Anal Bioanal Chem (2008) 391:885–894
DOI 10.1007/s00216-008-2083-z

TRENDS

Heteroatom(isotope)-tagged proteomics via ICP-MS: screening and quantification of proteins and their post-translational modifications

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Published online: 22 April 2008
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Introduction

Present drawbacks of MS-based techniques for quantitative proteomics

Mass spectrometry (MS) is one of the most powerful techniques at hand to investigate the protein players in biological systems. In fact, the number of studies involving molecular MS techniques, e.g. matrix-assisted laser desorption/ionisation (MALDI)-MS^n or electrospray ionisation (ESI)-MS^n, in the field of proteomics has rocketed in recent years [1, 2]. In any case, today's proteomics platform is built on technologies to identify large numbers of proteins in the same experiment. Characterization of such huge numbers of peptides and proteins in real-life complex mixtures, resulting from bottom-up or top-down proteomic strategies, is a rather difficult and intricate task.

Notwithstanding its present worldwide use, the great difficulties encountered could explain why proteomics results so far have been mainly *qualitative* in nature [3]. The need for quantitative approaches and data, however, cannot be overemphasised today: they are essential to obtain more precise information on the function of proteins, their temporal changes in the proteome, the kinetics of such changes, etc., allowing sound models to be established that are able to shed more light on the fundamental and cooperative roles of proteins in biological systems. In this vein, Ong and Mann recently published a seminal paper whose title, "MS-based proteomics turns quantitative" [3], stresses the new trend. The slow progress of quantitation in proteomics stems from the extreme difficulties associated with the accurate and precise determination of the absolute amount of a given protein in a complex mixture containing many other proteins and varied biocompounds. The main drawbacks making "quantitative" proteomics a tough challenge include:

1. The system to be analysed may be very small (e.g. a single cell with 0.5-pL volume and ca. 50-pg total protein content).
2. The possible proteins expression dynamic range is very large (e.g. 1–10^6 copies/cell) with proteins to be determined "buried" in a complex matrix.
3. The number of possible different proteins, e.g. in human samples, is huge.
4. Proteins show very large variations in their physicochemical properties (e.g. membrane proteins are highly insoluble and difficult to extract).
5. There are numerous and varied protein post-translational modifications (PTMs) that change in space and time the actual chemical nature and properties of a given scrutinized protein.

From the above it follows that accurate and robust determinations of proteins in real-life biological samples represents one of the greatest challenges in this field. To make matters worse, the MS signal obtained from a classical MALDI or ESI ion source is strongly affected by the species ionised (e.g. the considered peptide) and by the sample matrix and, of course, by the possible solvents used in the separations. In other words, MALDI or ESI sources are playing a phenomenal role in present proteomics, as

Robert Kellner Award Lecture. Presented at the Euroanalysis XIV Conference, Antwerp, Belgium, 13 September 2007.

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acknowledged by the 2002 Nobel Prize in chemistry, half of which was awarded to Koichi Tanaka and John Fenn for their introduction and application of the techniques. However, neither of those popular ion sources are able to provide a linear dependence between protein (or peptide) concentration in the sample and the measured MS signal. In fact, so far protein determinations are mostly "relative", that is, the relative change in protein amount observed between two biologically different states (e.g. relative measurements comparing diseased and control cells) is the measured analytical signal. However, quantification (determination) of "absolute" amount of the desired protein in the sample is always preferred in analytical chemistry and should be aimed at in the field of proteomics as well. At the end of the day, if just relative ratio information is desired for two different biological states, as is the case in "differential proteomics" experiments, that ratio can be easily obtained from the absolute amounts of the desired proteins (accurately quantified as is customary in analytical determinations).

So far, such "relative" quantifications of the desired proteins in the two different samples can be carried out using both typical 2D gel-based and gel-free approaches [4] to perform the required separations. The gel-free approaches, particularly those based on "stable-isotope labelling" of peptides and proteins are probably the most promising ones today. The required labelling may be achieved by a chemical reagent, as in the case of the known isotope-coded affinity tags (ICAT) method [5], 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 [5], but the same concept with other enriched isotopes has been reported now in several enhanced applications. For instance, an alternative approach of great potential is the use of stable isotope labelling in culture (SILAC), where cells are cultured in a medium containing a "heavy-isotope-marked" essential amino acid [6]. Another important relative quantification approach, developed subsequently, is isobaric tags for relative and absolute quantification (iTRAQ). This method can incorporate up to four mass tags that label the N-terminus of peptides [7].

It is important to stress that all such molecular MS methods and strategies are "relative" and have been originally introduced for "soft-type" ion sources where the energy of the source is actually controlled to produce the desired type of molecular ions: single-charged molecular ions, as MALDI provides, or multiple-charged molecular ions, as obtained using the ESI source.

The formidable challenge of protein determinations by conventional MS-based methods is of such magnitude that well-known experts in the field have reported that the task in real-life protein mixtures "is close to impossible" [8, 9].

An excellent revision of such "molecular" MS approaches to protein quantifications was recently published in this journal [10]. The "relative" character of the quantification approaches reviewed is undebatable.

A completely different scenario, however, can be envisaged by using "elemental" MS instead. The introduction of inductively coupled plasma (ICP) for ionisation allows for a most robust determination of the desired element (heteroelement) in a given peptide or protein. This ICP-MS-based elemental determination can be extended in many cases to calculate the corresponding peptide or protein concentrations, as we will illustrate later on in the different sections of this article.

Promising alternative for quantification: the use of ICP-MS

The exceptional abilities of ICP-MS to track any desired "heteroelement" (meaning in this context any bioelement of interest, except C, H, O or N) in a biomolecule at microgram per litre or better detection limits, in a very selective way, are today well documented.

By using a plasma at atmospheric pressure for ionisation (e.g. an ICP) the biomolecules are efficiently converted into elemental ions and, in this way, the fast and robust screening and determination of such elements in proteins is straightforward. Using ICP-MS for detection, very complex mixtures of biomolecules (e.g. metalloproteins in the most varied biological fluids or tissues) can be analysed in a much more tractable way: after the appropriate separation of the biocompounds, only the heteroelement in the protein (e.g. a metal) will be "seen" by such detector.

The exceptional characteristics of ICP-MS detection have proved in this way most advantageous for metals, semimetals (e.g. Se) and also some biologically very important nonmetals (e.g. S, P and I) providing a new powerful means for screening and/or quantification of any of those "heteroatoms" naturally occurring in a protein [11].

Also, the ability of ICP-MS for simultaneous isotope abundance measurements opens new avenues for metabolism studies and quantification of proteins, peptides and amino acids. This extended concept of "heteroatom(isotope)-tagged proteomics", previously introduced in *Analytical and Bioanalytical Chemistry* [11–13], does not exclude the extensive use and importance of molecular MS techniques. They will remain indispensable for identification and chemical characterization of the heteroatom-containing biomolecules (perhaps detected by ICP-MS for the first time in a typical HPLC-ICP-MS arrangement).

Of course, such concepts and analytical strategies can be directly applied (e.g. proteins and biomolecules naturally containing the "tag" or heteroelement) or after adequate derivatisations to introduce the heteroelement into the biomolecule (i.e. after "labelling"). This latter application,

requiring labelling, is much more problematic and it is still in its infancy for ICP-MS uses [14]. In any case, it is worth noting that labelling of biomolecules is known to be achievable by chemical, biochemical and metabolic means and the wide existing knowledge on such topics opens new research avenues to develop creative ICP-MS applications in future protein determinations and proteomic studies. No doubt, such studies could be enriched and strengthened by such “element-driven” MS research, enabled by on-line ICP-MS detection.

I hope to show here how typical strategies, born in the field of trace element speciation analysis [15], can be extended to the field of protein investigations and to proteomic analysis via “integrated” MS speciation. In particular, I envisage that ICP elemental source (complementing ESI and MALDI molecular ion sources) studies are called to play a crucial role in the near future by helping proteomics to become a more *quantitative* science.

Multielemental trace element screening in proteins

One of the most interesting applications of using an ICP-MS for biomolecules detection [10] is the possibility of a fast screening of the presence or absence of many heteroatoms in a given separated protein. That is, by using a classic HPLC-ICP-MS speciation arrangement the detection of several desired elements in the same chromatographic peak (i.e. protein) is straightforward. Moreover, the sensitivity and robustness of ICP-MS signals facilitate “visualisation” of the presence of certain metalloproteins “hidden” for a typical HPLC-ESI-MS system. As pointed out before, ESI-MS signals from a peptide are strongly dependent upon the sample matrix and the solvent used. In fact, the observed sensitivity may be orders of magnitude higher when the analyte is matrix-free (i.e. it has been adequately purified). As pointed out by Lobinski et al. [16], and in contrast to common knowledge in preliminary speciation papers, the observed sensitivity using ESI-MS can be equal or higher than that in ICP-MS, provided that analytes have been adequately purified prior to ESI-MS measurements.

In real-life proteomics analysis, however, appropriate sample purification and separations are difficult, cumbersome and usually lengthy. It is in such situations that the ICP source may come to the rescue. For analytes in samples “as they are” in nature (e.g. biological fluids or tissue extracts) the ICP-MS may provide, without such complex sample preparation, much higher signals than those observed by ESI-MS.

Thus, multielemental screening of trace metals by ICP-MS coupled on-line at the exit of the HPLC column can prove decisive to “visualise” the presence of a given metalloprotein (which should be declared “absent” by using ESI-MS only). Moreover, once that protein’s presence is uncovered, its appropriate preconcentration and purification in the corresponding fraction would allow its final molecular MS characterisation [17]. Figure 1 illustrates a work flow to “integrate” MS-based elemental and molecular techniques in protein studies.

The first step to investigate heteroatom-containing proteins could be a multielemental screening by HPLC-ICP-MS. Once the biomolecule(s) is(are) “visualised”, via the sought heteroatom, more or less complex treatments of that fraction for isolation and purification of proteins for final MALDI and ESI studies would follow. In this way, final characterisation and unequivocal chemical identification of the biomolecule are enabled (step 2 in Fig. 1). Once the biomolecule (e.g. metallocompound) is identified, the ICP-MS could be employed to determine the protein amount existing in the investigated biological sample, as will be illustrated in the following sections.

There are many examples in recent literature on bioinorganic speciation illustrating this concept of using ICP-MS to accomplish the first step in Fig. 1. I will here

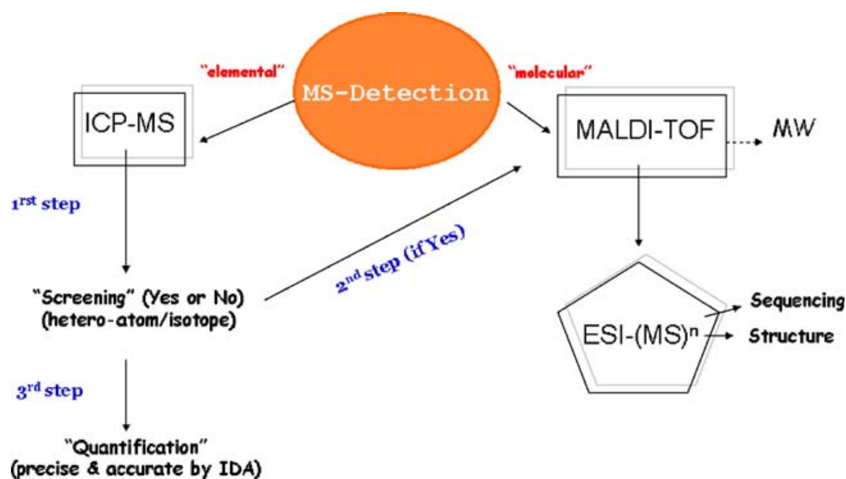

Fig. 1 Flow chart proposed in MS-based heteroatom(isotope)-tagged proteomics work

select the case of screening of metals in human transferrin, a good example investigated in my own laboratory for many years [18]. Figure 2a is a schematic of the experimental setup, whereas Fig. 2b shows the type of chromatograms we obtained for the corresponding metals just from one injection of a 1:1 diluted serum into the HPLC injector. In fact, similarly to Fe or Al (cf. Fig. 2b), many metals (including Ti, Cr, Mn, Co, V, Ga, Ni and La) are known to be bound to serum transferrin. Therefore, their presence (or absence) in normal, diseased or therapeutically treated individuals can be screened in a chromatographic run of the corresponding human serum sample. Another extremely useful example, this time in the field of nutrition, can be seen in our work on simultaneous screening of essential elements (including Fe, Cu, Zn, Se, and I) in different milk proteins in order to compare the nutritional value of commercial formula and maternal milks [19].

Quantitative analysis of proteins by ID-ICP-MS

Of course, once the sought protein has been characterised by molecular MS (step 2 in Fig. 1) its amount in the studied sample could be determined, again using the HPLC-ICP-MS arrangement, provided that certain critical conditions are met. The first condition is that the metal/protein stoichiometry is known (or has been established previously): as the ICP-MS destroys molecular information, only if the stoichiometry is known can the total amount of metalloprotein present be inferred from the elemental determination afforded by the ICP. A second condition derives from the above reasoning: the HPLC separation of the metalloprotein must be of the highest chromatographic resolution, otherwise we would obtain a sum of signals (positive errors) from the metal present in other coeluting biocompounds. Thirdly, any loss of the monitored metal during the analytical process (e.g. in the HPLC column) will bring about a negative error in the eventual metalloprotein determination. Thus, the column recovery for the investigated biocompound should be evaluated and/or possible losses corrected. Also, metal contaminations must be avoided for similar reasons.

Finally, the ICP-MS signals could be affected to a certain extent by changes in the composition of the mobile phases used in the HPLC separation and this effect should be known and corrected.

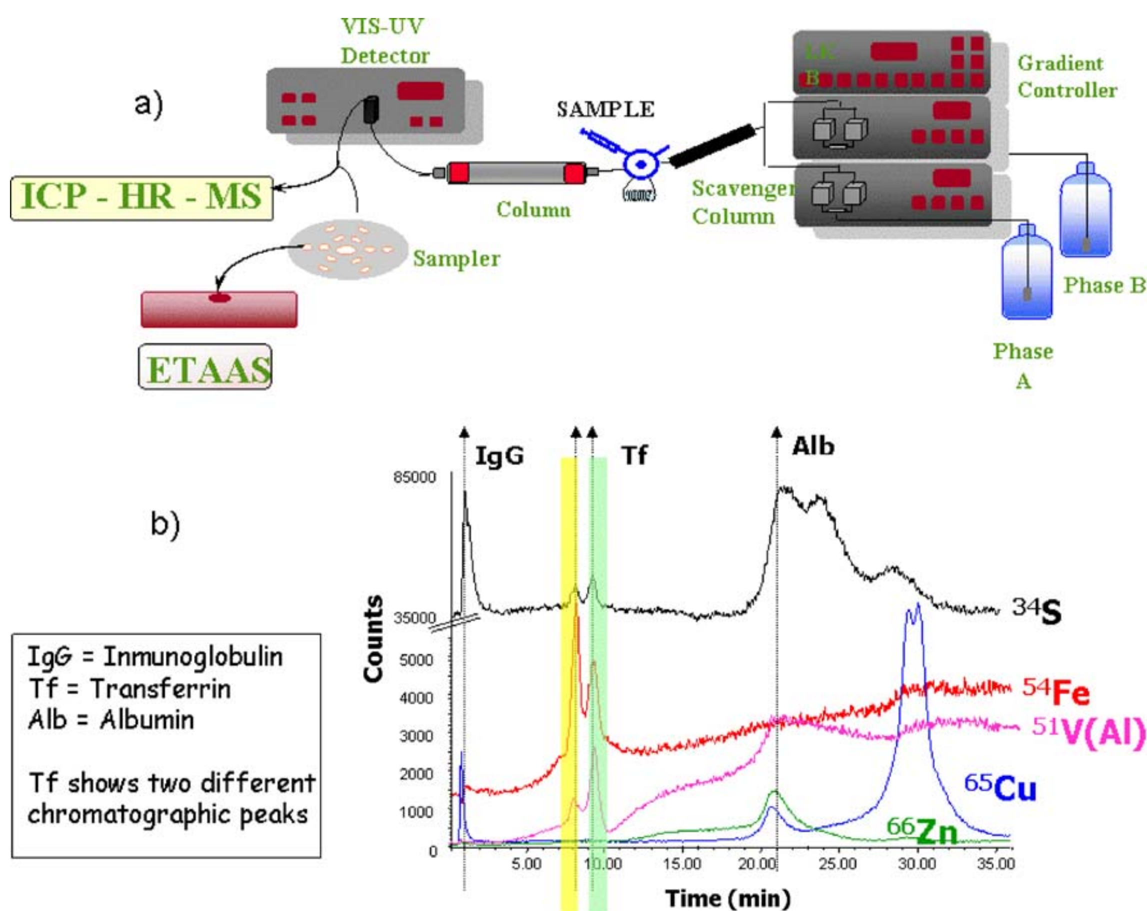

Fig. 2 **a** General setup for metal speciation (e.g. Fe^{3+}) in human serum. **b** Some elements, associated to human serum proteins, as detected by ICP-MS on-line with anion-exchange HPLC

Contamination and efficiencies in the column and also matrix effects in the ICP-MS signals are usually evaluated using analyte standards. Unfortunately, commercially available standards for possible metal–biomolecules to be found in our bioinorganic research are very scarce so far.

A way out or alternative approach for reliable metal determinations in speciation analysis consists of resorting to isotope dilution (ID) techniques, as pioneered by Heumann and coworkers [20] for speciation with HPLC-ICP-MS couplings. Although the term isotope dilution analysis (IDA) has been used in the organic/molecular MS literature for quantitative proteomics [21], such methodology is based on comparing ESI-MS^n peak areas of trypsin digested samples (peptides) with the related isotopically labelled peptides which are used as the internal standards. In other words, this operation is conventionally termed an "internal standard" approach in analytical chemistry [9, 22]. However, using the ICP-MS source, a selected isotope ratio can be measured in the heteroatom with great accuracy and it is this value what is incorporated in the well-known isotope dilution equation which will provide, without any previous calibration, the absolute quantity of the heteroelement in the analysed sample [20, 23]. In such HPLC-ICP-MS systems the selected isotope ratio is measured throughout the chromatogram and so the absolute amount of sought heteroatom in each point of the chromatographic peak can be calculated to obtain a "mass-flow" chromatogram [20]. In this way, the absolute amount of the heteroatom in each peak (ideally, in each isolated biomolecule) can be directly obtained.

Heumann et al. [20] proposed two different modes of operation: the "species-specific" and the "species-unspecific" spike modes. Of course, in principle, the species-specific spike mode would provide a more accurate IDA determination than the post-column or species-unspecific spike methods because any losses of the heteroatom in the column will occur the same for all isotopes and so they will be corrected for using the isotope ratio as the signal.

It should be pointed out, however, that in both modes "quantitative" speciation refers only to the metal(heteroatom) associated to the corresponding protein (e.g. two iron ions per molecule of Tf as in iron-saturated transferrin, Fig. 3). Therefore metal contamination should be avoided at any rate, high resolution metalloprotein separations are mandatory and the stoichiometry of metal (determined) to protein (to be evaluated) should be known for reliable "intact" or entire-protein determination.

Quantitative analysis of glycosylated metalloproteins: determination of transferrin sialylated isoforms

There are an increasing number of publications related to the use of post-column ID speciation applications to small

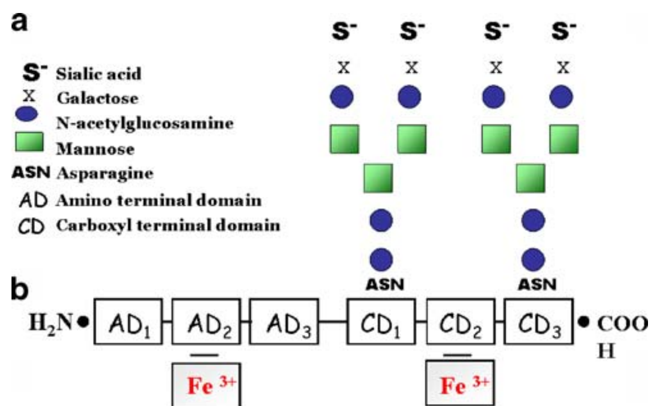

Fig. 3 Domain structure of the glycoprotein transferrin. The presence of a sialic acid at the end of each of the four glycosylated chains is clear and so if the protein is "iron-saturated" (two Fe^{+3} per protein molecule) the final charge will be dictated by the S^- groups present

molecules, particularly Se-containing amino acids. However, in the field of protein studies and proteomics the literature about such topics is rather scarce and refers mainly to accurate determinations of metal contents associated to metallothioneins [24]. Of course, as stressed before, the final aim of HPLC-ICP-MS systems for "absolute" protein determinations should be the determination of the "entire" protein (as mentioned before for transferrin by measuring $^{57}Fe/^{56}Fe$ isotope ratio).

Transferrin (Tf) is a well-known glycoprotein which transports many metals in human blood. Tf exhibits several glycoforms depending on its degree of glycosylation [25]. This post-translational modification is clinically most relevant because changes in the different known Tf glycoforms can be used as biomarkers for different disorders and diseases.

Normal Tf has two fully occupied N-glycosylation sites and it is a relatively homogeneous glycoprotein with more than 75% of its oligosaccharides being sialylated (S) structures with two branched glycoantennae (see Fig. 3). A daily alcohol intake above 60–80 g for more than 1 week results in a significant increase in the so-called carbohydrate-deficient transferrins (CDTs; forms containing none, one or two instead of the more common four sialic acid groups). In fact, measurement of CDTs in human serum constitutes the most specific marker for chronic alcohol abuse and congenital disorders of glycosylation in proteins. The development of new specific, precise and accurate analytical methods for individual CDT determinations is highly demanded these days because CDT-specific inmunoreactions or reliable CDT standards are not commercially available so far.

Work in our laboratory with HPLC-ICP-MS, using post-column ID (with ^{57}Fe) and "species-specific" ID, demonstrated the possibility of determinations of individual Tf glycoforms in human serum by ICP-MS [26], after HPLC separation of the glycoforms (Fig. 4). Each of those fractions

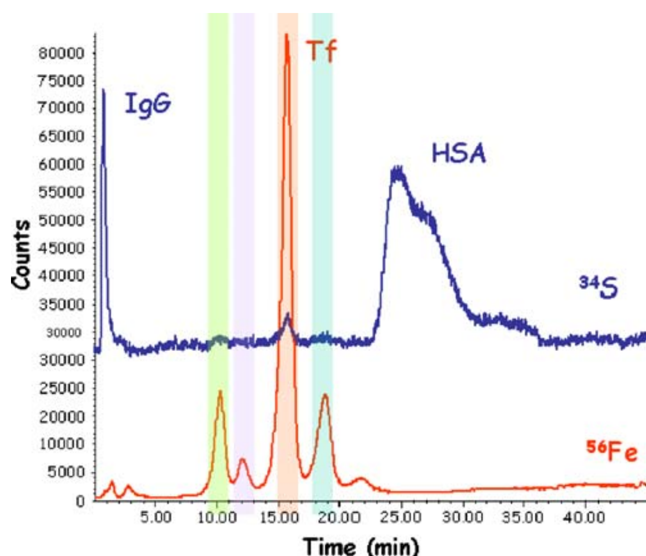

Fig. 4 Speciation of Tf glycoforms by HPLC-ICP-MS in human serum of a chronic alcoholic patient (saturated with $FeCl_3/NaHCO_3$ and diluted 1:1 before injection)

(isoforms) were isolated and examined in the MALDI-TOF (time of flight) to confirm the real nature of every Tf glycoform by its molecular mass determination [27].

It should be stressed that in our quantitative study [26] both post-column ID (species-unspecific spike) and species-specific spike modes provided successful results. Application to the determination of ten different healthy individuals' serum samples showed firstly that the precision of the proposed methods was excellent (e.g. ±1% for the most abundant glycoform, [S_4]). Secondly, values for the ten different individuals analysed from Asturias' Central Hospital showed good agreement with expected values for healthy subjects given as indicative values (as a normal range) in the literature and "relative" individual isoforms content in healthy people seems to be quite homogeneous (see Table 1).

Importantly, the levels of S_2 in the different serum samples of nonalcohol abusers turned out to be 1.4±0.4%, a value in the lower range of the thresholds established by other methods (probably due to the higher sensitivity of HPLC-ICP-MS).

All results in Table 1 agree with those expected by alternative methods, but values here are not just relative CDT figures. In fact, absolute amounts of individual glycotransferrin isoforms are determined using ICP-MS detection. Those absolute quantitative values showed that S_2 concentrations are much higher in alcoholic patients than in healthy people [26], a fact confirmed by ESI-MS (Q-TOF) but in a rather "qualitative" (or at most relative) measurement. It is worth mentioning that similar concepts and strategies have been recently extended in our group to develop a highly reliable methodology to achieve most accurate and precise determinations of clinical iron status parameters [28].

Other authors have also conducted the initial steps of "species-specific" ID in metalloproteins by synthesising ^{65}Cu-rusticianin [29], or ^{57}Fe-labelled ferritin [30] and/or ^{65}Cu, ^{64}Zn-superoxide dismutase (SOD) [31]. Although these standards have not been tested for determination of such proteins in real samples yet, the analytical potential of this strategy can be forecasted.

Quantitative analysis of phosphoproteins and phosphopeptides

The dynamic control of a protein's conformation and hence its function is achieved in nature mainly through chemical changes after it has been translated from messenger RNA. These so-called post-translational modifications (PTMs) in proteins are then of paramount importance. A good example is glycosylation, as illustrated for transferrin in the preceding section. Another example of a PTM of outstanding biological importance is phosphorylation. Protein phosphorylations are implicated in regulating protein activity and signalling pathways in cells. Therefore investigations on such PTMs have received enormous attention recently, owing mainly to the importance of signalling processes connected with cancer [32]. Unfortunately, robust phosphoprotein analysis is still a great analytical challenge due to a series of drawbacks hindering absolute quantitative analysis, including: (1) most of the signalling proteins are present at very low abundance within cells; (2) the stoichiometry of phosphorylation is generally low; (3) phosphorylation is a heterogeneous event: phosphorylated sites on proteins may vary; (4) phosphorylation dynamic range is very large; (5) phosphopeptide MS quantitative analysis (e.g. MALDI or ESI) is known to be problematic.

Classical approaches to analysing protein phosphorylation have consisted so far of labelling the proteins with radioactive ^{32}P or doing western blot analysis, which uses gel electrophoresis to separate the proteins according to

Table 1 Quantitative transferrin isoform results in ten different sera from healthy individuals determined by isotope dilution HPLC-ICP-MS (with ^{57}Fe)

Isoform	Reference values (%)	Obtained (%)[a]	Repeatability (%)[b]
S_2	<2.5	1.40±0.4	1.50±0.07
S_3	4.5–9	4.4±0.8	4.6±0.2
S_4	64–80	76±3	74.4±0.8
S_5	12–18	15±2	16.4±0.2
S_6	1–3	2.0±0.9	2.7±0.3

Note: see ref. [26] for details
[a] Mean of ten sera
[b] Three replicates

different mass and structure [33]. More recently the use of MS-based techniques has really boosted quantitative protein phosphorylation analysis. As stressed before in other examples, however, quantitative data in (phospho)proteomics are most often derived from "relative" phosphoprotein levels between two cell states [33].

As stressed before for general protein quantifications, molecular MS techniques for absolute phosphoprotein determinations are unfortunately constrained by the fact that they provide species-dependent and matrix-dependent signals. Conversely, as demonstrated by Lehmann's group, elemental ICP-MS provides an analytical signal that is specific for phosphorus in phosphoproteins [34]. Thus, it is important to note that the ICP-MS ^{31}P signal, under appropriate conditions, may be independent of the amino acid's nature and sequence in the phosphopeptide studied and of the matrix and solvents in which it is analysed. The first quantitative attempt to use such ICP-MS measurements for computing degrees of protein phosphorylation was reported by the same authors. They proposed using ICP-MS to measure the stoichiometric P/S ratio and convert it into the degree of phosphorylation using protein sequence information. Of course, such a measurement is clearly "relative" [35].

Thus, the usefulness of a capillary HPLC-ICP-MS speciation arrangement in providing a highly precise and accurate quantification of phosphopeptides in complex tryptic peptide mixtures just by using an elemental P standard (i.e. an internal standard, for calibration of any phosphopeptide) has been demonstrated [36]. Of course, a reversed-phase column is required to resolve such complex mixtures first. Chromatographic gradients of organic modifiers (e.g. acetonitrile/water) had to be used, affecting plasma stability. It is known that ICP-MS sensitivity (especially for those elements exhibiting high ionisation potentials like P) changes dramatically as organic content of mobile phases changes along the gradient. This fact ruins the possibility of direct species-independent calibrations in the ICP-MS. In our work [36] specific operating requirements to maintain a stable ^{31}P sensitivity along the gradient were worked out using the cap-HPLC-ICP-MS system and an optimised post-column makeup flow with a defined acetonitrile content.

It is worth emphasizing that the analytical performance characteristics of such absolute P determination in a separated model phosphopeptides mixture exhibited excellent overall accuracy (3–6%) and limit of detection (110 fmol of P). Accurate quantification of the phosphopeptides present in a real tryptic digest of β-casein of bovine milk was also achieved. After total P determination in the protein by continuous capillary flow ICP-MS, P recovery from the reversed-phase column was also evaluated (91±5%). Figure 5 shows the type of chromatogram obtained for β-casein

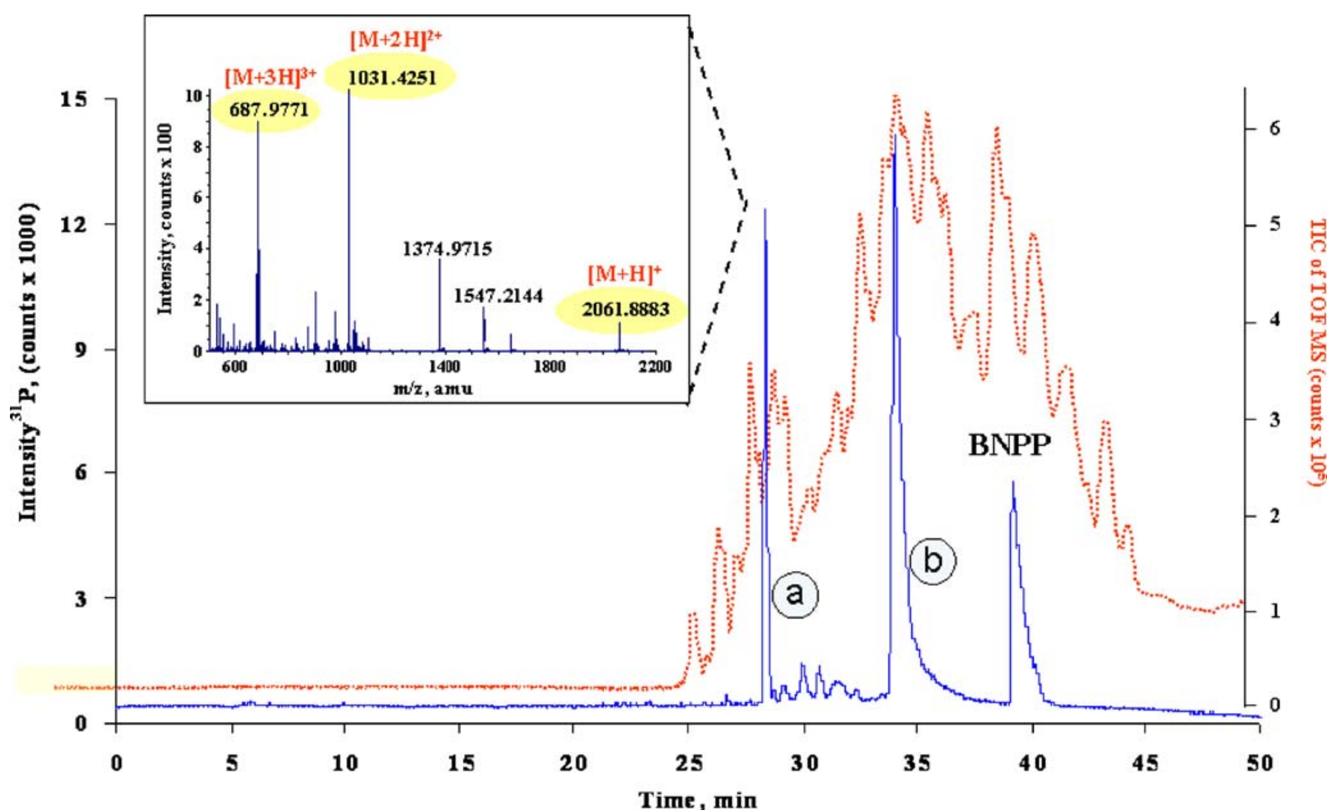

Fig. 5 Capillary HPLC chromatogram with ICP (*blue*) and ESI-MS (*red*) detection of a tryptic digest of β-casein. *Insert* MALDI-TOF mass spectrum of peptide *a* (TSTEPQY(PO_3H)QPGENL)

Table 2 Absolute quantification of phosphopeptides present in β-casein using BNPP as P standard

Peak	P/protein (pmol; *n*=3)	Sequence	pPep/protein[a]
a	0.99±0.02	FQ**p**SEEQQQTEDELQDK	0.99±0.02
b	3.20±0.05	RELEELNVPGEIVE**p**SL**p**S**p**S**p**SEESITR	0.80±0.01

[a] Degree of phosphorylation

tryptic digest spiked with the internal standard bis(4-nitrophenyl)phosphate (BNPP). As expected, BNPP eluted close to the elution window of the phosphopeptides (recognised selectively by the ^{31}P detection) and so assuring the same ICP-MS sensitivity in all the peaks. Also, Fig. 5 shows the two main peaks (phosphopeptides a and b) accounting for more than 90% of the total P present in the protein digest. Table 2 collects the calculated picomol of P per picomol of total protein digested for each of the two main peaks. Because the two main phosphopeptides (a and b) are quantified in absolute terms, we can express them as picomol of P per millilitre of tryptic digest or per pmol of protein (as expressed in the second column of Table 2).

Yet the robustness of ICP comes with a price: the loss of molecular information about each phosphopeptide. Thus, complementary use of "molecular" MS techniques is required to translate the total amount of P determined in each peak into an absolute amount of phosphopeptide. In this vein, the coupling of our capillary HPLC separation with ESI-MS^n allowed the unambiguous elucidation of the individual amino acids and their sequences in each phosphopeptide a and b (see Table 2, second column). Once the amino acid sequence is clearly known for each peak by ESI-MS^n (e.g. monophosphopeptides and tetraphosphopeptides), the quantitative data obtained by ICP-MS can be easily translated into "degree of phosphorylation" of each particular peptide at the moment of sampling the protein (last column in Table 2), a basic information parameter in quantitative phosphoproteomics. Moreover, this accurate and precise ICP-MS phosphopeptides determination method has also been demonstrated for proteins with a greater degree of complexity, as detailed in a very recent paper [37].

A particularly important application along this line will be to investigate kinetic signalling events (e.g. in cancer studies) [32]. As phosphorylation is dynamically regulated (i.e. there are distinct sites and phosphorylation degrees in a protein under a given set of conditions and they would change with time) the HPLC-ICP-MS technique proposed here provides and exceptional means to follow (de)phosphorylation kinetics (see Fig. 6 in which preliminary results from our laboratory demonstrate that discrimination power of phosphorylation states previously unachievable is now at hand).

To summarise, I believe that the proposed capillary HPLC-ICP-MS methods in conjunction with adequate P-containing "internal standards" could become a very powerful tool to: (a) optimise and validate quantitatively the plethora of procedures for phosphorylation determinations appearing in the literature; (b) optimize and validate the commonly used (but not quantitatively tested) sample preparation steps for phosphopeptides enrichment, such as those using immobilised metal affinity chromatography (IMAC) and TiO_2 minicolumns [37]; (c) allow robust phosphorylation data comparisons between different laboratories and address quality assurance issues, so urgently needed today for quantitative (phospho)proteomics; (d) finally, the demonstrated high precision and accuracy attainable make this approach ideal for the discrimination between very small temporal changes in phosphorylation levels (e.g. kinetic studies) as illustrated by preliminary results shown in Fig. 6.

A look into the future

All described applications of HPLC-ICP-MS techniques in the field of protein studies and proteomics have been carried out for "natural" heteroatom-containing proteins or those where the ICP-MS-monitored heteroatom is the key element for PTMs.

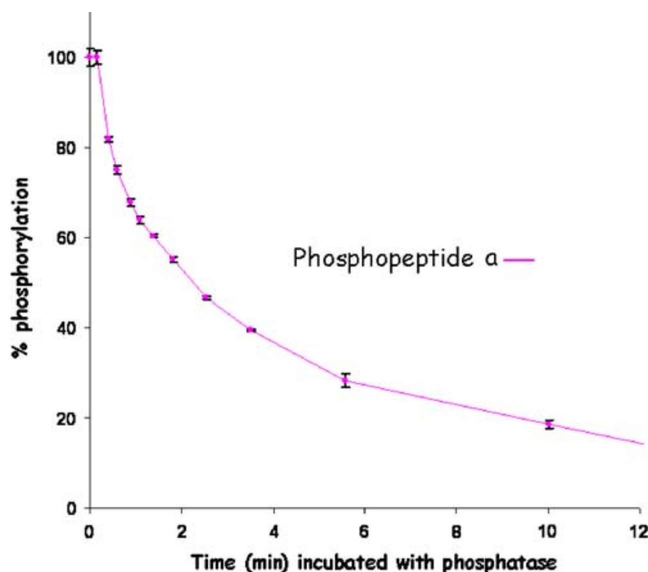

Fig. 6 Measurement of dephosphorylation kinetics observed by HPLC-ICP-MS for the model phosphopeptide a. Precision of each measurement 0.5–3.8% RSD. Discrimination of up to 12 different phosphorylation states in a 15-min experiment

Screening of heteroatoms and also absolute quantitative analysis of proteins and peptides, as explained with IDA techniques for multi-isotopic elements or with "internal standard" techniques for P, could also be achieved by resorting to the use of atom/isotope "labelling reagents" [14]. That is, not just natural "heteroatom-tagged" proteins or PTMs are amenable to ICP-MS determinations. Possibilities of using artificial reagents as "elemental labels" for rendering a given peptide or protein "visible" or quantifiable by ICP-MS have a great future.

If the additional requirements of the corresponding bioconjungation chemistry are met, it is envisaged that such labelling reagents, able to tag proteins and peptides with appropriate heteroatoms (to be detected and measured by ICP-MS), could tremendously expand the scope of quantitative heteroatom-tagged proteomics in the near future.

At this point, it could be argued that the examples given before refer mainly to the study of selected proteins and so the concept seems to fall short of investigating large numbers of peptides and proteins (typical in proteomics). Admittedly, so far "molecular" MS techniques are more suited than "elemental" techniques for such rather "qualitative" or at least "relative" global approaches. My point here is that ICP-MS is just a part of the integral approach proposed (see Fig. 1) and so if MS-based proteomics is a reality as a whole, "heteroatom-tagged proteomics" is just a smaller emerging field to help future proteomics investigations. What is more, I can envisage an experimental growth on the use of multidimensional separation techniques coupled with ICP-MS or laser ablation–ICP-MS [38], strategies extended to a large number of proteins in the near future. After dealing with stability issues [39] and working out appropriate treatments and multidimensional separations, a heteroatom such as sulfur (or perhaps appropriately introduced in such biomolecules as in ICAT [5], via a proper heteroatom-containing reagent) present in a great number of proteins could be measured by ICP-MS for selected quantification needs in larger numbers of biomolecules.

In any case, I regret to say that when attending well-established and internationally recognised MS conferences it becomes clear that a vast number of "molecular" MS people ignore the full potential and applications of ICP-MS to their field (not only proteomics practitioners). So the time has come to spread the word about the advantages of using ICP-MS and typical trace element speciation strategies, to complement their better-known molecular MS techniques (Fig. 1 shows a path to carry out that integrated work) in protein studies and quantitative proteomics problems.

Finally, crossfertilisation must be promoted; multidisciplinary and collaborative projects and ideas must be encouraged; and biologists, biochemists and chemists should meet together in order to achieve a real "integration" of elemental and molecular MS techniques. Together we will get further in present proteomic studies and efforts to clarify protein structures, their complexes, their interactions, their actual concentrations and eventually their functions in living organisms and in modern systems biology.

Acknowledgements The author is most grateful to his analytical spectrometry research group in general and to M. Montes-Bayón and J. Ruiz-Encinar in particular for most useful discussions.

Also, financial support from the Project CTQ2006–02309 from the Ministry of Education and Science, Madrid, is gratefully acknowledged.

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