Anal Bioanal Chem (2008) 390:3–16
DOI 10.1007/s00216-007-1615-2

REVIEW

Elemental mass spectrometry for quantitative proteomics

Alfredo Sanz-Medel · María Montes-Bayón · María del Rosario Fernández de la Campa · Jorge Ruiz Encinar · Jörg Bettmer

Received: 16 July 2007 / Revised: 5 September 2007 / Accepted: 6 September 2007 / Published online: 2 October 2007
© Springer-Verlag 2007

Abstract In the last decade mass-spectrometry-based proteomics has become an indispensable analytical tool for molecular biology, cellular biology and, lately, for the emerging systems biology. This review summarises the evolution and great potential of analytical methods based on elemental mass-spectrometric detection for quantitative proteomic analysis.

Keywords Bioanalytical methods · Genomics/Proteomics · Mass spectrometry/Inductively coupled plasma mass spectrometry · Speciation

Abbreviations

2D	two-dimensional
AFP	α-Fetoprotein
CE	capillary electrophoresis
ESI	electrospray ionisation
GE	gel electrophoresis
HPLC	high-performance liquid chromatography
ICAT	isotope-coded affinity tags
ICP	inductively coupled plasma
IDA	isotope dilution analysis
IgG	immunoglobulin G
LA	laser ablation
LC	liquid chromatography
μLC	capillary liquid chromatography
MALDI	matrix-assisted laser desorption and ionisation
MS	mass spectrometry
MT	metallothionein
SEC	size-exclusion chromatography
SILAC	stable isotope labelling by amino acids in cell cultures
SFMS	sector field mass spectrometry
T3	triiodothyronine
T4	thyroxine

A. Sanz-Medel (✉) · M. Montes-Bayón · M. del Rosario Fernández de la Campa · J. R. Encinar · J. Bettmer
Department of Physical and Analytical Chemistry,
Faculty of Chemistry, University of Oviedo,
C/Julián Clavería 8,
33006 Oviedo, Spain
e-mail: asm@uniovi.es

Introduction

In the last decade mass spectrometry (MS) based proteomics has become an indispensable analytical tool for molecular biology, cellular biology and, lately, for the emerging systems biology. So far, however, the field of proteomics is built mainly on technologies aiming at investigating large numbers of proteins, usually in a comparative manner, in the same experiment.

In fact, the term "proteomics" was coined in the context of two-dimensional (2D) gel electrophoresis (GE) and 2D-GE is today used extensively worldwide but mostly for rather qualitative experiments [1].

Thus, it is not surprising that the application of MS in proteomics has been driven by such "qualitative" character of typical proteomics research.

Classical limitations of 2D-GE include bias and poor resolution against certain proteins (e.g. membrane proteins), low sensitivity to the point that only the most abundant of all separated proteins can be visualised by staining, lack of a PCR-like technology to get around of this low abundance protein problem, etc. Such limitations can be superseded by resorting to MS-based techniques such as matrix-assisted laser desorption and ionisation (MALDI) MS and electrospray ionisation (ESI) MS, as shown recently in several papers [2–4]. A typical experiment these days starts out

with a crude and complex sample containing many proteins. This sample is often initially pretreated to enrich it with some protein characteristics or features which are then separated (e.g. phosphorylated proteins). The proteins in such an enriched separated product are then enzymatically digested (e.g. with trypsin) into their constituent peptides. Such peptides are further separated, e.g. by high-performance liquid chromatography (HPLC), followed by their molecular mass determination and perhaps sequencing with ESI tandem MS. Adequate MS (or MS^n) data mining in available databases allows final matching to their parent protein(s). In other words, we may say that protein identification is today almost straightforward using modern MS-based instrumentation.

The quantitative aspect and its problems

Of course, that statement above is too optimistic because it holds true only if the sensitivity of the MS method is high enough to detect those peptides. Otherwise we should draw the (false) conclusion that any undetected sought protein was absent in the sample. Moreover, as pointed out above, no PCR-like technology to amplify protein concentration levels is known. Thus, increasingly sophisticated preconcentration techniques should be called for to clean the sample and preconcentrate analytes to the concentration levels necessary for MALDI-MS and/or ESI-MS final measurements. What is more, concentration levels for determinations should be much higher than detection limits (the detection limit is $3\sigma_B$; the quantification limit is defined as $10\sigma_B$) [5]. In brief, the accurate determination of proteins in real-life biological samples is a great challenge today. Box 1 collects some of the most relevant problems to be faced in tackling such a challenging analytical goal.

To make matters worse using MS, the MS signal coming from MALDI or from ESI ion sources is very convenient for protein identification but rather problematic for their quantification. In fact, it is well known that the intensity of such MS signals is strongly affected by the sample matrix and by the species (i.e. the peptide) considered. Thus, no linear dependence between protein/peptide concentration levels and MS signals is observed.

Summarising, the challenge of protein determinations by common MS techniques is such that only 3 years ago well-known experts wrote: “The determination of a reliable and accurate amount of a protein among other proteins is close to impossible” [6, 7].

In spite of that situation, quantitative data of protein expression are being demanded more and more today [1]: for instance, any “modelling” effort in cellular or systems biology will require quantitative protein data and, in the same vein, the vast majority of changes resulting from an investigated perturbation of a cell (or biological system) will also require some type of quantification of the protein levels in comparative experiments.

So far, quantitative protein data are obtained in two forms: as the “absolute” amount of the protein in the sample or, much more frequently, as the “relative” change in the amount of protein observed between two biologically different states (e.g. altered and control cells). Of course, absolute quantification is always preferred in analytical chemistry. At the end of the day, if relative ratio information is desired for two compared biological states (e.g. in “differential” proteomics experiments), that calcu-

Box 1 Challenges to be tackled in accurate protein determinations

1. The system to be analysed may be very small (e.g. a single cell, with 0.5-pL volume and approximately 50-pg total protein content)
2. The possible protein expression dynamic range is very large (e.g. from 1 to 10^6 copies per cell) with proteins to be determined “buried” in a complex matrix
3. The number of possible different proteins is huge in comparison with the number of genes, especially considering the different post-translational modifications and/or associations with other biomolecules [105]
4. Proteins show very large variations in their physicochemical properties
5. There are numerous and most varied protein post-translational modifications changing in space and time the actual chemical nature of a given protein

lation is straightforward when absolute amounts of the desired proteins are determined and accurately known.

As a matter of fact, those summed-up limitations have prevented proteomics from being a truly quantitative science, but they have prompted many creative developments pursuing "relative" quantifications of proteins [8]. To get around the peptide and matrix signal intensity dependence in typical MS experiments "extracted ion current" methods have been proposed: the areas under the chromatographic peak obtained for the same peptide in the two different biological states under study are compared. The assumption is that the extracted ion current measured for exactly the same peptide and the same laboratory experimental conditions is nearly related to the amount of that peptide. So MS intensities of the same peptide observed in the two separate runs (states) are compared in order to determine the "relative" amount of peptide, providing sort of rough and ready quantitative information [9].

So far, relative quantification of desired proteins among different samples can be carried out using both typical 2D-GE-based and gel-free approaches [8]. These latter techniques, particularly those based on "stable-isotope labelling" of peptides are probably the most promising and useful approaches. The required labelling may be achieved by a chemical reagent, as in the case of the known isotope-coded affinity tags (ICAT) method, where the reagents consist basically of a thiol-specific protein reactive group, a linker (containing either a heavy or a light isotope) and biotin (for affinity purifications and preconcentrations). Initially the mass difference between the two reagents (tags) was generated with deuterium [10], but the same concept has been reported in several enhanced applications. An alternative approach of great potential is the use of stable isotope labelling by amino acids in cell cultures (SILAC), where cells are cultured in a medium containing a "heavy-isotope-marked" essential amino acid [11].

However, it is important to stress here that all such methods and strategies were originally introduced for "soft-type" ion sources where the energy of the source is actually controlled to produce the desired type of ions (molecular ions as MALDI and ESI usually provide).

The main intended contribution of this review is to focus attention on the use of a "high-energy" ion source instead. When an atomic ion source, such as an inductively coupled plasma (ICP), is employed, the plasma processes produce elemental ions produces elemental ions (mostly singly charged) from the biomolecules analysed. The use of such an energetic ion source allows the robust and efficient production of mainly the atomic ions coming from the elements present in the biomolecule. Box 2 summarises the most salient advantages of using ICP-MS for proteomics.

Absolute quantitative proteomics

Presently available stable isotope ratio based methods using molecular MS do not fulfil the increasing need for reliable methods of "absolute" quantification of proteins and, most importantly, for reliable discrimination between close expression levels of proteins. ICP-MS, being recognised as the detection method of choice for elemental determinations at trace and ultratrace levels, offers analytical signals directly proportional to the mass of the selected element (of a given biocompound) present in the plasma source. A remarkable advantage over soft-ionisation meth-

1. Specificity to the heteroatom (metals, semimetals or nonmetals)
2. Compound-independent detection sensitivity
3. High elemental sensitivity
4. Sample preparation and purity requirements comparatively low (robustness)
5. Direct isotopic information (heteroatoms with multiple isotopes)
   - Isotope dilution analysis
   - Metabolism and nutrition studies
6. Versatility for coupling with high-performance liquid chromatography, capillary electrophoresis, gel electrophoresis and gas chromatography

Box 2 Specific advantages of inductively coupled plasma mass-spectrometric detection in proteomics

ods is that the signal sensitivity is practically species- or compound-independent (Box 2). Consequently, elemental ion sources such as an ICP may open the door for real absolute quantification of protein traces in complex samples.

Present knowledge, which comes from fast progress on elemental speciation in biomolecules [12, 13], is paving the way for such challenging task. The so-called hybrid techniques, incorporating a powerful separation technique (e.g. chromatography) coupled to an elemental detection system (e.g. ICP-MS), have become today the tools of the trade for trace element speciation analysis [14]. On the other hand, many proteins and enzymes contain metals easily followed by ICP-MS [13], which determine their eventual biological activity. Thus, through such studies trace element speciation via elemental detection entered proteomics.

These days, this idea of element-driven research can be generalised to the term "heteroatom-tagged" biological research (considering the general concept of heteroatoms in organic compounds as elements other than C and H). The exceptional capabilities of ICP-MS to follow most heteroatoms (metals, semimetals and some important nonmetals such as halogens, sulphur or phosphorus) in complex biomolecules reliably has rendered such complicated structures and mixtures (e.g. of proteins) a more tractable problem. This is essentially the concept of "heteroatom-tagged proteomics" [13, 15].

Analytical strategies using natural elemental tags for quantitative proteomics

The possibility of using element-selective mass-spectrometric detectors such as ICP-MS systems, allowing the robust and specific monitoring of proteins containing one or more heteroatoms, has been mainly used via detection of metals and metalloids. However, some nonmetals such as phosphorous or sulphur are very interesting elemental tags, naturally occurring in proteins, that are today being intensively studied. Therefore, all types of heteroatoms investigated so far via ICP-MS will be reviewed in the following sections that are focused on *naturally occurring elemental tags* used for quantitative purposes or for metabolism studies.

However, we must keep in mind that the quantitative character of ICP-MS implies the loss of any structural information. The complementary use of molecular MS (MALDI and ESI sources) to elucidate the amino acid sequence of the peptide/protein or the knowledge beforehand of its identity is therefore mandatory to translate the determined amount of the heteroatom into the amount of protein.

General remarks

In general, most relevant proteins in cells and tissues are buried in complex matrices that make rather difficult their selective quantification. The complexity of such biological media demands the use of high-resolution separation methods (chromatographic or electrophoretic) before approaching the quantification step. Once the species have been separated and owing to the extraordinary characteristics of ICP-MS (Box 2), the quantitative determinations of heteroatoms present in such biomolecules can be done almost independently of the organic moiety they are buried in. Thus, quantitative evaluation of heteroelements present in individual fractions containing large biomolecules, such as proteins, can be performed by the *peak area normalisation method* using either inorganic standards or known species (different from the one sought) which contain the element(s) of interest [16, 17]. Such strategies have been used for quantitative purposes when the adequate standard was not available or when the elemental tag present in the biomolecule of interest is monoisotopic (such as As or P) and isotope dilution strategies cannot be applied. The most relevant applications of the use of these approaches will be illustrated in the following section. Additionally, if the heteroatom-containing proteins are well known and the standards are available, it is possible to obtain conventional calibration curves with matrix matched standard and/or standard addition quantification strategies.

On the other hand, ICP-MS provides not only element-specific but also isotope-specific information and these excellent capabilities have been exploited for quantification purposes. Isotope dilution analysis (IDA) has been successfully applied to the quantitative determination of known heteroatom-containing proteins with the single condition that the monitored element has at least two isotopes free of interferences to be measured by ICP-MS. Such strategies have permitted the improvement of the precision and accuracy of the quantitative data when analysis is performed in two different modes, speciated and nonspeciated isotope dilution. Both strategies will be described in detail in the following sections as well.

Direct calibration methods

As stated in the "Introduction", ionisation processes in molecular MS strongly depend on several factors, such as the physicochemical properties of the biomolecule and the presence of other components entering the ionisation source at the same time as the analyte. Therefore, standards for each biomolecule are strictly required in order to obtain accurate absolute quantification results. This requirement has traditionally resulted in the fact that molecular MS has been almost exclusively applied for relative quantifications as indicated in the "Introduction". Obviously, to have standards for the millions of different biomolecules (mostly proteins) contained in a particular biological system is virtually impossible. Additionally, most frequently the identity of the different biomolecules present in the sample

is not even known. In order to get around such limitations, many creative alternatives have recently been developed, most of them making use of the molecule-independent signal provided by elemental MS (ICP-MS).

Semiquantitative approaches

Many proteins and enzymes contain one or more functional centres able to coordinate (semi)metal ion(s). Additionally, metal ions may be bound to other sites when they are involved in protein allosteric regulations. Understanding the function of such metal sites and their important biological implications is nowadays a challenging biological goal [19].

As ICP-MS is a specific and sensitive detection system for (semi)metals and it is easily coupled with separation techniques, it becomes an extremely useful tool to screen for metal distribution in real samples [13, 20]. As a matter of fact, owing to its multielement detection capabilities, it allows pinpointing heteroelement-containing proteins from the rest of the proteins present in both a HPLC (or capillary electrophoresis, CE) eluent and a gel spot. Since these (semi) metal–protein interactions may be weak under the standard conditions usually employed during sample preparation and separation (chromatography, electrophoresis), extreme care must be taken to maintain the native conformation of the protein. In this sense, the preferred chromatographic mechanism is size-exclusion chromatography (SEC) because its mobile phases are easily compatible with the use of physiological ionic strength and pH [13, 21].

Different approaches have been employed when deeper insight into element quantitative distribution was sought. Quintana et al. [22] collected the SEC fractions containing Mn species of a defined molecular size and split them into two aliquots. One was used for total Mn determination by the standard additions method, whilst a second separation mechanism (capillary zone electrophoresis) was applied to the other one. In this way, the authors found that Mn present in porcine liver extracts was predominantly bound to enzymes such as arginase, isocitric dehydrogenase, galactosyltransferase, prolidase, pyruvate carboxylase and oxalate oxidase. Another possibility to obtain semiquantitative information from the SEC chromatograms consists of direct comparison of peak areas with the usual sensitivity observed for inorganic aqueous standards of the elements injected postcolumn. For instance, Wang et al. [23] found Mn at 3 μg kg^{-1} in the corresponding 13 kDa chromatographic peak, and Co at about 1 μg kg^{-1} at 155 kDa, in bovine liver extracts.

Of course, such rough quantitative data must be considered very carefully. First, the chromatographic purity of the fractions/peaks is doubtful and more than one metal-containing protein could easily be coeluted [20]. Second, the protein and the metal can be coeluted in SEC, but this is not a formal proof of their binding. Third, as it has already been stated [24], the stationary and mobile phases used could compete and displace the weakly bound metal from its natural sites in some proteins, leading to false metal distribution patterns. For example, it has been observed that Mn coordination to several enzymes was preserved, whereas the Mn–transferrin complex was degraded during SEC separations [22]. Unless element column recovery is complete, or at least determined, no reliable quantitative estimations can be obtained.

Quantitative information provided by ICP-MS has also been used to study the patterns of binding of metals to transferrin in human serum samples. Isolation of transferrin from the other major proteins as well as separation of four transferrin forms, namely apotransferrin, dimetallic transferrin and two monometallic transferrin forms, was achieved with an anion-exchange column. Nagaoka and Maitani [25] used peak areas/heights to follow the Al incorporation in transferrin in the presence of the iron naturally occurring in serum. Moreover, the ratio between ICP-MS and UV peaks provided a rough estimation of the metal–protein stoichiometry for each peak. Again, any quantitative conclusion is unrealistic without checking for element column recovery.

Sulphur as an internal standard

Once the amino acid sequence of a protein is known, and thus the number of sulphur-containing residues, the sulphur concentration obtained by ICP-MS can be easily translated into protein molar concentration. This approach has been extensively used to study phosphorylated proteins separated using HPLC and CE coupled with ICP-MS (Table 1). Differential influence of organic modifiers in S and P sensitivity along reversed-phase gradients has to be compensated. To do so, the two mobile phases A and B were spiked with the same amount of phosphorous- and sulphur-containing compounds and a standard capillary liquid chromatography (μLC) ICP-MS gradient was run while the $^{31}P^{+}$ and $^{32}S^{+}$ signals were continuously monitored [26]. Such data can be employed to compute the sensitivity function $^{31}P^{+}/^{32}S^{+}$ along the μLC-ICP-MS gradient, which can be subsequently used to calculate the correct P/S ratios in the sample. This corrected molar P/S ratio obtained can be easily converted into the degree of phosphorylation if the amino acid sequence is obtained by parallel μLC-ESI-MS^{n} analysis or it is known beforehand. The concept was first demonstrated with standard phosphoproteins such as α-casein and β-casein and synthetic phosphopeptides derived from protein kinase A catalytic subunit [26]. A detection limit of approximately 100 fmol, of total P injected, was achieved. Since this approach requires the presence of a sulphur-containing amino acid in the species analysed, its application

Table 1 Element to sulphur ratio determinations for quantitative heteroatom–protein stoichiometry

Isotope ratio	Technique	Application	Reference
$^{31}P^+/^{32}S^+$	μLC-ICP-SFMS	Phosphorylation degree in caseins and synthetic phosphopeptides	[26]
$^{31}P^+/^{32}S^+$	μLC-ICP-SFMS 1-D GE LA-ICP-SFMS	Standard phosphoproteins, cytoplasmatic proteome of bacterial and eukaryotic cells	[29]
$^{31}P^+/^{34}S^+$	μLC-ICP-SFMS	Phosphoproteome of different plant samples	[28]
$^{31}P^+/^{32}S^+$	GE-ICP-SFMS	Phosphorylation degree in caseins	[30]
$^{54}Fe^+/^{32}S^{16}O^+$ $^{56}Fe^+/^{32}S^{16}O^+$ $^{55}Mn^+/^{32}S^{16}O^+$ $^{56}Fe^+/^{32}S^+$ $^{55}Mn^+/^{32}S^+$	SEC-ICP-DRCMS SEC-ICP-SFMS	Biotechnologically produced and standard metalloproteins	[32]
$^{63}Cu^+/^{64}Zn^+/^{32}S^{16}O^+$	FI-ICP-CCMS	Superoxide dismutase	[33]
$^{64}Zn^+/^{32}S^+$	CE-ICP-SFMS	Zinc β-lactamase	[34]
$^{56}Fe^+/^{32}S^+$	GE-ICP-SFMS	Iron-containing standard proteins	[31]

μLC capillary liquid chromatography, *ICP* inductively coupled plasma, *SFMS* sector field mass spectrometry, *1-D GE* one-dimensional gel electrophoresis, *LA* laser ablation, *GE* gel electrophoresis, *SEC* size-exclusion chromatography, *DRCMS* dynamic reaction cell mass spectrometry, *FI* flow injection, *CCMS* collision cell mass spectrometry

is mainly restricted to intact proteins because the combined probability to find a sulphur-containing residue (cysteine or methionine) in a given tryptic phosphopeptide is rather low. Histidine phosphorylation in prokaryotes was also studied in a recombinant in vitro system derived from the bacterial chemotaxis protein CheA-H [27]. Recently, Krüger et al. [28] applied this approach to compare the average protein phosphorylation level in entire protein extracts from different plant organisms and different stages of plant development.

In-gel digestion and subsequent μLC-ICP-MS and protein blotting followed by laser ablation (LA) ICP-MS in the context of gel spot analysis were recently evaluated [29]. Quantitative results based on the P/S ratio obtained by both strategies were consistent, although the μLC-ICP-MS approach provides better sensitivity. However, it is clear that the lesser number of sample preparation steps involved during direct analysis of the intact phosphoproteins by LA-ICP-MS could reduce drastically the risk of error. In this case, the sensitivity factor of the $^{31}P^+/^{34}S^+$ ratio obtained using LA-ICP-MS was calculated by incubation of the membrane with known amounts of P- and S-containing amino acids. A rather constant value of 0.6 was found over the whole blot. All experimental data were subsequently corrected by this factor. The degree of phosphorylation observed in cytoplasmatic proteins was significantly higher in eukaryotic cells (approximately 0.8 mol P/mol protein) than in bacterial cells (approximately 0.01 mol P/mol protein) [29]. A recently developed online coupling of GE and ICP–sector field MS (SFMS) was furthermore used for the determination of the degree of phosphorylation in caseins following the concept of P/S [30] and for Fe/S ratio measurements in iron-containing proteins [31].

In addition, stoichiometry of biotechnologically produced metalloproteins is traditionally assessed by independent determination of the total metal in solution and the protein content by photometry. Obviously, the accuracy of this approach is critically compromised by metal contamination potentially produced during the protein isolation procedure. Lately, measurement of metal to sulphur ratios in SEC-ICP-MS has been also proposed as a promising tool to characterise already isolated metalloproteins [32]. Chromatography is still strictly required to separate the inorganic metal present as an impurity from the protein-bound metal. Relative sulphur/metal sensitivity factors could be determined by external flow injection (or SEC) ICP-MS calibration using inorganic standard solutions. The approach was applied to determine the Mn/S ratio in commercially available manganese superoxidase dismutase and arginase. A similar strategy was used for superoxide dismutase [33]. Here the purified protein was analysed by flow-injection ICP-MS and Cu/Zn/S ratios were determined. A high-resolution CE separation technique has been coupled with ICP-SFMS for the determination Zn/S ratios in zinc β-lactamase [34].

Iodine-containing proteins

Thyroglobulin is an iodoprotein which contains different iodo amino acid residues such as monoiodotyrosine and diiodotyrosine and the thyroid hormones triiodothyronine

(T3) and thyroxine (T4) [21]. The pioneer work of Takatera and Watanabe [35] used reversed-phase HPLC-ICP-MS to separate and quantify these iodine-containing species in a proteolytic digest of bovine thyroglobulin. Absolute detection limits ranged from 35 to 130 pg of iodine. CE-ICP-MS has been also tested for determination of iodine-containing molecules in human serum from healthy and thyroid-operated-on persons [36]. More recently, iodine, monoiodotyrosine and diiodotyrosine were determined by anion-exchange chromatography ICP-MS in an enzymatic digest of commercially available seaweed samples [37]. Wind et al. [38] used a modified direct-injection high-efficiency nebuliser in conjunction with capillary HPLC and an ICP-SFMS detection system. The lower detection limits obtained (high femtogram level) allowed quantification of an impurity trace of T3 (less than 0.25% of total I) present in a T4 standard.

Quantitative approaches

DNA adducts were the first small biomolecules quantified using the heteroatom signal ($^{31}P^{+}$) provided by liquid chromatography (LC) ICP-MS [39]. The injection of an internal standard containing P (phosphoric acid) at the end of each LC run allowed direct comparison of its $^{31}P^{+}$ peak area with those obtained for the DNA adducts. Two years later, the potential of ICP-MS as a generic detection system for LC in the quantification of unknown heteroatom-containing compounds occurring in pharmaceutical samples was also assessed [40]. Two equimolar mixtures of phospholipids and phosphopeptides, respectively, were analysed by LC with ICP-MS, UV and ESI-MS detection. ICP-MS response factors (ratio between peak areas) obtained for each species varied significantly, 1.0±0.2, likely owing to sensitivity changes along the gradient. One year later, Svantesson et al. [17] reported the first detailed study of plasma spectrometric (ICP atomic emission spectrometry and ICP-MS) responses of elements present in large biomolecules. The influence of the organic moiety to which the element was bound on the ICP signal was investigated in detail. Bovine serum albumin (S detection) and cyanocobalamin (Co detection) were assayed as test molecules. The effect of the organic moiety was almost negligible, only when the concentration was low enough, and therefore they suggested the use of inorganic elemental standards for biomolecule quantification (accuracy around 10%). Unfortunately, LC-ICP-MS led to less accurate results (below 15%) than the corresponding analysis by flow-injection ICP-MS.

The first successful proof of accurate and precise quantification of complex tryptic phosphopeptide mixtures using elemental phosphorus standards and ICP-MS was reported recently [41]. Reversed-phase separations were required to resolve the complex mixtures, which involved gradients of organic modifiers (mostly acetonitrile or methanol) affecting plasma stability. Unfortunately, ICP-MS sensitivity for every element, especially those exhibiting high ionisation potentials like P, changes dramatically as the organic content of the mobile phases is modified along the gradient [42]. This fact rules out the use of absolute species-independent calibrations. In order to compensate such P response variations, an acetonitrile make-up solution was used [41]. This concept had been already proved for μLC-ICP-MS [43] and nanoLC-ICP-MS [44] gradients applied to analysis of Co- and Se-containing biomolecules, respectively. In such conditions, the $^{31}P^{+}$ signal obtained was just directly proportional to the mass of P present in the compound (species) and was completely independent of its chemical structure. Thus, the simple addition of a commercially available P standard [bis(4-nitrophenyl)phosphate] to the sample allowed a computed mass response factor (picomoles of P per area unit of the chromatographic peak) to be applied in the quantification of every individual phosphopeptide separated simultaneously along the gradient (Fig. 1). The approach was first demonstrated in a phosphopeptide standards mixture with excellent results (accuracy below 4%) and was then applied to casein and β-casein tryptic digests. The combination of a total consumption nebuliser operated at low-flow levels and helium as the collision gas resulted in a decrease in $^{31}P^{+}$ background levels, leading to detection limits in the femtomolar range, similar to those reported by Pröfrock et al. [45]. Of course, it can be envisaged that these highly accurate and precise ICP-MS determinations hold great analytical potential as they will allow very small changes in protein phosphorylation levels to be followed (e.g. as observed during many signalling kinetic studies) [18].

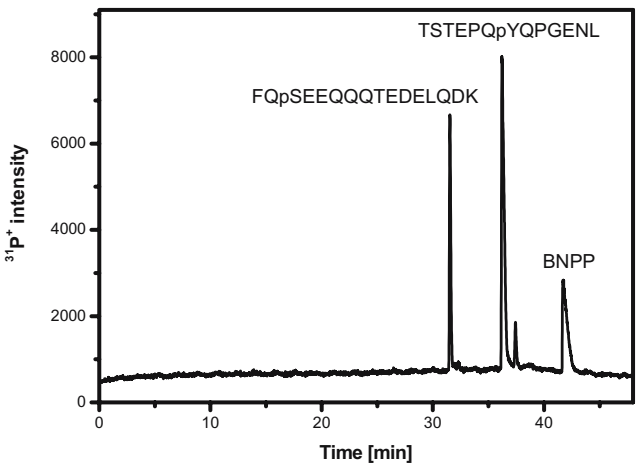

Fig. 1 Species-independent calibration for absolute protein quantification. Capillary high-performance liquid chromatography inductively coupled plasma (ICP) mass spectrometry (MS) chromatogram of two phosphopeptides and bis(4-nitrophenyl)phosphate (*BNPP*) added as an internal standard for species-independent calibration

The species-independent calibration concept has also been applied to protein gel spot quantifications. Since the pioneer work of Neilsen et al. [46] on identification of Co-binding serum proteins, different quantitative approaches have been developed to determine heteroatoms in GE-separated protein spots by LA-ICP-MS. Becker et al. [47] carried out quantitative determination of phosphorus in proteins extracted from yeast mitochondria by external calibration using one-dimensional gels containing different amounts of loaded ovalbumin (from 0.1 to 500 ng of protein). Sulphur was used as the internal standard to determine elemental (P/S) ratios. The procedure was further applied to in-gel screening of P and transition metals. Another possibility assayed to carry out the quantitative determination of P in protein spots was to prepare matrix-matched laboratory standards. Application of the approach to human brain proteins from patients with Alzheimer's disease led to quantification of P in 31 protein spots out of the 176 gel spots obtained after staining [48].

Elliot et al. [49] electroeluted phosphoproteins first separated by GE. The resultant whole gel elution fractions were collected and directly analysed by flow-injection ICP-MS via the $^{47}(PO)^{+}$ ion created in the reaction cell operated with O_2. Again, inorganic P contamination limited the approach. Lately, Feldmann et al. [50] investigated three different LA chamber geometries for quantitative imaging of heteroatoms (intensity distribution of an element in two dimensions by repetitive ablation line by line) using LA-ICP-SFMS. The $^{31}P^{+}$ calibration graphs obtained for β-casein and pepsin showed the same sensitivity, demonstrating the response of ICP-MS was independent of protein structure. Limits of detection of about 3 pmol for β-casein and of 5 pmol for pepsin were obtained.

Of course, there are some important weaknesses in a quantitative GE-LA-ICP-MS strategy:

1. A key requirement is to maintain the integrity of the element–protein binding during the isoelectric focusing. This is easily achieved in the case of S (Se) and P, but it is not so in metal–protein complexes. They should be studied under native rather than denaturing conditions in the second GE dimension [51–53]. In that case, the resolution is highly compromised.
2. Reagents and buffers typically used may be highly contaminated with trace elements, leading to high elemental backgrounds [54]. This contamination can be reduced by adding a washing step with $Ga(NO_3)_3$ or blotting the gel spots onto a membrane [55].
3. The quantitative character of the LA process is still not fully demonstrated [56].
4. Last but not least, the attainable reproducibility of the 2D-GE separation compromises its use for quantitative purposes.

Isotope dilution analysis methods

The term "isotope dilution analysis" (IDA) in the literature of quantitative proteomics reveals two different interpretations depending on the research area. A recent review describes how conventional IDA methods (widely used for determinations of drugs and small metabolites) have been extended for quantifying specific proteins in complex mixtures [57]. In this case, proteins are subjected to protease action and specific resultant peptides are then quantified by resorting to synthetic stable "isotope-labelled (using ^{2}H, ^{13}C, ^{15}N or ^{18}O) standard peptides". Such labelled peptides are chemically identical to their native counterparts formed by proteolysis, but are easily distinguishable by MS via a certain mass shift. Endogenous protein concentrations are determined by comparing ESI-MS^n peak areas of the peptides with those of the isotopically labelled peptides used as internal standards. This method is traditionally called IDA in analytical proteomics, even if it is more of a quantitative analysis "internal standard approach", from a pure analytical point of view [58].

With an ICP-MS system as an ion source, however, the concept of IDA for protein quantification is somewhat different. After combination of the naturally occurring and the isotopically labelled elements, the new elemental isotope ratios resulting from the atomisation in the plasma can be measured. The incorporation of such ratios in the well-known isotope dilution equation directly provides the absolute quantity of the element in the sample [59]. The postcolumn IDA methods developed first by Rottmann et al. [60, 61] for speciation have been adapted nowadays to meet the current needs in the analysis of large biomolecules (e.g. proteins containing metals and/or semimetals).

This is currently the most commonly applied mode of IDA, also called *nonspeciated IDA* or *species-unspecific IDA*, and it is conventionally used after separation of the proteins by chromatography or electrophoresis. Table 2 summarises the different methods for species-unspecific IDA applied to protein quantifications. In brief, a solution containing the element to be analysed (heteroatom) with altered isotopic abundances (spike) is continuously mixed with the eluent from the chromatographic column and both flows are merged and introduced into the ICP-MS instrument. Then, the selected isotope ratio can be measured by ICP-MS along the chromatogram. Finally, by applying the basic equation of IDA at each point, one can construct the so-called mass-flow chromatogram [59]. The absolute amount of the sought heteroatom in each chromatographic peak can be directly obtained by integration. Postcolumn IDA is well known as a precise and accurate quantification method, but it does not compensate for any losses during chromatographic separation (the column recovery has to be considered).

Table 2 The applications of species-specific and species-unspecific isotope dilution analysis (*IDA*) for quantification of heteroatom-tagged proteins

Sample	Elements	Method	Proteins	Reference
Species-unspecific IDA via ICP-MS detection				
Human brain cytosols	S, Cu, Zn, Cd	CE, HPLC	MTs (MT-3)	[68]
Commercial rabbit liver	S, Cu, Zn, Cd	CE	MTs	[69]
Commercial rabbit liver	Cd	RP-HPLC	MTs	[67]
Carp and eel tissues	Cu, Zn, Cd	SEC	MTs	[65]
Eel liver cytosols	Cu, Zn, Cd	AE-HPLC	MTs	[64]
Human serum	Fe, Cu, Zn	AE-HPLC	Tf, HSA, CP	[71]
Human serum	Fe	AE-HPLC	Tf sialoforms	[76]
Human serum	Se	Affinity LC	GPx, Sel P, HSA	[72]
Standard, yeast	S	nanoLC	HSA, SIP-18	[78]
Species-specific IDA via ICP-MS detection				
Human serum	^{57}Fe	AE-HPLC	Tf sialoforms	[76]
Selenised yeast	^{77}Se	μLC	12 kDa (HSP)	[77]

CE capillary electrophoresis, *HPLC* high-performance liquid chromatography, *RP* reversed phase, *AE* anion exchange, *LC* liquid chromatography, *MT* metallothionein, *Tf* transferrin, *HSA* human serum albumin, *CP* ceruloplasmin, *GPx* glutathione peroxidase, *Sel P* selenoprotein P, *SIP-18* salt induced protein 18, *HSP* heat shock protein

An increasing number of publications can be found in the literature regarding the use of postcolumn IDA with HPLC-ICP-MS, most of them related to the analysis of small Se-containing molecules of nutritional relevance for which no standards are available [44, 62, 63]. However, in the case of heteroatom-containing proteins, the existing literature is rather limited and most examples focus on the quantification of metals associated to a given type of protein (i.e. metallothioneins, MTs). In this regard, several examples can be extracted from the existing papers on the use of species-unspecific spiking for the determination of the metal content (namely Cd, Cu and Zn) in MTs coming from eel [64], carp [65] or rat liver [66, 67] (as an illustrative example see Fig. 2a).

Although most of these publications report the metal content of the different metalloproteins, the final aim is the quantification of the entire protein content in order to study protein concentration changes, e.g. investigating the level of MTs induction due to, for instance, the presence of cadmium. For this purpose, it is necessary to know the identity and stoichiometry of the metal–protein complexes formed, a task usually accomplished by molecular MS techniques (e.g. ESI-MS). The work of Prange et al. [68–70] has also demonstrated the utility of measuring metal to sulphur ratios obtained quantitatively through postcolumn IDA by CE-ICP-MS for determination of metal to protein ratios.

Beside this, some work has been done in human serum in order to obtain quantitative information of Fe, Cu and Zn associated to high molecular weight proteins by anion-exchange ICP-MS and postcolumn addition of the corresponding enriched isotopes (^{57}Fe, ^{65}Cu and ^{66}Zn) [71]. Additionally, by means of different affinity columns and ICP-MS detection, the determination of the main Se-containing proteins in human serum (selenoprotein P, albumin and glutathione peroxidase) has been accomplished [72].

The other strategy to conduct IDA for trace element speciation is the use of the *species-specific IDA* mode, whereby the sample is spiked with the same chemical species but containing an enriched isotope of the element to be analysed. Once the isotope equilibration is reached, the sample is separated by means of HPLC or CE and the isotope ratios are measured by ICP-MS. This mode is superior to the use of species-unspecific IDA, since any chemical or physical losses during the analytical procedure can be corrected in the final measurement. It is noteworthy that the species-specific IDA mode can be classified as an intermediate between protein tagging and labelling, since an occurring heteroelement is monitored in a specific molecule (tag) and the same protein is "artificially" loaded using isotopically enriched heteroatoms (label).

It is a requirement, in this case, that such interactions between the heteroelements and the organic moieties show enough thermodynamic and kinetic stability. Otherwise, some isotopic exchange can occur, which affects the final quantitative results.

Several methods have been reported for protein labelling by means of isotopically enriched amino acids (^{18}O, ^{15}N, etc.) [73]. Recently, the use of isotopically labelled metalloproteins, in which the isotopic composition of the metal is altered, has been proposed by several authors [74, 75]. The final aim is using these metalloproteins to conduct

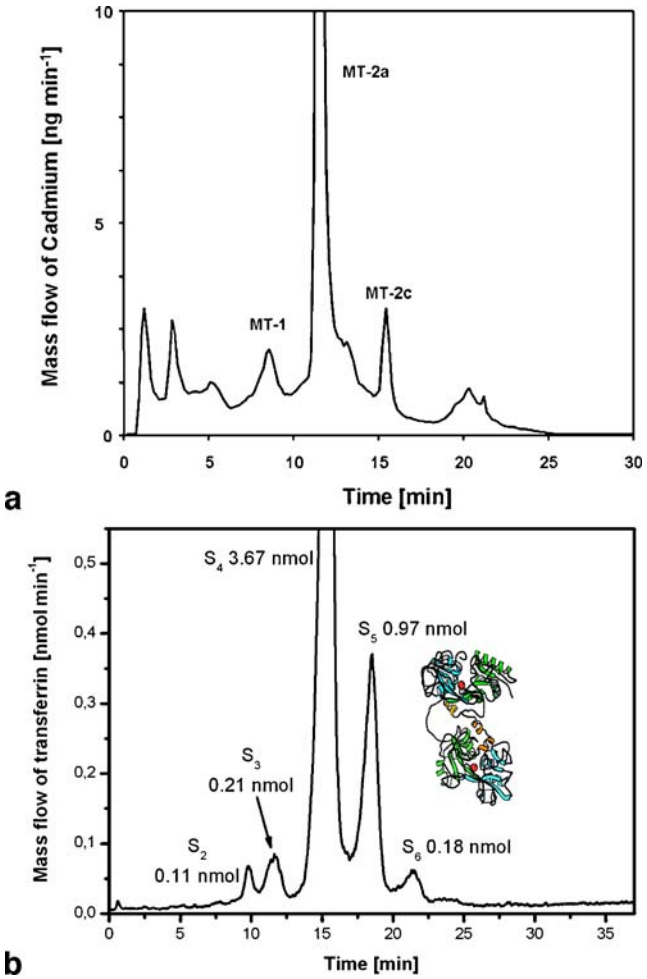

Fig. 2 From metal mass flow to protein mass flow: Postcolumn isotope dilution analysis as a tool in absolute protein quantification. **a** Mass-flow chromatogram of Cd in eel liver metallothioneins (*MT*) [79]. **b** Mass-flow chromatogram of transferrin in human serum. S_1, S_2, ..., S_6 indicate the different transferrin isoforms

quantification based on species-specific spiking to attain a high degree of precision and accuracy. Proteins that are isotopically labelled have the same chemical properties as the native proteins. Thus, the main limiting factor for performing species-specific spiking for metalloprotein quantification is the synthesis of the isotopically labelled species. In this regard, different strategies have been conducted, such as the use of a bacterial medium (*Acidothiobacillus ferrooxidans)* able to synthesise the Cu-containing protein rusticyanin [74] in the presence of enriched ^{65}Cu or the overexpression of recombinant Cu-containing plastocyanin in *Escherichia coli* [75]. However, most of these publications are restricted to the synthesis and characterisation of the isotopically labelled protein standards, while their application and validation are still pending for real quantitative analysis.

In this regard, a recent publication [76] has compared the two isotope dilution methods (species-specific and species-unspecific) for performing quantitative analysis of Fe-containing transferrin isoforms in human serum samples and furthermore validate the proposed method with a certified reference material. In this latter case, the protein is chemically saturated by incubating it with a solution of ^{57}Fe and the resulting product is quantitatively analysed by reversed isotope dilution analysis. The saturation of transferrin with Fe permits the conversion of the measured Fe concentration into protein concentration (2 mol Fe is equivalent to 1 mol transferrin), and so further structural characterisation of the metalloprotein by ESI-MS is not required (as an illustrative example see Fig. 2b).

Thus, by generating isotopically labelled proteins the traceability of the whole analytical protocol that is necessary to perform protein quantification (often time-consuming and prone to losses of analyte) is noticeably enhanced. Additionally, it provides a means to evaluate the analytical conditions under which the metal forms a stable complex with the organic moiety of the protein.

A different strategy to conduct quantitative analysis by isotope dilution was first presented by Polatajko et al. [77] using the species-specific spiking mode. In this case, a synthesised ^{77}Se-labelled selenopeptide (previously identified to be produced by the heat shock protein after tryptic digestion) is used for the quantification of the previously mentioned protein in selenised yeast by capillary-HPLC-ICP-MS. In this case, it is important to ensure that the enzymatic cleavage proceeds in the correct way and that miscleavages are avoided. Following a similar trend of protein quantification via accurate determination of peptide/s, the recent publication of precolumn isotope dilution strategies with nanoHPLC-ICP-MS analysis for the accurate absolute quantification of sulphur-containing peptides is noteworthy. In this latter work, a ^{34}S-labelled, species-unspecific sulphur spike (as sulphate) is added directly to the chromatographic eluents. Thus, a generic sulphur standard permanently present during analysis is used for peptide quantification and can be considered as a novel and interesting approach for protein quantification [78].

Stable isotopes for quantitative metabolism studies

ICP-MS has great potential in metabolism studies. In addition to previously mentioned advantages (Box 2), ICP-MS can be considered a powerful detection system for stable isotopes used as tracers of metabolic pathways. Those great advantages of ICP-MS open the way to investigate different aspects of metabolism of important biological compounds (e.g. heteroatom-tagged proteins), including absorption, availability, excretion and kinetics. So far, however, only a few quantitative applications can be found in the literature. In fact, most studies reported have focused on the distributions of the heteroelements among

proteins or metabolites, aiming just at "elemental information" without a projection to obtain protein quantitative data.

However, the extraordinary features of ICP-MS-based strategies have not been fully exploited yet in metabolism studies. Thus, a great future for such strategies can be envisaged in this research field.

The literature search reveals that, from a methodological point of view, the most common approach in metabolism studies via ICP-MS is feeding the living organisms with (or injecting) a highly isotopically enriched element (tracer). Subsequently, the element turnover in organs is evaluated by studying the changes of isotope ratios in the main proteins, where these tracers are incorporated. The main advantage of this strategy is that it allows preservation of the integrity and the activity of the biological species.

Some representative examples of what has been done so far deal with the de novo incorporation of cadmium into fish liver and kidney MTs, where ^{111}Cd was used as a tracer. The exposure of European eel (*Anguilla anguilla*) as a model organism to ^{111}Cd gave rise to the in vivo dilution of the natural previously existing Cd associated to MTs fractions in the selected tissues. This approach permitted the quantitative discrimination of the Cd-MTs isoforms induced by de novo incorporation of Cd in liver and kidney [79].

Similarly, Suzuki et al. [80] have conducted some studies on Se incorporation metabolism of different selenium compounds (tagged with stable enriched isotopes) administrated orally, attempting the identification of the proteins, where the selenium is incorporated. The use of two tracers ^{76}Se (as ^{76}Se-methyl selenocysteine) and ^{77}Se (as ^{77}Se-methionine) allowed it to be demonstrated that those two selenium species were equally incorporated in selenoprotein P. In later work, the same authors reported an interesting approach where rats were depleted of natural selenium, by feeding enriched ^{82}Se (more than 90%). After such treatment the previously described approach of stable isotopes is simplified [81].

Also, in order to investigate trace mercury-containing proteins in maternal rats, a tracer (^{196}Hg and ^{198}Hg) method has been investigated using SEC-ICP-isotope-dilution MS. Reliable qualitative and quantitative information on mercury-containing proteins in the organisms was reported in this work [82].

A second strategy consists of the direct separation of the target species, e.g. peptides, and its subsequent labelling with a heteroatom (not present in the target species) to be used for quantitative purposes. Obviously, this approach is more laborious and could change the integrity of the species and perhaps its activity depending on the site of labelling [83]. The only example in the literature of the use of a "labelling strategy" (see "Analytical strategies using artificial elemental labels for quantitative proteomics") in previously separated fractions containing proteins (i.e. bradykinin) was conducted in human and rat plasma [84, 85]. In these metabolism studies, synthetic bromobradykinin was used and subsequently detected and quantified via ICP-MS.

In conclusion, while qualitative metabolism studies based on stable isotope as markers are well-established, quantitative studies of metabolism of heteroatom-tagged proteins are now facilitated, and so, increasing applications of such strategies can be warranted in the near future.

Analytical strategies using artificial elemental labels for quantitative proteomics

General remarks

Labelling procedures have become an important tool for strategies aiming at quantitative protein determinations. They have been introduced into proteomics with the intention to isolate the analyte of interest from the biological matrix or to improve the protein detection itself, e.g. by introduction of fluorescent labels. In combination with mass-spectrometric techniques, in particular ESI and MALDI, several approaches have been developed during the last few years [2, 86–89]. They generally follow a concept of labelling molecules, which contain stable isotopes like ^{2}H, ^{13}C, ^{15}N or ^{18}O. Here, the ICAT concept pioneered this exciting branch of developments in protein quantification [10] Besides ICAT, the most promising developments are iTRAQ™ (isobaric tags for relative and absolute quantification) [90] and SILAC [11].

Although the great potential of protein labelling has been generally recognised and accepted, elemental mass-spectrometric detection of these bioconjugates is still quite scarce [83]. As summarised in the previous sections, sulphur and phosphorus are the most ubiquitous elemental tags, and are useable in ICP-MS analysis. And as their ICP-MS detection is mainly hampered by low ionisation efficiencies and spectral interferences, the introduction of an elemental label less prone to detection problems might be favourable. However, the chemical modification of a protein is somehow accompanied by serious problems, which hinder seriously its application in terms of quantitative determinations:

1. It has to be guaranteed that the labelling process shows at least satisfactory reproducibility in a certain matrix. As this derivatisation requires more or less complicated chemical reactions, its kinetics and thermodynamics are strongly influenced by manifold factors, e.g. the sample matrix.
2. The labelling selectivity has a great impact as well. For instance, a protein can have several reactive groups,

which are in principle available. Here, it has to be ensured that the stoichiometry is still known. Furthermore, the reaction yield can be affected by the labelling reaction selectivity too.

3. Finally, the labelling can influence the protein's recovery from a column, and as a consequence, it directly influences the accuracy of the quantitative result.

Against this background, it is not surprising that the combination of protein labelling and elemental MS is still in its infancy.

Quantitative proteomics based on inorganic labelling

Two different strategies for protein labelling have been developed for its use in elemental mass-spectrometric detection: (1) direct protein labelling and (2) antibody labelling with subsequent detection of antigen (protein)–antibody interaction.

Only a few examples of direct protein labelling can be found in combination with elemental MS. Nevertheless, an observable trend follows the first application of so-called element-coded affinity tags [91, 92] or metal-coded affinity tag [93]. These techniques are based on the lanthanide-containing chelates, e.g. 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid, covalently attached to sulphhydryl groups [91, 93] or primary amines [92] of a protein. As the application of different lanthanides as elemental labels has been suggested, these techniques might have great potential in absolute and relative protein quantification with low detection limits.

The use of isotopic labels was firstly studied for the example of ^{151}Eu/^{153}Eu [94]. Here, the label was cyclic diethylenetriaminepentaacetate, which directly forms an amide bond with the N-terminal amino acid of a protein or a peptide. In this preliminary study, the authors demonstrated the principal applicability for relative quantification of peptides (bradykinin, substance P).

The second labelling strategy relies on the chemical modification of an antibody, which can be then used for the specific interaction with the antigen in an immunoassay. A few examples of this approach have been published, often called "element-tagged immunoassay". The only difference from conventional immunoassays is the detection system used, in this case ICP-MS. To the best of our knowledge, the first report on such an attempt was described by Zhang et al. [95] in 2002. They described the indirect measurement of rabbit-anti-human immunoglobulin G (IgG) by the use of a sandwich-type immunoreaction. Here, the antibody (goat-anti-rabbit IgG) was modified with colloidal Au suspensions, and $^{197}Au^{+}$ detection was furthermore related to the antigen concentration. The results obtained were in good agreement with results of a conventional enzyme-linked immunoassay. Baranov et al. [96] presented a similar approach on the basis of commercially available NANOGOLD goat anti-human Fab' conjugates. As an alternative concept they suggested ICP-MS detection of europium, which is already present in the fluorescent labels for so-called AutoDELFIA (six to ten Eu ions per fluorescent label). Comparison of these two methods showed that fluorescent AutoDELFIA was still superior to ICP-MS in terms of detection limits. For simultaneous determination of at least two antigens they introduced the application of differently labelled antibodies (gold and europium) [97]. The authors pointed out this method might have broad multicapabilities through which many antigens, and also protein–protein interactions, can be quantitatively determined. A competitive immunoassay followed by ICP-MS detection of europium was further developed for the determination of total T4 in human plasma [98]. Based on a noncompetitive assay, the same group extended their concept to the simultaneous determination of α-fetoprotein (AFP) and free β-human chorionic gonadotropin [99]. Here, the monoclonal antibodies were labelled with Eu^{3+} and Sm^{3+}, to point out the attractiveness of ICP-MS for multianalyte immunoassays owing to its multielement capability. A flow-cytometry-based approach has recently been developed by Tanner's group [100, 101]. Specific antibodies each labelled with different rare-earth elements (Eu, Tb, Sm) were used for the detection of cell-surface

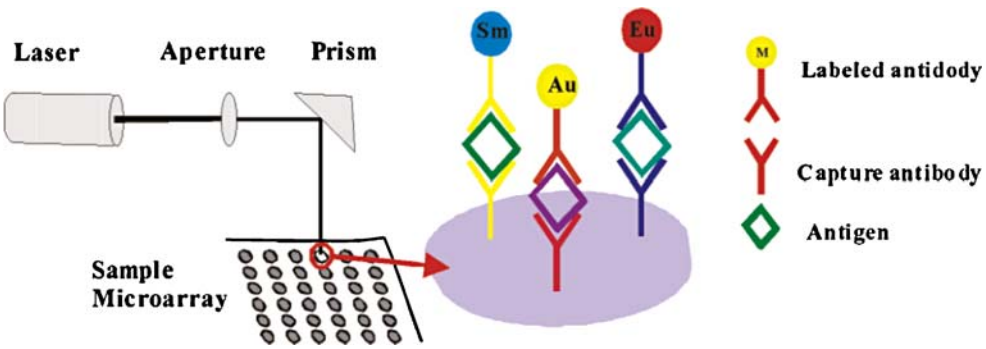

Fig. 3 Multiplexed protein quantification via immunomicroarray with heteroatom-labelled antibodies detected by laser ablation ICP-MS (from [103])

markers and the presence and the intensity of the ICP-MS signals could be directly correlated to the relative expression of the specific cell markers.

The abovementioned immunological methods were based on the acidic release of the metal from the antibody with subsequent solution-based ICP-MS determination. Recently, two different approaches were generated, in which LA-ICP-MS was applied to the quantitative characterisation of immunoreactions. Müller et al. [102] developed a method for the determination of Mre11 via gold-conjugated antibodies. One-dimensional sodium dodecyl sulphate polyacrylamide GE of cell lysates was followed by a transfer to a blotting membrane and protein marking with antibodies, which were covalently attached to gold nanoparticles (the number of gold atoms per antibody was calculated to be 53,350±1,600). The membranes were then ablated with the laser by continuous monitoring of the $^{197}Au^{+}$ signal. Although they observed quite high background levels, they calculated the detection limit to be 0.2 amol labelled antibody. Recently, Hu et al. [103] applied LA-ICP-MS for the detection of three different proteins (AFP, carcinoembryonic antigen, and human IgG) in immunomicroarrays (Fig. 3). They combined different labelling strategies for the three model proteins [96, 99] and LA-ICP-MS provided quantitative results by subsequent simultaneous determination of the elemental labels. The authors pointed out that the spatial resolution in the micrometre range may offer the possibility of high-density microarrays in the future. However, sensitivity could be amplified by using nanoparticles as elemental labels.

Although the development of antibodies with elemental labels has gained reasonable progress, their analytical performance, especially in terms of accuracy and precision, has to be critically assessed. Many approaches have made use of metal nanoparticles in order to improve the detection sensitivity and detection limits. This concept is clearly comprehensible, but a few limitations for quantitative approaches need to be considered carefully [83]:

1. Size control in nanoparticle synthesis is quite challenging, and even commercially available nanoparticles have shown a certain distribution in terms of particle size [104]. This uncertainty is directly accompanied by a variation of the number of atoms per nanoparticle serving as elemental labels.
2. The stoichiometry of protein to nanoparticle needs to be known exactly. Here, sophisticated bioconjugation strategies are necessary.

Nevertheless, extensive research activity can be expected in this exciting field of combining protein labelling with heteroatoms/isotopes and element-specific MS.

References

1. Ong SE, Mann M (2005) Nat Chem Biol 1:252–262
2. Aebersold R, Mann M (2003) Nature 422:198–207
3. Sadygov RG, Cociorva D, Yates JR (2004) Nat Methods 1:195–202
4. Wysocki VH, Resing KA, Zhang Q, Cheng G (2005) Methods 35:211–222
5. Currie LA (1995) Pure Appl Chem 67:1699–1723
6. Zhang H, Yan W, Aebersold R (2004) Curr Opin Chem Biol 8:66–75
7. Linscheid MW (2005) Anal Bioanal Chem 381:64–66
8. Unwin RD, Evans CA, Whetton AD (2006) Trends Biochem Sci 31:473–484
9. Chelius D, Bondarenko PV (2002) J Proteome Res 1:317–323
10. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Nat Biotechnol 17:994–999
11. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002) Mol Cell Proteomics 1:376–386
12. Szpunar J, Lobinski R, Prange A (2003) Appl Spectrosc 57:102A–111A
13. Sanz-Medel A, Montes-Bayón M, Fernández Sánchez ML (2003) Anal Bioanal Chem 377:236–247
14. Szpunar J (2005) Analyst 130:442–465
15. Sanz-Medel A (2005) Anal Bioanal Chem 381:1–2
16. Mestek O, Kominkova J, Koplik R, Kodicek M, Zima T (2007) Appl Organomet Chem 21:5–14
17. Svantesson E, Pettersson J, Markides KE (2002) J Anal At Spectrom 17:491–496
18. Pereira-Navaza A, Ruiz Encinar J, Sanz-Medel A (2007) J Anal At Spectrom. DOI 10.1039/b703555a
19. Messerschmidt A, Huber R, Poulos T, Wieghardt K (eds) (2001) Handbook of metalloproteins, vols 1–3. Wiley, Chichester
20. Lobinski R, Schaumlöffel D, Szpunar J (2006) Mass Spectrom Rev 25:255–289
21. Wind M, Lehmann WD (2004) J Anal At Spectrom 19:20–25
22. Quintana M, Coluda AD, Gondikas A, Ochsenkühn-Petropoulou M, Michalke B (2006) Anal Chim Acta 573–574:172–180
23. Wang J, Dreessen D, Wiederin DR, Houk RS (2001) Anal Biochem 288:89–96
24. Sanz-Medel A, Soldado Cabezuelo AB, Milacic R, Bantan Polak T (2002) Coord Chem Rev 228:373–383
25. Nagaoka MH, Maitani T (2000) Analyst 125:1962–1965
26. Wind M, Wesch H, Lehmann WD (2001) Anal Chem 73:3006–3010
27. Wind M, Wegener A, Kellner R, Lehmann WD (2005) Anal Chem 77:1957–1962
28. Krüger R, Wolschin F, Weckwerth, Bettmer J, Lehmann WD (2007) Biochem Biophys Res Commun 355:89–96
29. Krüger R, Kübler D, Pallissé R, Burkovski A, Lehmann WD (2006) Anal Chem 78:1987–1994
30. Helfrich A, Bettmer J (2007) J Anal At Spectrom 22:1296–1299
31. Garijo Añobe M, Messerschmidt J, Feldmann I, Jakubowski N (2007) J Anal At Spectrom 22:917–924
32. Hann S, Koellensperger G, Obinger C, Furtmüller PG, Stingeder G (2004) J Anal At Spectrom 19:74–79
33. Hagège A, Baldinger T, Martin-Jouet M, Zal F, Leroy M, Leize E, Van Dorsselaer A (2004) Rapid Commun Mass Spectrom 18:735–738
34. Van Lierde V, Chéry CC, Strijckmans K, Galleni M, Devreese B, Van Beeumen J, Moens L, Vanhaecke F (2004) J Anal At Spectrom 19:888–893
35. Takatera K, Watanabe T (1993) Anal Chem 65:759–762
36. Michalke B, Schramel P (1999) Electrophoresis 20:2547–2553
37. Shah M, Wuilloud RG, Kannamkumarath SS, Caruso J (2005) J Anal At Spectrom 20:176–182

38. Wind M, Eisenmenger A, Lehmann WD (2002) J Anal At Spectrom 17:21–26
39. Siethoff C, Feldmann I, Jakubowski N, Linscheid M (1999) J Mass Spectrom 34:421
40. Axelsson B-O, Jörnten-Karlsson M, Michelsen P, Abou-Shakra F (2001) Rapid Commun Mass Spectrom 15:375–385
41. Pereira Navaza A, Ruiz Encinar J, Sanz-Medel A (2007) Angew Chem Int Ed 46:569–571
42. Kovacevic M, Goessler W, Mikac N, Veber M (2005) Anal Bioanal Chem 383:145–151
43. Yanes EG, Miller-Ihli NJ (2004) Spectrochim Acta Part B 59:883–890
44. Giusti P, Schaumlöffel D, Ruiz Encinar J, Szpunar J (2005) J Anal At Spectrom 20:1101–1107
45. Pröfrock D, Leonard P, Ruck W, Prange A (2005) Anal Bioanal Chem 381:194–204
46. Neilsen JL, Abildtrup A, Christensen J, Watson P, Cox A, McLeod CW (1998) Spectrochim Acta Part B 53:339–345
47. Becker JS, Zoriy M, Krause-Buchholz U, Becker J, Pickhardt C, Przybylski M, Pompe W, Rödel G (2004) J Anal At Spectrom 19:1236–1243
48. Becker JS, Zoriy M, Becker J, Pickhardt C, Damoc E, Juhacz G, Palkovits M, Przybylski M (2005) Anal Chem 77:5851–5860
49. Elliot VL, McLeod CW, Marshall PS (2005) Anal Bioanal Chem 383:416–423
50. Feldmann I, Koehler CU, Roos PH, Jakubowski N (2006) J Anal At Spectrom 21:1006–1015
51. Lustig S, De Kimpe J, Cornelis R, Schramel P (1999) Fresenius J Anal Chem 363:484–487
52. Ma RL, McLeod CW, Tomlinson K, Poole RK (2004) Electrophoresis 25:2469–2477
53. Kastenholz B (2006) Protein Pept Lett 13:503–508
54. Marshall P, Heudi O, Bains S, Freeman HN, Abou-Shakra F, Reardon K (2002) Analyst 127:459–461
55. Wind M, Feldmann I, Jakubowski N, Lehmann WD (2003) Electrophoresis 24:1276–1280
56. O'Connor C, Landon MR, Sharp BL (2007) J Anal At Spectrom 22:273–282
57. Mayya V, Han DK (2006) Expert Rev Proteomics 3:597–610
58. Lin SH, Shaler TA, Becker CH (2006) Anal Chem 78:5762–5767
59. Rodríguez-González P, Marchante-Gayón JM, Alonso JIG, Sanz-Medel A (2005) Spectrochim Acta Part B 60:151–207
60. Rottmann L, Heumann KG (1994) Anal Chem 66:3709–3715
61. Heumann KG, Rottmann L, Vogl J (1994) J Anal At Spectrom 9:1351–1355
62. Huerta VD, Sanchez MLF, Sanz-Medel A (2006) Anal Bioanal Chem 384:902–907
63. Huerta VD, Reyes LH, Marchante-Gayón JM, García-Alonso JI, Sanz-Medel A (2003) J Anal At Spectrom 18:1243–1247
64. Rodríguez-Cea A, de la Campa MRF, Gonzalez EB, Fernandez BA, Sanz-Medel A (2003) J Anal At Spectrom 18:1357–1364
65. Goenaga Infante H, Van Campenhout K, Schaumlöffel D, Blust R, Adams FC (2003) Analyst 128:651–657
66. Polec-Pawlak K, Schaumlöffel D, Szpunar J, Prange A, Lobinski R (2002) J Anal At Spectrom 17:908–912
67. Ferrarello CN, Ruiz Encinar J, Centineo G, Alonso JIG, de la Campa MRF, Sanz-Medel A (2002) J Anal At Spectrom 17:1024–1029
68. Prange A, Schaumlöffel, D, Brätter P, Richarz AN, Wolf C (2001) Fresenius J Anal Chem 371:764–774
69. Schaumlöffel D, Prange A, Marx G, Heumann KG, Brätter P (2002) Anal Bioanal Chem 372:155–163
70. Wang Z, Prange A (2002) Anal Chem 74:626–631
71. Sariego Muñiz C, Marchante-Gayón JM, García-Alonso JI, Sanz-Medel A (2001) J Anal At Spectrom 16:587–592
72. Hinojosa Reyes L, Marchante-Gayón JM, García-Alonso JI, Sanz-Medel A (2003) J Anal At Spectrom 18:1210–1216
73. Havlis J, Schevchenko A (2004) Anal Chem 76:3029–3036
74. Harrington CF, Vidler DS, Watts MJ, Hall JF (2005) Anal Chem 77:4034–4041
75. Hann S, Obinger C, Stingeder G, Paumann M, Furtmüller PG, Koellensperger G (2006) J Anal At Spectrom 21:1224–1231
76. Del Castillo Busto ME, Montes-Bayón M, Sanz-Medel A (2006) Anal Chem 82:8218–8226
77. Polatajko A, Ruiz Encinar J, Schaumlöffel D, Szpunar J (2005) Chem Anal (Warsaw) 50:265–278
78. Schaumlöffel D, Giusti P, Preud'Homme H, Szpunar J, Lobinski R (2007) Anal Chem 79:2859–2868
79. Rodríguez-Cea A, Fernández de la Campa MR, García Alonso JI, Sans Medel A (2006) J Anal At Spectrom 21:270–278
80. Suzuki KT, Doi C, Suzuki N (2006) Toxicol Appl Pharmacol 217:185–195
81. Suzuki KT, Somekawa L, Kurasaki K, Suzuki N (2006) J Health Sci 52:590–597
82. Shi JW, Feng WY, Wang M, Zhang F, Li B, Wang B, Zhu MT, Chai ZF (2007) Anal Chim Acta 583:84–91
83. Bettmer J, Jakubowski N, Prange A (2006) Anal Bioanal Chem 386:7–11
84. Marshall P, Heudi O, Mckeown S, Amour A, Abou-Shakra F (2002) Rapid Commun Mass Spectrom 16:220–228
85. Heudi O, Ramirez-Molina C, Marshall C, Amour A, Peace S, Mckeown S, Abou-Skakra F (2002) J Pept Sci 8:591–600
86. Goshe MB, Smith RD (2003) Curr Opin Biotechnol 14:101–109
87. Tao WA, Aebersold R (2003) Curr Opin Biotechnol 14:110–118
88. Leitner A, Lindner W (2004) J Chromatogr 813:1–26
89. Leitner A, Lindner W (2006) Proteomics 6:5418–5434
90. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ (2004) Mol Cell Proteomics 3:1154–1169
91. Whetstone PA, Butlin NB, Corneillie TM, Meares CF (2004) Bioconj Chem 15:3–6
92. Liu H, Zhang Y, Wang J, Wang D, Zhou C, Cai Y, Qian X (2006) Anal Chem 78:6614–6621
93. Linscheid M, Pieper S, Ahrends R, Weisshof H, Hamester M, Lindemann T, Scheler C (2006) Presentation at the 54th ASMS conference on mass spectrometry and allied topics, Seattle, WA, 26 May–1 June 2006
94. Patel P, Jones P, Handy R, Harrington C, Marshall P, Evans EH (2007) Anal Bioanal Chem. DOI 10.1007/s00216-007-1432-7
95. Zhang C, Zhang Z, Yu B, Shi J, Zhang X (2002) Anal Chem 74:96–99
96. Baranov VI, Quinn Z, Bandura DR, Tanner SD (2002) Anal Chem 74:1629–1636
97. Quinn ZA, Baranov VI, Tanner SD, Wrana JL (2002) J Anal At Spectrom 17:892–896
98. Zhang C, Wu F, Zhang X (2002) J Anal At Spectrom 17:1304–1307
99. Zhang S, Zhang C, Xing Z, Zhang X (2004) Clin Chem 50:1214–1221
100. Ornatsky O, Baranov VI, Bandura DR, Tanner SD, Dick J (2006) J Immunol Methods 308:68–76
101. Tanner SD, Ornatsky O, Bandura DR, Baranov VI (2007) Spectrochim Acta B 62:188–195
102. Müller SD, Diaz-Bone RA, Felix J, Goedecke W (2005) J Anal At Spectrom 20:907–911
103. Hu S, Zhang S, Hu Z, Xing Z, Zhang X (2007) Anal Chem 79:923–929
104. Helfrich A, Brüchert W, Bettmer J (2006) J Anal At Spectrom 21:431–434
105. Griffin TJ, Aebersold, R (2001) J Biol Chem 276:45497–45500