying the tag are shifted on average from chemical noise derived from sample impurities and unlabelled peptides by approximately −0.1 amu per mass defect atom. Mass defect shifted peptides can be readily resolved from the chemical noise with any mass spectrometer with a resolution ≥10 000 on the basis of the incorporation of one mass defect element in the labelling reagent. Since the labelled species can be easily discriminated from the unlabelled ones in the mass spectrometer, any extensive front end separation of peptides prior to analysis can be reduced to a minimum level.

As shown in Fig. 5b, the reagent features an amino-reactive group (*N*-hydroxysuccinimidyl ester), two carbons, which can be selectively labelled with ^{12}C or ^{13}C to create a light and a heavy version of the reagent for the differential quantitation of peptide up or down regulation, the mass defect element (bromine) and a positive charge for high mass spectral ionisation efficiency.[55,56]

This new reagent might also be used in a complementary setup which utilises molecule specific detection techniques such as ESI or MALDI MS combined with inductively coupled plasma mass spectrometry (ICP-MS) as an element specific detection technique. Since bromine represents a further interesting (hetero) element tag, various groups have already demonstrated the sensitive determination of brominated compounds using ICP-MS coupled to different chromatography separation techniques.[57–59]

As a complementary technique ICP-MS could be used for the absolute protein quantification *via* measuring the bromine content of every separated labelled peptide. Based on the known tag stoichiometry obtained during the protein identification *via* peptide sequence information, the absolute protein/peptide amount can be calculated.

Although the labelling reagent has already been announced, it is still not yet commercially available.

2.3.4 iTRAQ™ (isobaric tag for relative and absolute quantitation). While most other techniques allow only the comparison of two sample states, the *isobaric tag for relative and absolute quantitation* (iTRAQ™) currently allows the multiplex analysis of up to four samples at the same time (4 plex assay).

As shown in Fig. 5c, the four chemically identical iTRAQ™ labels consist of a reporter group (*N*-methylpiperazine moiety), a balancer group (C═O moiety) and a reactive group targeting all primary amines (*N*-hydroxysuccinimide).

The labelling process of the four samples, which takes place at the peptide level after enzymatic digestion, yields labelled peptides identical in mass and hence also identical in the single MS mode. However they produce strong diagnostic, low mass signature ions at masses 114, 115, 116 and 117 in the MS/MS mode, allowing the simultaneous comparison of up to four sample states.

The multiplexing capability, the better protein sequence coverage due to the amine specific labelling and the improvement of the MS and MS/MS spectra quality due to the iTRAQ™ label are the most important advantages of this approach. However, no information on the protein level can be obtained and it is difficult to detect post translational modifications as well as protein degradation. Furthermore, this approach cannot be used with all ion trap MS's since the reporter group ions appear in the range 114 to 117 Da and many instruments show a low mass cut-off during MS/MS.

A number of examples showing the successful application of the iTRAQ™ approach can be found in the literature.[60–65]

However, detailed fragmentation experiments on multiple charged proteolytic peptides have shown that the efficient liberation of iTRAQ™ reporter ions from lysine residues only occurs at higher collision energies, which results in a loss of sequence informative fragment ions.[65]

Reagents for an 8 plex assay are currently under development.

2.3.5 PROTEIN-AQUA™ (protein absolute quantification). The application of stable isotope (^{2}H, ^{13}C, ^{15}N, ^{18}O) labelled analogues of the compounds under investigation as internal standards has a long tradition in mass spectrometry especially for the accurate quantification of small organic molecules.

The AQUA (*absolute quantification*) strategy, as introduced by Gerber and co-workers in 2003, can be described as a variation of the well known isotope dilution mass spectrometry technique and includes the application of synthetic, stable isotope labelled peptide analogues as internal standards. These correspond to selected native peptides formed during proteolytic digestion of the sample investigated.[10] Covalent modifications, such as phosphorylation sites, can also be introduced into the synthetic labelled peptide allowing the precise, absolute quantification of either proteins or post-translational modified proteins after enzymatic digestion of the spiked sample. Depending on the labelled amino acid selected for incorporation into the synthetic peptide analogues, mass shifts of 6 Da (L-proline-$^{13}C_5$,^{15}N/L-valine-$^{13}C_5$,^{15}N), 7 Da (L-isoleucine-$^{13}C_6$,^{15}N/L-leucine-$^{13}C_6$,^{15}N), 8 Da (L-lysine-$^{13}C_6$,$^{15}N_2$) or 10 Da (L-arginine-$^{13}C_6$,$^{15}N_4$/L-phenylanaline-$^{13}C_9$,^{15}N) can be obtained.

The labelled internal standard peptides are analysed by MS/MS to acquire their fragmentation patterns followed by the design of selected reaction monitoring experiments (SRM) in which a specific precursor/product transition is measured. During sample analysis the abundance of a specific fragment ion from both the native peptide and its labelled analogue are measured in an LC-SRM experiment. Since the absolute amount of the labelled peptide spike is known, the absolute concentration of the corresponding native protein can be calculated by comparing the signal abundance of the AQUA internal standard peptide with the native peptide.

The main advantage of the AQUA strategy is its capability to allow absolute protein quantification of either "normal" as well as post-translational modified protein species. Since the internal standard peptides are spiked into the sample before the enzymatic digestion procedure, extraction efficiency, absolute

loss during sample handling and variability during introduction into the LC-MS system do not affect the determination of the ratio of the native and the AQUA peptide abundances.

However, due to the need to synthesise specific labelled peptide analogues this approach is more useful for a targeted proteomics approach focusing on a limited number of known proteins rather than for the large scale identification and quantification of large sets of (unknown) proteins.

2.3.6 GIST (global internal standard technology). The *global internal standard technology* (GIST), as introduced by Chakraborty and Regnier, is based on the derivatisation of peptides with *N*-acetoxysuccinimide or *N*-acetoxy-$^{2}D_{3}$succinimide, respectively, which react with all primary amine groups (lysine residues and N-termini).[66]

In comparison to other techniques, the different protein sets (sample and control) are enzymatically digested separately prior to the differential isotopic labelling of the resulting peptides. Both sets are then mixed, fractionated by reversed phase chromatography, and this is followed by isotope ratio measurements using mass spectrometry. The main advantage of this approach is that almost all tryptic peptides are labelled due to the presence of primary amine groups in virtually every peptide so a redundant set of peptides will represent the different proteins within the sample.

The main disadvantage of the GIST technique is that the use of deuterium labels causes retention time shifts during reversed phase chromatography as already described in connection with the original ICAT® reagent.[42,43] Since the two samples under investigation are digested separately and the labelling and the combination of the two samples takes place at a rather late stage of the workflow, specific losses and other errors can not be compensated.

2.4 Chemical labelling due to derivatisation of selected amino acid residues using bi-functional chelating agents and lanthanides as mass markers

As an alternative to using numbers of atoms of the stable isotopes ^{2}D, ^{13}C, ^{15}N or ^{18}O to generate a significant mass difference between the light and the heavy version of the labelling reagent, naturally monoisotopic lanthanides can be used to prepare a variety of mass tags allowing multiplex experiments to be performed that exceed the current possibilities of established technologies such as iTRAQ™ (quadruplex) or ICPL™ (triplex).

Furthermore, lanthanides are heavy elements whose mass defects give the masses of tagged peptides exact values not normally shared by molecules that contain only the light "standard" elements (H, C, N, O), which are the main constituents of every bio molecule.

Table 1 gives an overview of mass differences between pairs of natural *monoisotopic* lanthanides which could be used as mass markers. The number of combinations highlights the possibility to design highly multiplexed experiments.

The field of radiopharmacology pioneered the controlled labelling of peptides, proteins or antibodies with radioactive nuclides by the development of appropriate reaction chemistry. Meanwhile nearly every nuclide of the periodic table and thus also their stable counterparts can be attached to biomolecules, either covalently or due to the formation of extraordinarily stable complexes. These complexes have been used to specifically target tumor cells for radioimmunoimaging or radioimunolacalization in cancer research but also for the transport of radiation with respect to a highly tumor-specific immunoradiotherapy.

Table 1 Mass differences between different natural monoisotopic (>97 %) rare earth elements used for labelling

Element	^{89}Y	^{139}La	^{141}Pr	^{159}Tb	^{165}Ho	^{169}Tm	^{175}Lu
^{89}Y		50	52	70	76	80	86
^{139}La			2	20	26	30	36
^{141}Pr				18	24	28	34
^{159}Tb					6	10	16
^{165}Ho						4	10
^{169}Tm							6
^{175}Lu							

In particular, DOTA (1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) or DTPA (diethylentriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid) based reagents targeting different functional sites of a protein or peptide have often been used for labelling with lanthanides due to the high stability of the metal chelate complexes formed. The following section will highlight the latest organic MS based strategies which use bi-functional chelating agents targeting different amino acid residues and lanthanides as differential mass tags.

2.4.1 ECAT (element-coded affinity tag). Whetstone *et al.* introduced a further cysteine specific labelling strategy termed *element-coded affinity tag* (ECAT). The reagent consists of a derivative of the bi-functional chelating agent 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA), which contains a bromoacetamido reactive group to specifically target cysteine residues (see Fig. 5d).

DOTA forms outstandingly stable complexes with lanthanides, which provide a variety of choices for different mass tags. As with ICAT®, the ECAT approach also includes an affinity purification step performed after the enzymatic digestion of the differently labelled proteins. In contrast, the affinity purification step does not utilise the frequently used antibody–antigen interaction between biotin and avidin, which often shows unspecific binding reactions leading to an enrichment rather than an isolation of the tagged species. Whetstone and co-workers used the monoclonal antibody 2D12.5 which binds selectively to DOTA-rare earth chelates.

As proof of principle they digested an unlabelled as well as Tb and Y labelled synthetic peptides mixed at different ratios. They could show that the antibody selectively binds the DOTA labelled peptides independent of the chelated rare earth element and that the differentially labelled peptides show co-elution during reversed phase chromatography. During MS/MS experiments neither fragmentation of the tag nor detrimental effects on the fragmentation behaviour of the labelled peptide have been observed, which are prerequisites for the comparative multiplex analysis and relative quantification of more complex samples.[67]

2.4.2 MECT (metal element chelate tag). Recently Liu *et al.* described a comparable attempt. In contrast, their method called

metal element chelate tag (MECT) is based on the labelling of primary amines with a metal chelate. To introduce a rare earth metal chelate tag they used the bicyclic anhydride diethylentriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), which is covalently coupled to peptide amine groups and finally chelates the rare earth elements (see Fig. 5d).

The MECT approach features some advantages since DTPA is a quite inexpensive, well known reagent often used in the field of radiopharmacology for the covalent modification of proteins, peptides and antibodies. Since DTPA labels all primary amine groups, theoretically every generated peptide will be recognized by this approach. Furthermore, a DTPA modified N-terminus could promote a cleaner fragmentation, since mainly y-series ions are generated, which increases the confidence level of protein identification.[68]

3 Quantitative analysis of bio-molecules *via* natural element tags and chemical labels using inductively coupled plasma mass spectrometry (ICP-MS)

Besides the number of recent developments in the quantitative analysis of proteins using organic MS techniques, a number of groups are working in the field of plasma mass spectrochemistry to push the development of new methodologies for the quantification of bio-molecules using ICP-MS. This section will highlight this progress and the potential of elemental mass spectrometry, namely ICP-MS, as a complementary tool especially for the quantification of bio-molecules in life-sciences related research.

Since many proteins contain one or more metal ions coordinated at their catalytical or functional domain, ICP-MS has been widely used to detect a selected number of such proteins *via* their natural metal label.

Metals are often coordinated due to the presence of histidine (*via* N), cysteine (*via* S bonds) or carboxyl (*via* O) residues.[69] Most of these metal-protein complexes are quite fragile under standard analytical conditions, so they can mainly be used as tags under mild native conditions such as during CE or SEC experiments. Denaturating conditions, organic solvents, extreme pH levels or the presence of enzymes results in the loss of the quarternary structure of a protein and often leads to the release of the weakly bound metal tags, thus making them useless for ICP-MS detection.

A number of good reviews can be found in the literature dealing with the idea of using trace elements as tags for a limited number of selected bio-molecules.[15,16,70] However, the focus in this review will be placed on elements which are *covalently* bound to proteins or peptides and which show a much wider distribution in these bio-molecules allowing their general application for the detection and quantification of nearly all proteins.

The two main approaches emerging for the complementary application of ICP-MS for life-science orientated research can be defined, as follows:

– the utilisation of *natural* (hetero) element tags for the determination of bio-molecules,

– the *chemical labelling* of bio-molecules with rare earth elements using bi-functional chelating agents or element containing nano particles

In comparison to the ESI or MALDI ionisation process, the main advantage of the ICP as an ion source is its matrix independency, since its sensitivity is proportional to the number of detectable atoms inside the molecule investigated. Therefore, a compound independent calibration can often be performed using simple inorganic standards allowing the quantification of unknown compounds without the need for specific standards.[71]

Moreover, the wide dynamic range of up to 9 orders of magnitude (allowing the simultaneous detection of high and low abundant compounds), its multi-element capabilities as well as the high sensitivity (detection limits of 0.1–1 ng mL^{-1} or better) for most elements of the periodic table make ICP-based mass spectrometry an attractive complementary future key tool in a number of selected life-science orientated applications—especially within the field of quantitative bio-analysis.

3.1 Utilisation of naturally occurring inductively coupled plasma mass spectrometry (ICP-MS) detectable, covalently bound (hetero) element tags

The most interesting and promising, naturally occurring element tags are phosphorus, sulfur, selenium and iodine since they are covalently incorporated into proteins. Statistically, at least one of them can be found in all proteins. The following section will give a critical overview on the recent application of different *natural* (hetero) element tags used for the ICP-MS guided sample fractionation and the quantification of selected bio-molecules. Table 2 provides a comprehensive overview of the current applications, which utilized phosphorus as a natural element tag.

3.1.1 Phosphorus. Phosphorus has been recognised as a biologically important element in terms of cell biology. It is an essential part of the ribose-deoxyribose phosphorus backbone in the RNA or DNA chain and also a component of phospholipids, which are the main buildings blocks of all prokaryotic and eukaryotic cell membranes. Moreover, protein phosphorylation is one of the most important *post-translational* modifications of a protein.

From an analytical point of view, phosphorus also opens up different possibilities in both the qualitative and quantitative determination of bio-molecules since it shows a widespread distribution in biological systems.

Unfortunately, phosphorus is not a showpiece element in terms of its ICP-MS detectability, since it features a high first ionisation potential (10.484 eV), a low ionisation efficiency in an argon based plasma and various ubiquitous, polyatomic interferences such as $^{14}N^{16}O^{1}H^{+}$.

Since it is mono-isotopic (main stable isotope ^{31}P) it is not possible to switch to an isotope with less interference. However, the commercial availability of more stable, high resolution instrumentation and more recently the introduction of collision/reaction cell ICP-MS has helped to overcome the interference problem and has allowed phosphorus to be utilised as a natural (hetero) element tag for the determination of selected bio-molecules.

3.1.1.1 Determination of the total phosphorus content in proteins. As mentioned above, the sensitive and reliable measurement of phosphorus as a tag for the degree of phosphorylation of selected proteins is still not straightforward. Bandura and co-workers described, for the first time, the application of

Table 2 Overview on different applications using phosphorus as a natural element tag for the detection and quantification of bio-molecules

Year	Sample type	Separation technique	Detector	Detection limits	Comments	Ref.
DNA						
1999	Styren oxide–DNA adducts	RP-HPLC, normal bore (Nucleosil 25 cm × 4 mm i.d.), methanol gradient	HR-ICP-MS with O_2 addition and parallel ESI-MS	One modification in 3.5×10^5 unmodified nucleotides corresponding to 920 fmol P	After mathematical correction of changes in the instrumental response due to the LC gradient, phosphorus standards can be used for the quantification of the different adducts	75
2003	DNA nucleotides Calf thymus DNA Nuclease P1 digests	CZE and RP-HPLC, normale bore (Discovery C16 Amide, 15 cm × 4.6 mm i.d.), isocratic elution ammonium acetate	CC-ICP-MS with He as cell gas and ESI-MS	CZE: 87 μgL^{-1}/1 pg HPLC: 6 μgL^{-1}/60 pg	First report on the application of He as cell gas for interference reduction during ^{31}P analysis	79
2005	Styren oxide–DNA adducts	RP-HPLC, micro bore (Multisphere RP18, 25 cm × 1 mm i.d.) ammonium acetate-methanol gradient	Hexapole collision cell ICP-MS and membrane desolvation	Three modifications in 10^7 unmodified nucleotides corresponding to 45 fmol P	Bis(4-nitrophenyl)phosphate) used as internal standard for adduct quantification, with no mathematical correction necessary	77
2006	Melphalan DNA adducts	RP-HPLC, micro bore (Multisphere RP18, 25 cm × 1 mm i.d.) ammonium acetate-methanol gradient	Hexapole collision cell ICP-MS and membrane desolvation ESI-Q-TOF	Three modifications in 10^7 unmodified nucleotides corresponding to 45 fmol P	Bis(4-nitrophenyl)phosphate) used as internal standard for adduct quantification, with no mathematical correction necessary	76
2006	DNA standards	Agarose GE	HR-ICP-MS		First reports on the on-line hyphenation of GE and ICP-MS	81,82
Phosphorylated proteins						
2001	Synthetic phosphopeptides, beta casein digests, MAP kinase	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS, hexapole and collision cell ICP-MS with membrane desolvation and ESI-Q-TOF	0.1 pmol P injected	First paper on the application of ICP-MS in combination with capillary LC as complementary tools for protein phosphorylation analysis	96
2001	Synthetic phosphopeptides, alpha and beta casein and their tryptic digests	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS and ESI-Q-TOF		Simultaneous measurement of sulfur and phosphorus allows the calculation of the degree of phosphorylation of intact proteins	95
2002	Beta casein	1D SDS-PAGE and membrane blotting	Hexapole collision cell ICP-MS and laser ablatin	^{31}P: 16 pmol	First report on the application of membrane blotting for LA-ICP-MS	104
2002	Beta casein, alpha casein	DRC-ICP-MS	0.06 $\mu g\ L^{-1}$ $^{31}P^{16}O^+$	0.19 $\mu g\ L^{-1}$ ($^{32}S^{16}O^+$)	Oxygen used as cell gas to measure S and P simultaneously as their oxides with less interference	72
2002	Synthetic phosphopeptide mixture	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS and ESI-Q-TOF	40 fmol P (Spray chamber Nebuliser) 50 fmol P (modified DIHEN)	Introduction of a new interface based on a modified DIHEN nebuliser	94
2002	Polo-like kinases Plx1 and Plk1	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS and ESI-Q-TOF-MS	0.1 pmol ^{31}P injected		99
2003	Myoglobin, alpha casein, fibrinogenprotein kinase A	1D SDS-PAGE	HR-ICP-MS and laser ablation	5 pmol ^{31}P	Phosphorylation analysis LA-ICP-MS of gel-separated proteins significantly improved by the application of a blotting step and a novel cleaning procedure to eliminate inorganic phosphorus contamination	105
2003	CRM-BCR-273 Tau protein Alpha casein Beta casein		HR-ICP-MS and ICP-Q-MS	40 fmol ^{31}P (Spray chamber Nebuliser) 50 fmol ^{31}P (modified DIHEN)	FIA used for the analysis of the phosphorus content of small sample volumes	74

Table 2 (*Contd.*)

Year	Sample type	Separation technique	Detector	Detection limits	Comments	Ref.
2003	Tryptic digests of human fibrinogen, bovine fetuin	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS and ESI-Q-TOF-MS	0.1 pmol P injecte	The complementary application of elemental mass spectrometry helps to identify four new phosphorylation sites in bovine fetuin	98
2003	Beta casein Human tau protein	2D SDS-PAGE	HR-ICP-MS with laser ablation MALDI/ESI-FT-ICR-MS		Quantification of single protein spots not possible due to the high background of P and S in the gel matrix	163
2004	Human brain sample extracts	2D SDS-PAGE	HR-ICP-MS and laser ablation	^{31}P: 0.6 mg kg^{-1} ^{32}S: 150 mg kg^{-1}	2D spots screened by LA-ICP-MS for the presence of P and S as well as selected trace elements	164
2004	Human cancer tissue samples	1D SDS-PAGE	DRC-ICP-MS with oxygen as cell gas and laser ablation,	^{31}P: 0.6 mg kg^{-1}		73
2005	Beta casein	1D SDS-PAGE with whole gel elution	DRC-ICP-MS with oxygen as cell gas and laser ablation, FIA	0.09 mg kg^{-1} ^{31}P^{16}O^{+}	Whole gel elution combined with FIA for the determination of the phosphorus content in the different fractions helps to overcome contamination problems often seen during the direct LA of gel spots.	106
2005	Tryptic digests of beta casein	RP-HPLC, capillary, (Agilent Zorbax SB C18 15 cm × 0.3 mm and Trap column Agilent Zorbax SB C18 5 mm × 0.3 mm), TFA-methanol gradient	CC-ICP-MS with He as cell gas and ESI-MS-MS	1.95 μg L^{-1} ^{31}P (capillaryLC conditions) coresponds to 1.95 pg absolute Below 400 fmol for the singly phosphorylated tryptic fragment of beta casein	Introduction of a new interface system for the coupling of capillary and nano LC with ICP-MS detection	100
2005	Chemotaxis protein CheA	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm), TFA-acetonitril gradient	HR-ICP-MS and ESI-Q-TOF-MS			97
2006	Ovalbumin, lactoperoxidase, alpha casein, myoglobin, cytoplasmatic proteome of *Corynebacterium glutamicum* and *Mus muculus* cells	1D SDS-PAGE, RP-HPLC, capillary (Vydac C18, 15 cm × 0.2 mm), TFA-acetonitril gradient	HR-ICP-MS and Laser ablation	^{31}P: 0.2–30 pmol ^{32}S: 10–400 pmol (depending on the use analytical strategy)	First report on the global phosphorylation degree analysis of a complex sample mixture using HR-ICP-MS as detector	109
2006	Beta casein, Pepsin	1D SDS-PAGE, membrane blotting	HR-ICP-MS and laser ablation	3 pmol beta casein, 5 pmol pepsin	First report on a new laser ablation chamber allowing the ablation of blot membranas up to 12 × 12 cm	107
2007	Alpha and beta casein	1D SDS PAGE	HR-ICP-MS and laser ablation	1.5 pmol ^{31}P	Introduction of a new ablation cell setup which can carry a whole blotting membrane	108
2007	Tryptic digests of beta casein	RP-HPLC, capillary, (Agilent Zorbax SB C18 15 cm × 0.3 mm), formic acid-acetonitril gradient	CC-ICP-MS with He as cell gas		BNPP used as internal standard for absolute phosphor peptide quantification. Post column sheath flow applied to compensate for sensitivity drifts during gradient elution	102
2007	Protein extracts from *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*	RP-HPLC, capillary (Vydac C18, 15 cm × 0.2 mm), TFA-acetonitril gradient	HR-ICP-MS		The simultaneous measurement of P and S allows the calculation of the global degree of phosphorylation of the different sample species	101

Table 2 (*Contd.*)

Year	Sample type	Separation technique	Detector	Detection limits	Comments	Ref.
2007	Casein standards	Agarose GE	HR-ICP-MS		First report on the application of on-line gel electrophoresis for the analysis of protein phosphorylation degrees	83
Miscellaneous						
2005	Carboxylic acids	RP-HPLC, normal bore (Phenomenex Luna C18, 10 cm × 4.6 mm), formic acid-acetonitril gradient	HR-ICP-MS with membrane desolvation and ESI-IT-MS	7.8 ng ^{31}P absolute	The reagent used allowed the labelling of carboxylic acids with phosphorus making them detectable with elemental mass spectrometry	165

a dynamic reaction cell ICP-MS system for the quantitative determination of phosphorus in protein samples.[72,73] Polyatomic interferences which have a detrimental effect on the phosphorus specific detection have been eliminated by using oxygen as reaction gas. It is then possible to analyse phosphorus as its corresponding oxide (as $^{31}P^{16}O^{+}$). However new interferences at *m*/*z* 47 have to be considered when using this approach. In parallel they also measured sulfur (as $^{32}S^{16}O^{+}$ at *m*/*z* 48) allowing the determination of the degree of phosphorylation of different standard proteins.

Detection limits down to 0.06 ng mL^{-1} for P and 0.2 ng mL^{-1} for S have been obtained. The measured degree of phosphorylation for beta-casein (4.9 phosphorus atoms/molecule) and regular alpha-casein (8.8 phoshorus atoms/molecule) were in good agreement with the structural data for the proteins.[72]

Becker *et al.* described the determination of the total phosphorus content in small sample amounts using flow injection analysis (FIA) hyphenated to high resolution ICP-MS. The results obtained for the quantitative phosphorus determination were in good agreement with the values specified for the certified reference material used for method validation.[74]

3.1.1.2 Application to DNA analysis. Siethoff and co-workers pioneered the utilisation of phosphorus as a natural (hetero) element tag.[75] As an example they showed the quantitative determination of *in-vitro* generated adducts of styrene oxide and the four nucleotides 2′-deoxyguanosine-5′-monophosphate (dGMP), 2′-deoxythymidine-5′-monophosphate (dTMP), 2′-deoxycytidine-5′-monophosphate (dCMP) and 2′-deoxyadenosine-5′-monophosphate (dAMP) by the complementary use of high resolution ICP-MS and ESI-MS coupled to normal bore RP-HPLC.

Oxygen additions to reduce the carbon build-up within the system and the application of a mathematical correction function to compensate for changes in the instrumental phosphorus response during the HPLC gradient were necessary. Inorganic phosphorus standards were used for the quantification of the different adducts allowing the detection of one modified nucleotide within 3.5 × 10^5 un-modified nucleotides. Using a different chromatographic setup, even higher sensitivities have been achieved which are comparable with radioactive post-labelling techniques.[75]

Recently Edler *et al.* used a hexapole collision cell ICP-MS combined with a membrane desolvation system and a microbore HPLC setup for the quantification of DNA adducts generated due to the reaction with either styrene oxide or melphalan.[76] SPE was applied for sample preparation and sample enrichment. Gradient elution and bis(4-nitrophenyl)phosphate (BNPP) as internal standard were used for the separation and quantification of the different, modified nucleotides. Unfortunately no ESI-MS data for characterisation of the different styrene oxide DNA adducts was shown. However the authors achieved comparable analytical figures of merit to those presented by Siethoff *et al.*[75–77] Furthermore, no mathematical corrections were necessary to compensate for changes in the instrumental response due to the application of membrane desolvation.

Besides using LC for the separation of RNA nucleotides, Yeh *et al.* used capillary electrophoresis hyphenated to ICP-MS for their separation and phosphorus specific detection.[78]

More recently Pröfrock *et al.* demonstrated the use of capillary electrophoresis (CE) and normal bore HPLC hyphenated to an octopole based collision/reaction cell ICP-MS for the separation and element specific detection of mono-phosphorylated deoxynucleotides.[79] In parallel, they used HPLC-ESI-MS to identify the separated compounds. Helium was used as cell gas to minimise polyatomic ions which interfere with the sensitive phosphorus specific detection. In contrast to other applications described in the literature no membrane desolvation was necessary.[79]

Mueller-Spitz *et al.* described the application of SEC-ICP-MS to investigate the interaction of chromium with DNA extracted from metal-contaminated soil samples. A collision cell system with helium as cell gas was used to reduce polyatomic interferences on the masses of phosphorus (^{31}P) and chromium (^{52}Cr). Phosphorus was monitored to trace the DNA containing fraction during the SEC separation.[80]

Recently Brüchert and Bettmer introduced the online coupling of gel electrophoresis and ICP-SFMS for the separation and quantification of DNA strands of different lengths.[81] Detection limits of 1 ng DNA absolute could be achieved, which corresponds to 96 pg of phosphorus. Overall their approach is mainly limited by the (expensive) instrumentation needed and the relatively poor separation efficiency that can be obtained with a 1D agarose gel. They proposed their approach as an alternative for the precise quantification of DNA strands especially in certification exercises, where alternative independent methods are urgently needed.[81,82]

In addition, the same authors recently extended their approach to the determination of the degree of phosphorylation of selected model proteins.[83]

3.1.1.3 Protein phosphorylation analysis. Whereas the basic function of a protein is genetically encoded, the real time dynamics and regulation of the protein structure and its function are generally dependent on specific post-translational modifications due to chemical reactions, which cause, for example, their phosphorylation or glycosylation.[2]

Currently more than 200 different kinds of post-translational protein modifications have been described and characterised.[84]

The *reversible phosphorylation* of proteins at their *serine*, *threonine* and *tyrosine* residues is one key modification in eukaryotic organisms that ultimately regulates cell signalling as well as the enzymatic activity, subcellular localisation, complex formation, and degradation of proteins and therefore the function of a biological system.[85,86]

Phosphorylation is a highly dynamic process which is regulated by the complex interplay of kinases and phosphatases that catalyse protein phosphorylation and de-phosphorylation, respectively.[87]

Up to 30% of all proteins in a eukaryotic cell are supposed to be phosphorylated at the same time indicating the importance of this process.[86]

However, despite the outstanding methodological and instrumental developments especially within the field of mass spectrometry, the analysis of protein phosphorylation is still not straightforward.

Different approaches have been used for the detection and quantification of protein phosphorylation. Standard procedures include *in-vivo* or *in-vitro* incorporation of radioactive ^{32}P as phosphate or *via* labelled adenosine tri-phosphate (ATP) followed by gel electrophoresis or thin layer chromatography, Edman sequencing and the use of phosphospecific antibodies.[88,89]

Besides these traditional strategies for protein or peptide phosphorylataion analysis, ESI and MALDI based MS techniques have gained increasing interest since they can provide sequence as well as phosphorylation site specific information.

The comparison of the peptide mass fingerprint obtained by MALDI-MS of a sample before and after the treatment with enzymes such as *alkaline phosphatase*, which will cleave the ester bonds between the phosphate groups and the amino acid residues serin, threonine and tyrosine, provides information on which peptides carry phosphorylation sites due to the observation of characteristic mass shifts within the mass spectrum.[90]

ESI based tandem MS systems also provide various scan modes which can be used for phosphorylation analysis. Neutral loss scanning in the positive ion mode after collision induced dissociation (CID) experiments for H_3PO_4 (−98 Da) or HPO_3 (−80 Da) provide characteristic information on the presence of phosphoserin or phosphothreonine containing peptides. Phosphotyrosine containing peptides generate a diagnostic immonium ion at *m/z* 216.043. Precursor ion scanning in the negative ion mode for fragments that generate the loss of PO_3^- (−79 Da) represents a further sensitive approach for the detection of phosphorylated peptides.[2,91,92]

There is a growing need for the quantification of phosphorylation events especially in signal transduction pathways. Quantitative data on phosphorylated residues in selected signalling proteins will permit a true systems biology approach which will facilitate the understanding of regulatory pathways and networks based on phosphorylation.[92]

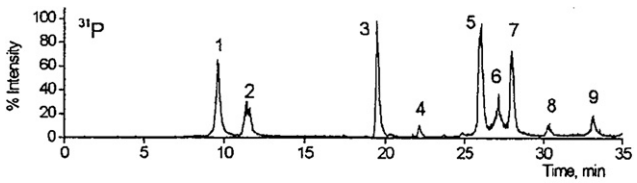
Therefore ICP-MS represents a valid complementary approach for protein phosphorylation analysis with respect to the fast screening of complex samples for phosphopeptides as well as their absolute quantitative determination, since it allows a selective phosphorus specific determination.

Wind and co-workers pioneered this field with a number of papers, which initiated a significant change in the field of plasma spectrochemistry.[93–96] Firstly, they introduced the successful application of capillary LC hyphenated to ICP-MS. This is a prerequisite for the real complementary application of ESI and ICP based MS techniques in life sciences related research due to their high compatibility with the optimal solvent flow rates of electrospray ionisation ($\leq$1 μL min^{-1}) and their superior chromatographic properties with respect to separation efficiency, peak sensitivity, adsorption effects and sample dilution.

Additionally, the application of low flow separation techniques helps to reduce problems related with the introduction of organic solvents such as acetonitrile or methanol into the inductively coupled plasma, which are frequently used for the reversed phase gradient separation of proteins or peptides.

In their initial paper, they used capillary LC hyphenated to either high resolution ICP-MS and a hexapole collision-reaction cell ICP-MS as well as ESI-MS-MS for the phosphorus specific detection of different synthetic phosphopeptides and tryptic digests of β-casein or activated MAP kinase. Fig. 6 shows the results of one of the initial experiments by Wind *et al.* using capillary LC and HR-ICP-MS for the separation and ^{31}P specific detection of 9 phosphopeptides. Membrane desolvation was used to reduce the organic solvent load within the plasma and to further reduce the number of spectral interferences to an acceptable level especially when using the hexapole ICP-MS system. However, they observed some sample loss within the desolvation systems especially of late eluting compounds.[96]

In a second paper they extended their approach to the simultaneous measurement of the phosphorus and sulfur content of the eluting peptide fractions to investigate the degree of phosphorylation of different model proteins. It was also possible to



peak no.	component	mol wt (mono)
1	SPE-pS-SDTEEN	1173.37
2	EAQAA-pS-AAQAK	1124.49
3	V-pS-INEK	768.34
4	LRRA-pY-LG	927.47
5	KG-pS-EQESVKEFLAK	1658.79
6	FKGPGDTSNFDDYEEEEIRV-pS-INEK	2997.29
7	TW-pT-LCGTPEY	1249.47
8	impurity present in 7	
9	TW-pT-LCGTPEYLAPEIILSK	2214.07

Fig. 6 Capillary LC-ICP-HRMS (resolution 4000) run with ^{31}P detection of a mixture of synthetic phosphopeptides with phosphoserine, phosphothreonine, and phosphotyrosine residues. Assignment of the chromatographic peaks was performed by a separate LC ESI-MS run and is summarised in the Table. (Taken from ref. 96, reprinted with permission from *Anal. Chem.* 2001, **73**, 29–35. Copyright 2001 American Chemical Society.)

investigate compound specific discrimination effects of the electrospray ionisation process between non-phosphorylated and phosphorylated sample species.[95]

To overcome the problem of post column band broadening effects during capillary or nano flow separations of phosphor peptides, Wind *et al.* modified a direct injection nebuliser (DIHEN) to minimise the dead volume of the device. In comparison with previously used spray chamber nebulisers, the DIHEN was slightly less sensitive but showed better chromatographic resolution, superior signal stability and more robustness with respect to changing gradient conditions. Different phosphorus peptides were used as model compounds to compare the performance of the different devices.[94]

In a number of further papers, the same group demonstrated the complementary application of elemental and molecule specific MS for the investigation of protein phosphorylation of real samples, such as the polo-like kinases Plx1 and Plx2, human fibrinogen and fetuin subunits or the phosphorylation state at His-48 of the chemotaxis protein CheA, which influences the stability of this protein.[97–99]

The commercially available nebulisers used by Wind *et al.* (PFA microflow, DIHEN) were a compromise in terms of the hyphenation of capillary or even nano LC to ICP-MS, since they are designed for highest sensitivity at higher flow rates (typically 20–100 μL min^{-1}). Furthermore, the need to operate the HR ICP-MS system in the medium resolution mode to eliminate the polyatomic interferences at the *m/z* ratio of phosphorus results in a reduced overall sensitivity of *ca.* 90 % for some elements compared to the low resolution mode.

To overcome some of these problems Pröfrock *et al.* introduced a new interface system allowing the successful direct hyphenation of capillary and nano HPLC to an octopole collision cell ICP-MS system. This interface system facilitates the direct introduction of organic gradients into the ICP-MS without the need for using membrane desolvation or oxygen addition.[100] Helium was used as cell gas to minimise polyatomic interferences at the mass of phosphorus while maintaining a good overall instrumental sensitivity. The new interface, based on a modified capillary electrophoresis interface and a specially designed spray chamber, allows direct nebulisation inside the ICP torch without the detrimental effects of *e.g.* DIHEN nebulisers. It features 100% transport efficiency, good nebulisation stability (≤1% signal RSD for 10 μg L^{-1} Y at a flow rate of 3 μL min^{-1}) and minimised dead volumes at capillary as well as nano LC flow rates (below 1 μL min^{-1}).

As a model protein, β-casein was used to demonstrate the potential of the proposed setup for the phosphorylation profiling of tryptic protein digests. As a complementary approach, capillary LC-ESI-MS-MS was used to further characterise the peptides pre-selected due to their phosphorus content. Fig. 7 shows the different working steps of the complementary application of ICP and ESI-MS for the investigation of protein phosphorylation. Down to 400 fmol of the singly phosphorylated peptide FQpSEEQQQTEDELQDK derived by the tryptic digestion of β-casein could be detected, while detection limits of 1.95 μg L^{-1} (1.95 pg absolute) for phosphorus were obtained.[100]

In one of the latest papers on protein phosphorylation analysis using elemental mass spectrometry, Krüger and co-workers analysed protein phosphorylation levels of *Arabidopsis thaliana* and the algae *Chlamydomonas reinhardtii* as representatives for multicellular and unicellular, green, photosynthetically active organisms.[101] Capillary LC and high resolution ICP-MS in the medium resolution mode were employed for the separation and element specific detection of phosphorylated peptides after

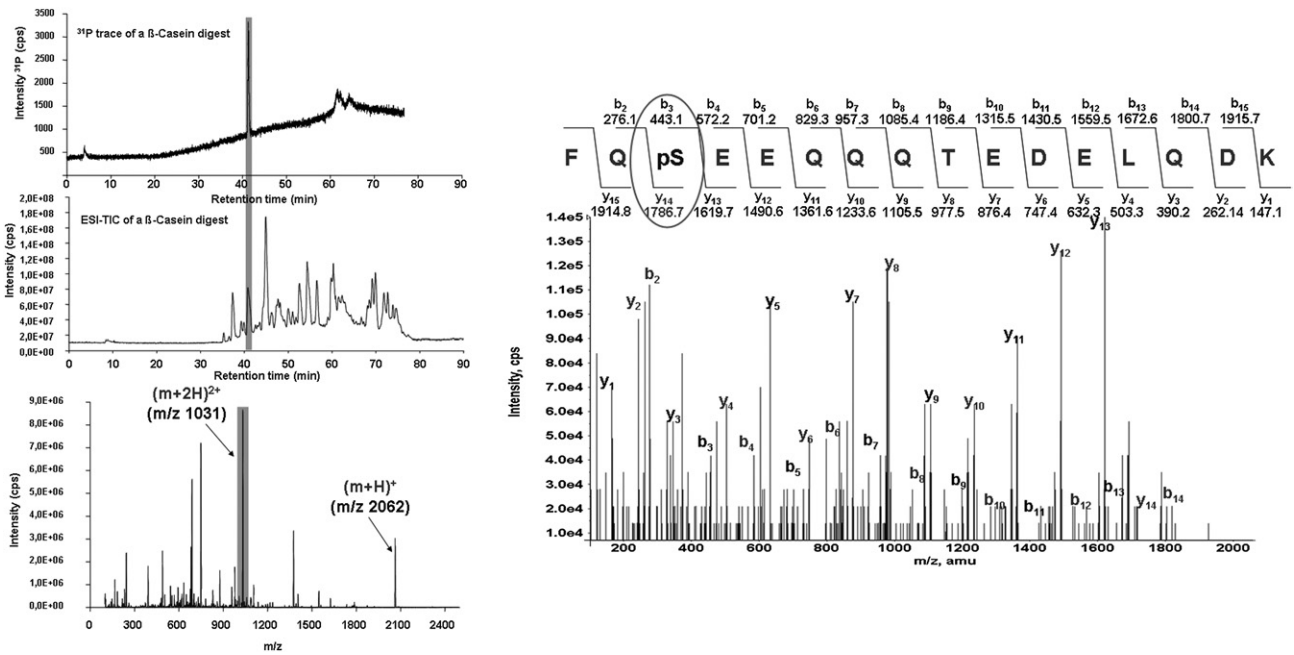


Fig. 7 Procedure steps using ICP-MS and ESI-MS-MS as complementary tools in protein phosphorylation analysis: (a) Alignment of the ^{31}P trace and the ESI-TIC of β-casein separated by CapLC. (b) Data deconvolution of the ESI-TIC at the retention time of the previously detected peak in the ^{31}P trace. (c) On-line CapLC-ESI-MS-MS spectra of the doubly charged peptide at *m/z* 1031. (Taken from ref. 100, reprinted with permission from *Anal. Bioanal. Chem.*, 2005, **381**, 194–204. Copyright Springer–Verlag 2005.)

enzymatic digestion of the protein extracts. Besides monitoring phosphorus they also monitored the corresponding sulfur traces to quantify the cellular protein phosphorylation levels of the different organisms and their compartments respectively. Based on the genome data of both organisms they estimated the average number of sulfur containing amino acids to convert the molar phosphorus to sulfur ratio into a stoichiometric protein phosphorylation degree. Due to the generally low abundance of phosphoproteins in complex samples they used metal oxide affinity chromatography (MOAC) to specifically enrich phosphoproteins from the different plant extracts. Again, elemental mass spectrometry was used to monitor the enrichment procedure as well as to determine the enrichment factor obtained. Unfortunately, the authors did not present additional ESI results for phosphoprotein/peptide identification even though the ICP-MS based screening of the samples indicated the presence of a number of phosphorylated peptides.[101]

While most examples of ICP-MS based quantitative analysis of protein phosphorylation have utilised mathematical correction functions to compensate for sensitivity drifts during the LC gradient, Pereira Navaza *et al.* recently introduced a new strategy for the accurate quantification of protein phosphorylation using simple organic compounds as a phosphorus standard.[102] They used a post column sheath flow with a constant acetonitrile content to buffer gradient composition changes, which influence the ionisation efficiency of phosphorus within the plasma even at capillary LC or nano LC flow rates. As a result, a partly constant elemental response over the capillary LC gradient has been achieved (10–50 % B), allowing the application of a single phosphorus containing compound, such as bis(4-nitrophenyl) phosphate (BNPP), as standard for the phospho-peptide quantification.[102]

The application of laser ablation ICP-MS (LA-ICP-MS) as an interface for the utilization of slab gel techniques such as 1 or 2 dimensional gel electrophoresis as a high resolution separation technique has also gained much interest, especially in protein phosphorylation analysis.

Initially Neilsen *et al.* described the combination of gel electrophoresis and LA-ICP-MS as a new strategy for the speciation of metal binding serum proteins.[103] Since metal–protein complexes often show limited thermodynamic stability, special emphasis has to be placed on the integrity of the metal–protein complexes during electrophoresis, thus favouring investigation under native rather than denaturating conditions. However, since phosphorylation sites are covalently attached to selected amino acids, denaturating separation conditions can also be utilised.

In a communication, Marshall and co-workers described, for the first time, the investigation of phosphorylated proteins blotted on a membrane by LA-ICP-MS.[104] The well known β-casein was used as model protein and detection limits of 16 pmol were obtained. They also attempted the direct analysis of gels containing the destained protein spots. However, due to the high phosphorus background during the ablation process of the gel matrix it was not possible to determine the location of the phosphorylated proteins.[104]

Wind *et al.* improved this approach by adding a washing step after the blotting process into their strategy for the LA-ICP-MS based analysis of gel separated phosphoproteins.[105] The aim was to remove any inorganic, non-covalently bound phosphate from the blot membrane, which often shows non-specific interactions with other non-phosphorylated sample constituents leading to false positive results. A $Ga(NO_3)_3$ solution was used as complexing agent to remove any non-covalently bound phosphate from the membrane. Detection limits of 5 pmol phosphorus were obtained. They also presented some quantitative data indicating the possibility of using this approach for the quantification of the separated phosphor protein species.[105]

Elliott and colleagues introduced a new strategy for the analysis of gel separated phosphoproteins by combining whole gel elution with flow injection ICP-MS detection. Phosphorus was measured as $^{31}P^{16}O^+$ at *m*/*z* 47. In comparison, they also applied LA-ICP-MS to the phosphorus specific screening of 1D PAGE gels.[106]

Recently Feldmann and co-workers developed and optimised a new laser ablation cell for the detection of phosphoproteins blotted on to nitrocellulose membranes.[107] In comparison to other ablation cells, where the membrane had to be cut into pieces, their new device allowed the direct analysis of whole blot membranes with dimensions up to 8.5 cm height and 10 cm length. This approach helps to overcome some of the problems related to the direct ablation of native or SDS gels, especially the high background levels for elements such as phosphorus and sulfur caused by the gel matrix and the chemicals used. Furthermore, the blotting process results in an enrichment of the protein species in a thin layer within the membrane which can be easily ablated by each single laser shot. Detection limits for the model proteins pepsin and β-casein down to 5 and 3 pmol respectively were calculated. The quantitative results obtained for a commercially available protein marker standard calculated from the acquired calibration function were also in good agreement with the concentrations given by the manufacturer. Overall, this approach is mainly limited by the possibility of additional sample loss and the additional time needed for the blotting process.[107]

Based on the new ablation cell setup described by Feldmann and co-workers, Venkatachalam *et al.* introduced a new calibration approach for quantitative detection of phosphorylated proteins blotted on to membranes.[108] Different parameters, such as scanning conditions, reproducibility and membrane type, were investigated and optimised. For the calibration procedure they compared two approaches: The first based on the dotting of different standards (β-casein, pepsin, phosphate solution) containing different, known amounts of phosphorus directly on the blotting membrane. Unfortunately, the results showed that dotting leads to underestimation of the amount of phosphorus in the test protein and a poor recovery of only around 55%. As an alternative, the standards were separated and blotted together with the test sample. A total amount of 126 pmol of phosphorus was determined with the latter approach, which is in good agreement with the known calculated total phosphorus amount of 139 pmol of the test sample. Additionally, the authors achieved a higher dynamic range in comparison to their previous work. The calculated number of phosphorylation sites was also in good agreement with the theoretical value. Taking into account the whole spot area, the standard deviation of the whole method (electrophoretic separation, blotting process, laser ablation procedure) was 6%.[108]

However, most of the current applications using LA-ICP-MS for a phosphorus specific detection of electrophoretically separated proteins can be considered as proof of principle, since mainly standard phosphoproteins, such as the well known α and β caseins, have been repeatedly used as model proteins.

In a recent publication Krüger *et al.* showed, for the first time, the protein and proteome phosphorylation stoichiometry analysis of the cytoplasmatic proteome of bacterial cells (*Corynebacterium glutamicum*) and eukaryotic cells (*Mus musculus*) by a combined approach based on 1D gel electrophoresis, in-gel digestion and capillary reversed phase LC-ICP-MS as well as an additional strategy, which includes the application of 1D gel electrophoresis, protein blotting and LA-ICP-MS.[109] Both approaches revealed consistent quantitative results, however higher sensitivities were achieved with the capillary LC based setup. Overall, in their study the eukaryotic proteome was found to be significantly more phosphorylated in comparison to the bacterial proteome (around 0.8 mol of P per mol of protein *vs.* around 0.01 mol of P per mol of protein). They showed for the first time the direct and fast deduction of a global phosphorylation degree of a complex protein mixture.[109]

A very valuable review article on the application of ICP-MS to protein phosphorylation analysis has been recently published by Navaza *et al.*[110]

3.1.1.4 Phospholipid analysis. Phospholipids are the main membrane constituents of all pro- and eukaryotic cells. Besides their structural function, they play an important role in various biological processes and biochemical pathways such as in cellular signalling systems. Phospholipids have also gained special attention as biomarkers in chemotaxonomic studies or as components of pharmaceuticals and cosmetic products.

Due to their heterogeneity and their complexity in biological samples, neither the qualitative nor the quantitative analysis of membrane phospholipids is straightforward.

Despite the advantages of using elemental mass spectrometry as a detector for the phosphorus specific phospholipid analysis, up to now this application has not gained much interest in comparison to other applications such as measuring DNA adducts or protein phosphorylation states.

Since phospholipids are only soluble in organic solvents, normal phase liquid chromatography is mostly used for the separation of complex phospholipid mixtures. To deal with the high organic solvent load, which is necessary for the chromatographic separation of these compounds, special techniques have to be employed, such as membrane desolvation, oxygen addition or low flow LC systems, to reduce the carbon load in the plasma.

Phospholipid analysis using phosphorus as an element tag was first described by Axelsson *et al.*, who used liquid chromatography hyphenated to a hexapole collision/reaction cell ICP-MS system *via* an ultrasonic nebuliser and a membrane desolvator.[111] Different phospholipid standards were used for method development and the authors clearly showed the advantages of this generic detection approach over other techniques such as UV, RI, ELSD or MS in terms of sensitivity and linearity.[111]

More recently Kovacevic *et al.* described the application of normal bore HPLC hyphenated to an octopole reaction cell ICP-MS system for the separation and phosphorus specific detection of different phospholipid standards as well as lipid extracts from the yeast *Sacharomyces cerivisiae*.[112] Solvent splitting, spray chamber chilling down to −5 °C and the addition of oxygen were employed to prevent the extinction of the plasma as well as carbon deposition on the cones during analysis. Helium was used as a cell gas to reduce polyatomic interferences at the nominal mass of phosphorus. Absolute detection limits for phosphorus between 0.21 and 1.2 ng phosphorus were obtained.[112]

3.1.2 Sulfur. Sulfur also represents another promising naturally occurring elemental tag detectable by ICP-MS that can be found in bio-molecules. Sulfur is statistically present in most proteins and peptides due to the sulfur containing amino acids methionine or cysteine. Recent calculations on the basis of the human proteome have revealed that 26.6% and 25.5%, respectively, of the resulting tryptic peptides contain at least one of the amino acids cysteine or methionine within their sequence.[9] Overall these peptides represent 96.1% and 98.9% respectively of all human proteins, so it is obvious that it might be possible to determine the majority of proteins on the basis of their natural sulfur tag. This approach allows the absolute quantification of proteins or peptides once their sulfur stoichiometry is clarified by the complementary application of ESI or MALDI based MS approaches.[93]

Recently, Wind *et al.* proposed sulfur as a key element for the quantitative analysis of proteins and peptides.[93] As an example they demonstrated the quantification of insulin as a model protein using thiamin as internal standard. They also showed the complementary application of capillary LC HR-ICP-MS and capillary LC ESI-MS-MS for the quantification and characterisation of tryptic protein digests of two functional domains of the bacterial chemotaxis protein cheAH (3–137) and cheA-C (257–513) overexpressed in *E.coli*.[71,93]

At first glance, sulfur would seem to be a nearly ideal tag since it is covalently incorporated into bio-molecules, it shows a widespread distribution in nearly all proteomes and it features four stable isotopes (^{32}S, ^{33}S, ^{34}S, ^{36}S see Table 3) which can be utilised for its detection using isotope dilution analysis or for the development of multiplex assays based on metabolic labelling with amino acids (methionine, cysteine) isotopically enriched in one of the mentioned isotopes.

In a very recent paper, J. G. Martinez-Sierra and co-workers have demonstrated the biosynthesis of a ^{34}S labelled yeast and its characterisation by multicollector ICP-MS.[113] Even though this initial study only highlights the precise measurement of the total sulfur concentration within the ^{34}S labelled yeast as well as the precise investigation of the different sulfur isotope ratios, it already indicates the possibility of using the differently,

Table 3 Overview of the different sulfur isotopes and interfering molecular ions

Isotope	Abundance (%)	Main Interference
^{32}S	94.93	$^{16}O_2^+$
^{33}S	0.76	$^{32}S^1H^+$, $^{16}O_2{}^1H^+$
^{34}S	4.29	$^{33}S^1H^+$,
^{36}S	0.02	$^{35}Cl^1H^+$

metabolically ^{34}S labelled yeast strains for comparative proteomic studies (SILAC approach) as well as the absolute protein quantification based on the sulfur tag.

Unfortunately, sulfur is also one of the more difficult elements with regard to its detectability by plasma-based spectrometric techniques. Sulfur features a high first ionisation potential (10.357 eV) and also a relatively low ionisation efficiency in an argon-based plasma. Moreover, various polyatomic molecules such as $^{16}O_2^+$ or $^{16}O^{18}O^+$ which are formed inside the plasma, interfere with the sensitive determination of sulfur due to isobaric overlap with the main isotopes ^{32}S and ^{34}S respectively. To overcome this problem, a spectral resolution of at least $m/\Delta m$ 1800 is necessary in order to resolve, for example, the mass ^{32}S (at exactly 31.97207 amu) from its main interference $^{16}O_2^+$ (31.98983 amu).[114] The physical separation of the analyte ions from the interfering ions thus requires either high resolution ICP-MS or techniques such as collision cell or dynamic reaction cell ICP-MS as highlighted within the following sections.

Despite the possibilities that arise due to the quantification of proteins or peptides using their natural sulfur tag, the overall number of examples that can be found in the literature is quite limited.

Heumann and co-workers introduced the application of isotope dilution analysis into the field of elemental speciation analysis as an accurate quantitative approach.[115–118] Prange and Schaumlöffel *et al.* picked up this attempt and pioneered the field of sulfur isotope dilution analysis by combining post column isotope dilution and capillary electrophoresis hyphenated to sector field ICP-MS for the highly resolved separation of different metallothionein (MT) isoforms as a model system. This allowed the determination of both the metal stoichiometry of the different isoforms and their absolute quantification.[119,120] A species unspecific element spike containing ^{34}S, ^{65}Cu, ^{68}Zn and ^{116}Cd introduced *via* the make-up flow of the CE interface was used for on-line isotope ratio measurement of $^{32}S/^{34}S$, $^{63}Cu/^{65}Cu$, $^{64}Zn/^{68}Zn$ and $^{114}Cd/^{116}Cd$. Mass flow calibration of the spike solution was performed by reversed isotope dilution using a standard solution containing S, Cu, Zn and Cd with natural isotopic abundances. 2,6-diacetylpyridine-bis(*N*-methylenpyridinohydrazone) was used to stabilise the metal ions in solution at the given pH of 7.4 used during the electrophoretic separation. Based on the known sulfur stoichiometry, molar ratios between sulfur and the different metals as well as the absolute amounts of the different isoforms were quantified.[119,121] Wang and Prange extended this approach by using surface modified CE capillaries to further improve the separation efficiency of the setup.[122] Overall, the approach is mainly limited by the small sample amounts used in CE and the necessity to use high resolution ICP-MS at medium resolution to overcome the sulfur interference problem, which both result in relatively high detection limits.

While the initial methodological developments of the CE-ICP-IDSF approach were carried out using commercially available protein standards Polec-Pawlak *et al.* demonstrated the application to real samples. Metallothionein complexes in rat liver extracts were investigated during their study. In addition, ESI-MS was used as a complementary technique to further identify the separated protein species.[123]

More recently Van Lierde *et al.* used an CE-ICP-SF-MS setup and simple species unspecific element standards such as albumin (for S) and $ZnCl_2$ for the quantification and determination of the stoichiometric Zn/protein ratio for the *Aeromonas hydrophila metallo beta lactamase AE036*.[124] This again demonstrates the nearly compound independent response of ICP-MS. Detection limits of 8 ng of the protein calculated on the basis of sulfur were obtained.[124]

In the past, the sensitive determination of sulfur using its main isotope ^{32}S as a natural element tag for the quantification of bio-molecules was restricted to high resolution ICP-MS due to the omnipresent interfering molecule ion $^{16}O_2^+$.

To overcome this problem Pröfrock *et al.* demonstrated, for the first time, the application of a quadrupole based collision/reaction cell ICP-MS with xenon as cell gas to reduce the interferences at the ^{32}S isotope.[125] Overall they could decrease the spectral background by about six orders of magnitude enabling the determination of sulfur with quadrupole based ICP-MS. Instrumental detection limits of 1.3 ng mL^{-1} (^{34}S) and 3.2 ng mL^{-1} (^{32}S) were obtained. They also showed the sulfur specific detection and quantification of metallothionein isoforms isolated from non-incubated fish samples after on-line CE-ICP-MS using species unspecific element standards. The total element content calculated after integration of the whole electropherogram was in good agreement with the concentrations measured with total X-ray fluorescence analysis as an independent method.[125]

As demonstrated by Bandura and co-workers, quadrupole ICP-MS with a dynamic reaction cell can also be used for the determination of sulfur.[72] To overcome the $^{16}O_2^+$ interference problem they used oxygen as cell gas to generate $^{32}S^{16}O^+$ ions. Due to the oxidation of $^{32}S^+$ it is possible to generate a mass shift of +16, allowing the detection of sulfur at m/z 48, which suffers less interference.[72,73] Based on this approach, Yeh *et al.* showed the determination of sulfur containing amino acids by using capillary electrophoresis coupled to dynamic reaction cell ICP-MS.[126]

Hann and co-workers published a comparison between SEC coupled to either ICP-DRCMS (oxygen as cell gas) and ICP-SFMS (medium resolution at $m/\Delta m$ 4000) for the determination of metal sulfur ratios in selected metalloproteins such as myoglobin or Mn superoxide dismutase.[127] Excellent detection limits for sulfur of 4.3 μg L^{-1} were obtained with the ICP-DRCMS approach in comparison to 14 μg L^{-1} that have been obtained with ICP-SFMS instrumentation. Different sample species-unspecific calibrants such as Fe^{3+}, Mn^{2+} or SO_4^{2-} were checked, however in terms of uncertainty of measurement better results were obtained by using metalloprotein standards. Since the method is based on an SEC separation with limited chromatographic resolution, it must be mentioned that its application may be restricted to pure or purified protein samples.[127]

In a further publication, the same group described the complementary application of LC coupled to either ICP-MS and ESI-TOF-MS for the investigation of native and recombinant copper proteins derived from the cyanobactterium *Synechocystis*. Sulfur was measured as $^{32}S^{16}O^+$ using oxygen as cell gas. They used this approach for the determination of the metal stoichiometry of the recombinant expressed protein species. Overall, they could show, *via* an altered sulfur/metal ratio caused by the removal of an N-terminal methionine, that two of the recombinant proteins show heterogeneous expression. Depending on

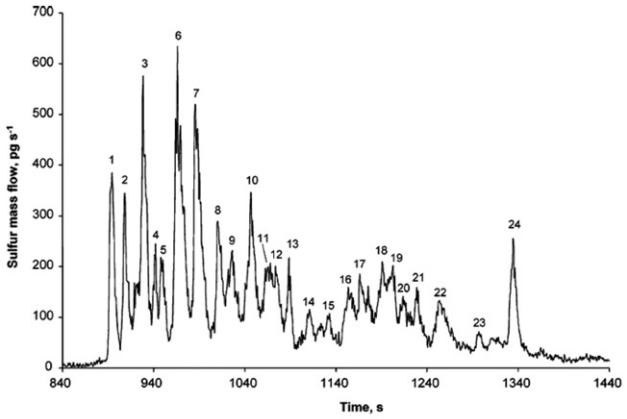
the chromatographic separation method, detection limits for sulfur between 4.6 μg L^{-1} (SEC-ICP-MS) and 16 μg L^{-1} (IC-ICP-MS) were obtained.[128]

Krüger *et al.* described the characterisation of a gadolinium-tagged modular contrast agent for magnetic resonance imaging (MRI) by a combined approach based on elemental and molecular mass spectrometry.[129] This new agent allows the cross membrane transport of gadolinium complexes, which are widely used as paramagnetic contrast agents for the imaging of intracellular compartments. SEC coupled to high resolution ICP-MS was used to calculate the Gd saturation of the synthetic cross membrane transporter complex based on the measurement of the sulfur to gadolinium ratio. Additionally the contrast agent was further characterised by nanoESI-QTOF-MS.[129]

Recently Garijo Anobe and colleagues used sulfur as an additional natural tag during the investigation of different iron containing metalloprotein standards such as haemoglobin, cytochrome c, ferritin and transferrin.[130] They introduced a new interface comparable to those initially described by Brüchert and Bettmer for the on-line coupling of 1D gel electrophoresis and ICP-MS. Iron–sulfur ratios were measured to monitor possible iron loss during the SDS-PAGE separation.[81,82,130]

Resuming, it can be stated that up to now most of the published methods on the application of sulfur as a natural (hetero) element tag for the detection and quantification of bio-molecules can also be only considered as proof of principle, since most applications have focused on the quantification of selected, intact, model proteins rather than on more proteomics relevant, complex samples such as tryptic digests. Despite this limitation they have all helped to supply a number of valuable approaches which should now be used in more interdisciplinary, more complex topics.

The most recent approach to using sulfur as a tag for peptide quantification has been published by Schaumlöffel *et al.* who showed the promising application of pre-column isotope dilution analysis in nanoHPLC-ICP-MS for the absolute quantification of sulfur containing peptides.[131] As a complementary technique the authors used nanoHPLC-ESI-QTOF-MS to identify and to elucidate the sulfur stoichiometry of the separated peptides, which is a prerequisite for the precise, absolute quantification based on the natural sulfur tag. As introduced by Pröfrock *et al.*, they also utilised xenon as cell gas to overcome the interference problems related to the sensitive sulfur detection using the main isotopes ^{32}S and ^{34}S. Different sulfur containing peptides as well as tryptic digests of human serum albumin (HAS) and the salt induced protein (SIP 18) isolated from selenium-rich yeast were used as model compounds for method development and validation. The detection limit for sulfur was 45 μg L^{-1} which corresponds to 1–2 pmol of the individual peptide species. The method developed has high accuracy, with recovery rates of 103%, and good precision of the quantification, with an RSD of 2.1 %. Sodium taurocholate was used as the sulfur compound to calibrate the mass flow during pre-column isotope dilution. This again indicates the compound independency of the ICP ionisation process and therefore the potential of using simple element containing standard compounds for the quantification of complex protein or peptide mixtures. Fig. 8 shows a sulfur mass flow chromatogram obtained from an HAS tryptic digest allowing the quantification of the different



peak no.	sulfur mass, pg	peptide amino acid sequence[b]	position in the HSA amino acid sequence[c]	peptide mass, ng
1	2427.2	TCVADESAENCDK	52–64	52.4
2	1868.6	ETCFAEEGKK	565–574 mc	66.5
3	4382.0	CCAAADPHECYAK	360–372	62.9
4	1035.2	CASLQKFGER	200–209 mc	36.7
5	1397.1	ADDKETCFAEEGK	561–573 mc	62.8
6	5615.1	AAFTECCQAADK	163–174	
		YICENQDSISSSK	263–274	
7	4929.2	CCTESLVNR	476–484	
		ETYGEMADCCAK	82–93	
8	2250.8	QEPERNECFLQHK	94–106 mc	116.8
9	2571.8	ECCEKPLLEK	277–286	47.7
10	3435.1	NECFLQHK	99–106	
		LKECCEKPLLEK	275–286 mc	
11	2150.3	VHTECCHGDLLECADDR	241–257	42.8
12	1254.7	NECFLQHKDDNPNLPR	99–114 mc	75.9
13	1305.1	AACLLPK	175–181	29.1
14	999.6	LCTVATLR	74–81	27.3
15	772.0	VHTECCHGDLLECADDRADLAK	241–262 mc	19.4
16	1770.7	RPCFSALEVDETYVPK	485–500	102.3
17	1126.0	QNCELFEQLGEYK	390–402	56.2
18	1765.5	LVRPEVDVMCTAFHDNEETFLKK	115–137 mc	74.9
19	1737.6	SLHTLFGDKLCTVATLR	65–81 mc	101.6
20	1069.4	LVRPEVDVMCTAFHDNEETFLK	115–136	44.2
21	1436.1	EFNAETFTFHADICTLSEK	501–519	98.6
22	2364.1	AVMDDFAAFVEK	546–557	98.9
23	665.8	SHCIAEVENDEMPADLPSLA ADFVESK	287–313	30.3
24	2134.8	TYETTLEKCCAAADPHECYAK	352–372 mc	
		MPCAEDYLSVVLNQLCVLHEK	446–466	
		ALVLIAFAQYLQQCPFEDHV K	21–41	

[a] Absolute quantification of sulfur-containing peptides in 1 mL of HSA tryptic digest via sulfur quantification. [b] Sulfur-containing amino acids: C cysteine, M methionine. [c] mc: miscleavage.

Fig. 8 Peptide quantification using pre-column sulfur isotope dilution analysis. (a) Sulfur mass flow chromatogram obtained from HAS tryptic digest by the precolumn isotope dilution technique. (b) Peak assignment and quantitative data of the different peak. (Taken from ref. 131, reprinted with permission from *Anal. Chem.* 2007, **79**, 2859–2868. Copyright 2007 American Chemical Society.)

sulfur containing peptides.[71] A comprehensive overview regarding the application of sulphur as a natural (hetero)element tag can be found in Table 4.

3.1.3 Selenium and iodine. Selenium as an essential trace element in human nutrition has gained much interest and a number of studies on its chemical forms (speciation) have been performed.[132] Here, selenium has been widely used as a tag to facilitate the recognition of the selenium containing species in complex sample mixtures as well as to allow their quantitative determination as summarised in various reviews.[133,134]

Currently, the work of various groups is focused on proteinaceous species, where selenium is covalently incorporated as a constituent of the amino acids *selenomethionine* and *selenocysteine*, which are derivates of methionine and cysteine respectively.[135–137]

While the incorporation of selenocysteine into proteins is genetically encoded by the UGA codon, selenomethionine is often incorporated into proteins instead of methionine under sulfur deficient conditions.[138]

Up to now, 22 mammalian selenoproteins have been found.[139] Current *in-silico* studies estimate that the human seleno proteome would consist of at least 25 selenoproteins.[140,141]

Therefore selenium serves as a tag only for a well defined number of proteins not suitable for general large scale protein quantification.

Table 4 Overview on different applications using sulfur as naturally element tag for the detection and quantification of bio-molecules

Year	Sample type	Separation technique	Detector	Detection limits	Comments	Ref.
Protein quantification						
2003	Thiamin, Insulin	RP-HPLC, capillary (Vydac C18, 25 cm × 0.3 mm or 12.5 cm × 0.3 mm), TFA-acetonitril gradients	HR-ICP-MS and ESI-Q-TOF	Peptides: 10 pmol ^{32}S Proteins: 25 pmol ^{32}S	Application of thiamine as internal standard for insuline quantification	93
2004	Myoglobin, Hemoglobin, Cytochrom c, Catalase-peroxidase, Mn-SOD, Arginase	SEC-HPLC, normal bore (Sephadex G25, 35 cm × 2 mm), ammonium acetate	HR-ICP-MS and DRC-ICP-MS	14 μg g^{-1} ^{32}S	In comparison with inorganic standards the use of a metalloprotein as calibrant shows better results in terms of uncertainty	127
2004	L-cystine, L-cysteine, DL-homocystine, L-methionine	CZE	DRC-ICP-MS	59 μg L^{-1} (measured as $^{32}S^{16}O$)	Oxygen used as cell gas to measure sulfur as oxide at *m/z* 48 with less interference	126
2004	Aeromonas hydrophila metallo-beta lactamase AE036	CZE	HR-ICP-MS	8 ng (absolute detection limito f the protein calculated on the basis of sulfur)	Sample species unspecific standards used for quantification (Albumin for S, $ZnSO_4$ for Zn)	124
2004	Gadolinium tagged modular contrast agent	SEC-HPLC, normal bore, (TSKgel Super SW3000, 30 cm × 4.6 mm), sodium acetate buffer	HR-ICP-MS and ESI-Q-TOF-MS		Measurement of sulfur/gadolinium ratios allows the determination of the metal saturation level of the contrast agent	129
Peptide quantification						
2007	Tryptic digests of human serum albumin, salt induced protein SIP 18	RP-HPLC nano bore (Dionex C18 PepMap 100, 15 cm × 0.075 mm with Dionex Acclaim C18 300 as trap column 5 cm × 0.3 mm), TFA-acetonitril gradient	CC-ICP-MS with Xe as cell gas and ESI-Q-TOF-MS	4 μg L^{-1} ^{32}S (Instrumental detection limit) 1 mg L^{-1} ^{32}S (transient signal under nano LC conditions)	Application of pre-column isotope dilution for absolute quantification of sulfur peptides with a homemade nano LC-ICP-MS interface	131
Metalloprotein quantification						
2002	MT standards	CZE	HR-ICP-MS	6.8 mg L^{-1} (^{32}S)	First report on the species unspecific isotope dilution analysis for the absolute quantification as well as the stoichiometric characterisation of protein/MT sample *via* their natural sulfur and metal tags	119
2002	MT standards	CZE with surface modified capillaries	HR-ICP-MS		Species unspecific isotope dilution analysis allows the quantification as well as the stoichiometric characterisation of MT samples *via* their natural sulfur and metal tags	166
2002	Rat liver MT	CZE	HR-ICP-MS and ESI-MS	7 mg L^{-1} (^{32}S)	First application of species unspecific isotope dilution analysis for the quantification of real protein samples *via* their natural sulfur tag	123
2003	MT standards, *Abramis brama L.* metallothionein like proteins	CZE	CC-ICP-MS with Xe as cell gas	3.2 μg L^{-1} ^{32}S, 1.3 μg L^{-1} ^{34}S, (Instrumental detection limit) 15 mg L^{-1} ^{32}S (transient signal under CZE conditions)	First report on the application of Xe as cell gas to minimize interferences at the ^{32}S isotope	125
Mixed applications						
2002	Beta casein, alpha casein		DRC-ICP-MS	0.06 μg L^{-1} $^{31}P^{16}O^{+}$ 0.19 μg L^{-1} ($^{32}S^{16}O^{+}$)	Oxygen used as cell gas to measure S and P simultaneously as their oxides with less interference	72
2004	Human brain sample extracts	2D SDS-PAGE	HR-ICP-MS and laser ablation	^{31}P: 0.6 mg kg^{-1} ^{32}S: 150 mg kg^{-1}	2D spots screened by LA-ICP-MS for the presence of P and S as well as selected trace elements	164

Table 4 (*Contd.*)

Year	Sample type	Separation technique	Detector	Detection limits	Comments	Ref.
2005	Styren oxide- DNA adducts	RP-HPLC, micro bore (Multisphere RP18, 25 cm × 1 mm i.d.) ammonium acetate-methanol gradient	Hexapole collision cell ICP-MS and membrane desolvation	Three modifications in 10^7 unmodified nucleotides corresponding to 45 fmol P	Bis(4-nitrophenyl)phosphate) used as internal standard for adduct quantification with no mathematical correction necessary	77
2006	Ovalbumin, lactoperoxidase, alpha casein, myoglobin, cytoplasmatic proteome of *Corynebacterium glutamicum* and *Mus muculus* cells	1D SDS-PAGE, RP-HPLC, capillary (Vydac C18, 15 cm × 0.2 mm), TFA-acetonitril gradient	HR-ICP-MS and Laser ablation	^{31}P: 0.2–30 pmol ^{32}S: 10–400 pmol (depending on the use analytical strategy)	First report on the global phosphorylation degree analysis of a complex sample mixture by using HR-ICP-MS as detector	109

However research on selenium and selenoproteins has promoted new analytical techniques, namely further interface approaches for the coupling of capillary and nano HPLC with elemental MS, thus allowing the utilisation of the same chromatographic conditions generally used in ESI experiments (flow rates below 1 μL min^{-1}) and therefore a real complementary application of elemental and organic MS.

To overcome some of the limitations of the first interface systems for coupling capillary LC to ICP-MS introduced by Wind *et al.*,[94,96] the application of a nebuliser originally developed by Prange and Schaumlöffel[142,143] for the coupling of capillary electrophoresis (CE) with ICP-MS was introduced for the coupling of capillary LC and ICP-MS by Schaumlöffel *et al.*[144] The mapping of selenopeptides derived from water extracts of selenised yeast demonstrated the potential of the new interface approach in comparison with results previously obtained with normal bore LC techniques. Overall the authors achieved 100–150 times lower detection limits for selenium in comparison to results obtained with conventional HPLC setups and ICP-MS detection.[144]

The same interface has been used by Giusti *et al.* to couple nanoHPLC with ICP-MS.[145] To compensate for the higher solvent flow rates necessary for the stable operation of the nebuliser, they applied a post-column sheath flow to obtain a total flow of around 4 μL min^{-1}. As demonstrated, this approach also facilitates on-line species unspecific isotope dilution analysis and therefore the absolute quantification of selenium containing peptides. The quantified amount of selenium present in the sum of all peaks (calculated by on-line isotope dilution analysis) was in good agreement with the total selenium concentration present in the tryptic digest injected.[145]

Recently Giusti *et al.* presented a further interface designed for the coupling of nano HPLC and elemental mass spectrometry.[146] To generate a stable spray at flow rates of 300 nL min^{-1}, they used a fused silica needle with a pulled tip (i.d. 10 μm, o.d. 20 μm) comparable to those used in nano ESI sources as the nebuliser capillary.

At flow rates higher than 200 nL min^{-1}, they obtained signal RSD's of better than 5 %. Oxide formation (measured as the ratio CeO^+/Ce^+) remained stable at around 0.2% at flow rates between 50 and 450 nL min^{-1}. The formation of doubly charged ions (measured as the ratio Ce^{2+}/Ce^+) remained stable at around 1.5% at flow rates between 50 and 450 nL min^{-1}. Absolute detection limits for ^{80}Se of 25 fg were obtained. A tryptic digest of a high molecular weight fraction isolated from a water extract of selenised yeast was used as test sample to demonstrate the complementary application of nanoHPLC-ICP-MS and nanoHPLC-ESI-QTOF-MS for the investigation of selenium containing peptides.[147]

In an additional paper the same author realised a setup which includes an on-line pre-concentration step allowing large volume injection resulting in a 100-fold enrichment and therefore further improvement of the sensitivity.[148]

Iodine is part of the thyroid hormones 3,3′,5,5′-tetraiodothyronine (T4) and 3,5′,5-triiodothyronine (T3), which are synthesised by the thyroid gland. Both are an essential part of the thyroglobulin protein. Already, in 1993 Takatera and Watanabe used iodine as a natural tag to detect and to quantify the contents of iodine-containing amino acids in an enzymatic digest of bovine thyroglobulin.[149]

Shah *et al.* detected different iodine containing compounds after the enzymatic digestion of seaweed samples using HPLC-ICP-MS.[150] However, both of these examples on using iodine as a tag are focused on the application of ICP-MS as a detector for iodine speciation rather than on utilising iodine for the quantification of the protein which has incorporated the different iodine species. No data are given with respect to the characterisation of the iodine containing protein species. Since the number of natural iodine containing protein species is also quite small, iodine has nearly no significance for protein quantification.

However the radiolabelling of proteins with ^{125}I is a well known approach to track proteins in an organism. Recently Venkatachalam *et al.* used stable iodine isotopes for the labelling of different antibodies targeting different forms of the cytochrome P450 enzyme family. They used laser ablation ICP-MS for the detection of membrane blotted microsomal cytochromes P450 *via* the differential labelled (iodinated) antibodies.[151]

3.2 Chemical labelling of bio-molecules with rare earth elements using bi-functional chelating agents or element containing nano particles

The field of radiopharmacology pioneered the controlled labelling of peptides, proteins or antibodies with radioactive

isotopes with the development of appropriate reaction chemistry. In the meantime, nearly every nuclide in the periodic table and therefore also their stable counterparts can be attached to a bio-molecule either covalently or due to the formation of extraordinarily stable complexes. These complexes have been used to specifically target tumor cells for radio immuno imaging or radio immuno localisation in cancer research but also for the transport of radiation with respect to a highly tumor specific immuno radiotherapy.

The controlled labelling of bio-molecules which contain no natural tag offers some advantages since elements can be used that show better detection capabilities in terms of sensitivity and which are not prone to interferences.

Currently, labelling with heavy metal containing nanoparticles or labelling with lanthanides, complexed by chelating agents which have been covalently attached to proteins *via* selected reaction chemistry, represent the most promising strategies for the future, elemental MS based quantification of nearly every protein despite the lack of a natural (hetero) element tag.

3.2.1 Application of bi-functional chelating agents. Zhang *et al.* described the first application of an element label for the quantitative determination of the thyroid-stimulating hormone (TSH) in human plasma samples by using ICP-MS and an Eu^{3+} labelled antibody. *N′-(p-isothiocyanatbenzyl)diethylentriamine-*N^1,N^2,N^3,N^4*-tetraacetate-*Eu^{3+} has been used as bifunctional chelating agent for the controlled labelling of the TSH antibody. Good correlation between the ICP-MS based assay and an already established radioimmunoassay was achieved.[152]

Quinn *et al.* extended this approach to a novel ICP-MS-linked immunoassay, which can be used to measure at least two antigens simultaneously. They used an antibody, labelled with Eu by using the Wallace AutoDELFIA™ reagent, which was originally designed for the fluorescence detection of antibodies in an automated fluoroimmunometric system. The second antibody was labelled by using the NANOGOLD® reagent, which is often used in electron microscopy to visualise and localise cellular proteins. They conclude that such an approach has broad multi-analyte capabilities through which many different antigens as well as protein–protein interactions can be quantitatively measured through elemental analysis, provided that distinguishable tags are used.[153]

In a recent paper Ornatsky *et al.* presented the first 4-plex assay for the simultaneous determination of the expression of intracellular oncogenic kinase BCR/Abl, myeloid cell surface antigen CD33, human stem cell factor receptor c-Kit and integrin receptor VLA-4 in a human leukaemia cell line model.[154] Au, Sm, Eu and Tb were conjugated to secondary antibodies by using the NANOGOLD® and different labelled Wallace AutoDELFIA™ reagents. They could show that the sensitivity of the ICP-MS linked immunophenotyping approach based on commercially available element tags is comparable with that of flow cytometric analysis (FACS).[154]

Ornatsky and co workers have also described the detection of messenger RNA (mRNA) in leukemia cell lines by using metal-tagged *in situ* hybridisation for the detection of specific RNA sequences allowing multiple gene expression analysis by ICP-MS based flow cytometry.[155] This approach is quite promising with respect to the development of multiplex assays with up to 50 antibodies providing a fingerprint of the expression level of leukaemia related proteins in a single cell.[155]

Recently Tanner *et al.* reviewed progress within the field of using ICP-MS as a detector for multiplex bio-assays based on differential element labelling.[156]

Current elemental MS based labelling strategies have been used to quantify a number of selected compounds defined by the availability of differently labelled antibodies.

In parallel to the approach published by Whetstone *et al.*, Krause and co-workers applied for a patent for a DOTA based reagent which features a cysteine reactive malemide group and a further position, which can be modified with other functional groups facilitating affinity purification and enrichment, named metale coded affinity tag (MeCAT®).[157] Fig. 5d shows the structure of the MeCAT® reagent together with other reagents used for the labelling of proteins with lanthanides.

Similar to the ICAT® reagent, the current MeCAT® reagent contains a biotin residue which allows the utilisation of biotin–avidin affinity chromatography, so that crudely labelled protein mixtures can be purified and labelled peptides where specifically enriched.

Recently Ahrends *et al.* published a first paper using bovine serum albumin and proteins of the *Sus scrofa* eye lens as model system. Different parameters such as labelling conditions, reaction efficiency and the reproducibility of the labelling process have been optimised and evaluated. They also showed that differentially labelled tryptic digests can be separated and detected with LC-MS-MS systems and that the same differential labelled peptides show co-elution, which is a prerequisite for multiplex comparative proteomics experiments.[158]

Already, in 2001 Zhang and co-workers demonstrated the potential of lanthanide labelling of bio-molecules combined with the high detection capabilities of ICP-MS.[152]

Krause *et al.* and Ahrends *et al.* showed in their work for the first time that lanthanide based labelling approaches combined with the introduction of elemental mass spectrometry based on inductively coupled plasma mass spectrometry (ICP-MS) into their proteomic workflow opens up new possibilities, since ICP-MS shows a large linear dynamic range, nearly no matrix effects, especially when working with low flow sample introduction techniques such as capillary HPLC or nano HPLC, and a high sensitivity. Furthermore, simple sample unspecific metal standards can be used for the absolute quantification of the differently labelled compounds. The number of monoisotopic lanthanides present in the periodic table allows the design of multiplex experiments for quantitative protein expression analysis which extends the possibilities of the currently available approaches.

Unfortunately, up to now they have only shown the application of flow injection analysis (FIA) ICP-MS experiments for the quantification of differently labelled protein spots obtained from soluble 2D gels.

3.2.2 Application of element containing nano-particles. Zhang *et al.* demonstrated for the first time the determination of antibodies labelled with colloid gold nanoparticles by ICP-MS. Good correlation was achieved with an enzyme linked immunosorbent assay (ELISA) used in a comparative study.[159]

As demonstrated by Baranov *et al.*, a gel filtration ICP-MS based immunoassay can also be used to separate and quantify different labelled antibodies *via* their metal tag. They showed the determination of human gamma globulin (IgG) by using a commercially available goat anti human Fab′ covalently labelled with a 1.4 nm nanogold cluster. The authors conclude that the immunoassay linked ICP-MS determination of elemental labelled antibodies offers a number of advantages over other detection methods. Since impurities have less impact on ICP-MS detection, detection limits can be improved by multiple labelling with a single isotope. In addition the equivalent sensitivity for equally labelled compounds, the wide linearity of the response and the ability to provide absolute quantitation represents further advantages of such approaches.[160,161]

While most applications dealing with the determination of labelled bio-molecules have been carried out in solution, another interesting application of LA-ICP-MS for the analysis of gel-separated protein species has been recently demonstrated. Since direct determination of a specific protein is not possible with ICP-MS due to the loss of any structural information in the 7000 K argon plasma, Müller *et al.* used Western Blots to specifically detect selected protein species by using gold cluster labelled antibodies.[162] After the 1D SDS PAGE separation and the blotting process, the selected protein species were subsequently marked with the gold labelled antibody due to the highly specific antigen–antibody reaction process.

The covalent labelling of the antibodies with a cluster of gold atoms helps to improve the sensitivity of this approach in comparison with other approaches, which mostly utilise single covalently bound heteroelements, such as phosphorus or sulfur, for the element specific detection process. Gold is less susceptible to interferences and gold contamination of a sample is quite rare leading to overall low background levels.

Detection limits for the gold cluster labelled antibodies down to 0.2 amol have been achieved. In comparison to other antibody detection methods, such as using alkaline-phosphatase conjugated secondary antibodies, the LA-ICP-MS approach shows much better linearity. They applied the methodology to the analysis of the Mre11 protein in crude lysates of CHO-K1 fibroblasts, which is involved in DNA repair and recombination. Furthermore, this protein is essential for mediating genome stability in mammalian cells.[162]

Beside the advantages of labelling approaches over other traditional detection methods, it has to be kept in mind that accurate label based quantification is strongly influenced by the complexity of the labelling chemistry, which might result in variable labelling efficiencies and therefore different stoichiometries of the label-protein complex. This will influence the quantification process. Furthermore, in the case of the NANOGOLD® reagent the stoichiometry of the label is not totally clear.

4 Conclusions and outlook

Both the need for accurate, relative and absolute quantification of bio-molecules and the detection of their quantitative, time dependent changes during dynamic processes within living systems have been recognised as vitally important to further understanding key cellular processes such as protein expression or their post-translational modification.

Elemental mass spectrometry, namely ICP-MS, has been proposed as a powerful complementary tool to be used alongside ESI or MALDI MS techniques in the emerging field of life-science related research, with special emphasis on the field of proteomics. ICP-MS provides quantitative elemental information while molecule specific MS facilitates compound characterisation and identification.

Work in the field of elemental speciation analysis has pushed this progress over the last decade by the development and publication of suitable technologies and methods.

In the future, much communication will be necessary to bring the possibilities of elemental mass spectrometry more into the focus of the proteomic community. A closer look into the literature and the citation behaviour reveals that still only a few authors outside the elemental MS community have realised the potential of this technique, especially for proteomics orientated applications, *e.g.* protein phosphorylation analysis.[86,87]

To overcome this problem the elemental MS community clearly has to show where this powerful technique has its reliability and where it really can help to solve problems that cannot be solved with ESI or MALDI techniques with all their facets, which are still the established gold standards within the field of protein biochemistry or proteomics.

A number of groups have recognised that ICP-MS has matured to become a real complementary tool for life sciences orientated research. However, at this time, many of the published methods can only be considered as proof of principle.

The challenge for the future will be to demonstrate the suitability of the proposed methods for real samples. All the approaches described will have to be further developed and the fitness for purpose, applicability and robustness of the methods will have to be validated.

Besides the still important relative quantification with respect to a comparative proteome analysis, the future ability to perform absolute protein quantification for large numbers of proteins, which is still impossible with most of the current stable isotope based labelling methods, will become increasingly important.

At present the utilisation of ICP-MS for protein phosphorylation studies and for the absolute protein quantification based on natural sulfur tags represents the most promising application field for the complementary use of ICP-MS in the life sciences. However, both fields belong also to the most challenging applications for ICP-MS, especially due to the interference problems, which both elements have to deal with.

New possibilities for the absolute quantification arise due to the implementation of strategies already established in radio pharmacology allowing the covalent chemical labelling of virtually every protein or peptide with an ICP-MS detectable (hetero) element tag, such as lanthanides. This will especially allow the design of large scale multiplex assays. Promising approaches tackling this strategy are within the focus of current developments and will possibly help to open up new application fields, where the complementary application of elemental mass spectrometry becomes vitally essential.

Elemental mass spectrometry has the potential to further mature to one of the key technologies especially within the field of precise bio-molecule quantification.

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