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Detection of Proteins by Hyphenated Techniques with Endogenous Metal Tags and Metal Chemical Labelling

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Abstract.

The absolute and relative quantitation of proteins plays a fundamental role in modern proteomics, as it is the key to understand still unresolved biological questions in medical and pharmaceutical applications. Highly sensitive analytical methods are required to quantify proteins in biological samples and to correlate their concentration levels with several diseases.

Enzyme-linked immunosorbent assay (ELISA) and Western blot represent specific strategies for protein quantitation. However, these approaches are impractical for quantitative studies: the availability of high quality ELISAs for biomarker candidates is limited, and the performance characteristics of many commercially marketed ELISAs are poorly documented or unknown. The development of ELISA or Western blot is also expensive and time-consuming.

The limitations of these strategies, combined with the large numbers of biomarker candidates emerging from genomic and proteomic discovery studies, have created the need for alternative strategies for quantitation of targeted proteins.

In recent years, a widely explored approach to identify and quantify intact proteins is based on (i) the detection of endogenous metals covalently bound to the protein structure or (ii) the labelling of proteins with metallic probes. The development of several hyphenated analytical techniques for metals quantitation has led to new possibilities for the quantitative analysis of proteins.

In the present review, we attempt to provide a full coverage of current methodologies for proteins quantitation based on the detection of endogenous metal(loid)s or chemical labeling with metal(loid)s, highlighting, to the best of our knowledge, their merits and limits.

Introduction.

The quantitative analysis of protein mixtures is essential to understand the variations in the proteome of living organisms. Quantitative proteomics is a rapidly increasing, important research field since many specific functions in the cell are controlled by changes in protein expression levels under different physiological conditions. Thus, the quantitation of the proteome can reveal alterations of the normal biological state or even point out biological markers in important diseases. Figure 1 shows the number of published research articles in proteomics using a search query with keywords "protein* and quantitation" in title from 2000 to 2013, and reflects the growing interest in absolute and relative protein quantitation techniques.

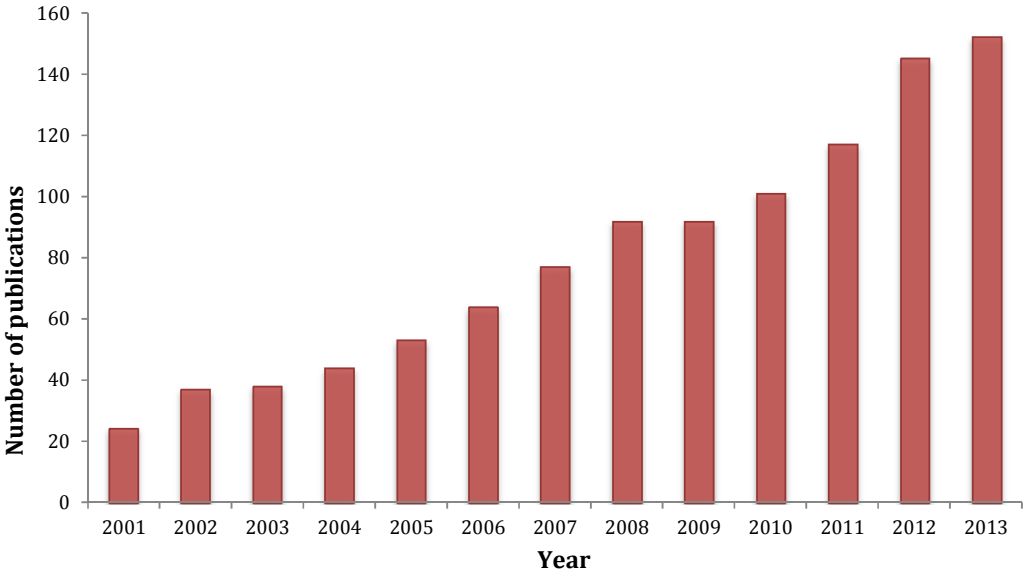


Fig. 1 Number of published research articles in proteomics using a search query with keywords "protein* and quantitation" in title from 2000 to 2013 via Scopus excluding reviews and abstract proceedings.

For a clinical biomarker, quantitative information is mandatory in order to use the protein/peptide routinely in clinical diagnosis.[1]

Consequently, sensitive analytical methods are needed, which allow the quantitation of individual proteins synthesized by a cell at a given moment and under specific conditions. One of the main goals of developing protein quantitation strategies is their clinical applications to quantify candidate proteins in biological matrices as biomarkers or putative marker proteins associated with a variety of diseases, for the development of new drug and personalized medicine.

In an analytical framework the expression "relative quantitation" refers to the quantitation of relative ratios of proteins and/or peptides in two or more samples. In contrast, the expression "absolute quantitation" refers to the quantitation of proteins and peptides in units of weight, concentration or total amount of substance in one or more samples.[2]

Traditionally, the enzyme-linked immunosorbent assay (ELISA) has been the method more widely applied for the targeted quantitation of proteins, providing good sensitivity and throughput.[3]

When ELISA assays or high quality antibodies already exist, the process of validation of a biomarker candidate can be relatively straightforward as, to date, it remains the "gold standard" for targeted protein quantitation.[4]

However, for many or most of the novel protein candidates discovered in recent proteomics studies, the ELISA approach is limited by the lack of availability of antibodies with high specificity, and the development of a high quality ELISA assay requires a significant investment in time and resources.[3]

Mass spectrometry (MS) with electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) are currently the major technique for protein identification.[5,6] Advances in the use of mass spectrometry over the last 5 years opened the door to the identification and quantitation of proteins with an unprecedented speed.

Although MS techniques are crucial in the identification of peptides and proteins, their application to quantitative analysis presents some important drawbacks such as the differential response of proteins and peptides depending on size, hydrophobicity, matrix, or solvents.[7]

To overcome these disadvantages and obtain better analytical results various types of tags have been developed to label proteins for their detection and quantitation. Additionally, since standards

for most biomolecules of natural origin are unavailable, their tagging using different derivatization approaches is a valuable alternative for their quantitation.[8]

The variety of chemistry available to modify reactive groups in a typical peptide (Figure 2) combined with the numerous structures possible for a quantitative tag creates a large number of possibilities to chemically incorporate a labeling agent.

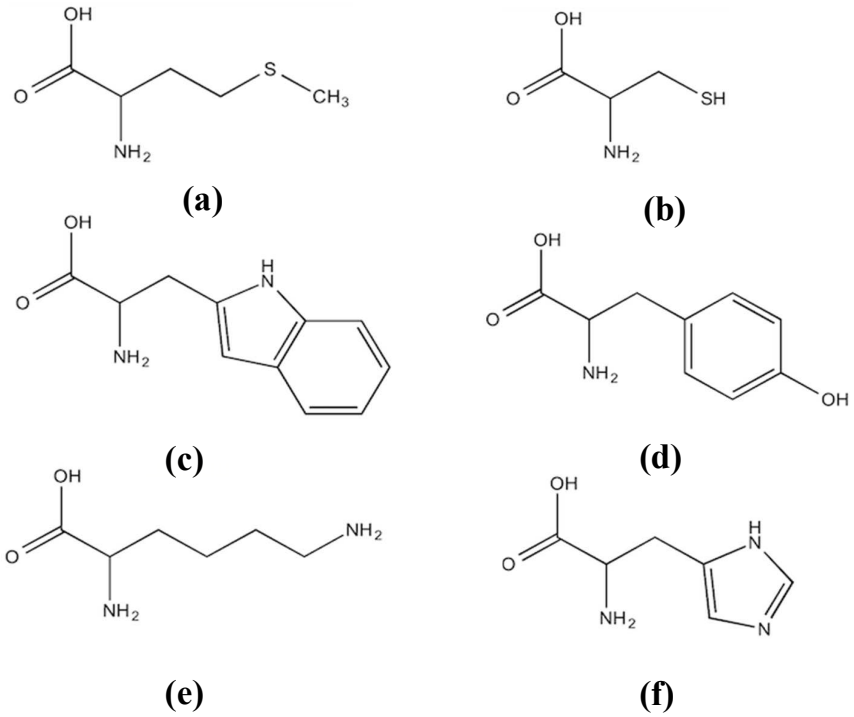


Fig 4. Structures of the most-reactive amino acids used to functionalize proteins. (a): methionine; (b): cysteine; (c): tryptophan; (d): tyrosine; (e): lysine; (f): histidine.

The so-called "global" approaches aim to target common functional groups, i.e., amino groups at the N terminus of a peptide or protein and on lysine (Lys) side-chain, or carboxyl groups at the C terminus and on aspartic (Asp) and glutamic (Glu) acid residues.[9] The labeling agent for a relative quantitation of proteins or peptides may be introduced in this way to ensure the highest possible coverage, so that almost every peptide will carry the tag. Global strategies have to rely on more sophisticated separation steps like multidimensional chromatography or high-resolution mass spectrometry to deal with the higher complexity of the mixtures.

More specific approaches are frequently directed towards peptides carrying rare amino acids. Cysteine (Cys) is a relatively rare amino acid, with an average relative abundance ranging between 2.26% in mammals and 0.5% in bacteria.[10] Cys is very frequently used as probe target because its thiolic group can be specifically modified, and many different stable-isotope labeling reagents for Cys have been reported.[11] With the exclusion of the thiolic group of Cys, few other functional groups of amino acids can be specifically modified, e.g. the specific tagging of lysine (via amidination/guanidination) or tryptophan (modification of the indol system) has been reported.[9] These methods, restricted to those proteins that have these amino acids, are advantageous because they lead to the determination of target proteins in complex matrices.

In the case of Cys labeling, reduction of disulphide bonds in proteins is usually a necessary step. This can be achieved using dithiothreitol (DTT), mercaptoethanol or 3,3',3''-phosphanetriyltripropanoic acid (TCEP), the last preferred in order to avoid additional thiol groups in the mixture that may react with the labeling reagent.

For quantitative analysis, the ideal labeling reagent should provide high detection sensitivity, specificity, quantitative labeling reaction and it must/should not be susceptible to major matrix interfering reactions. The selected labeling agent should not require solvent extraction steps to remove reagent excess prior to the separation step.

Analytical methods using colorimetric labeling reagents and UV/fluorescence detection are simpler as compared with MS technique but they have lower sensitivity. Fluorescence leads to much lower detection limits than UV absorbance detection, and the use of lasers (mainly argon, or in some studies mercury or krypton ion lasers) to induce fluorescence is associated with further improvements.

Many proteins have been labeled with probes that target the Cys reactive thiolic group. Typical derivatization reagents are imidazole[12], monobromobimane[13], 5,5-dithio-bisnitrobenzoic acid[14], maleimide[15], 3-diazole-4-sulfonate[16], 3-iodoacetylaminobenzanthrone.[17]. For example, Nygren et al. presented a dual-labeling approach of a binding protein using N-iodoacetyl-N-(5-sulfo-1-

naphthyl)ethylenediamine and succinimidyl-6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate to subsequently label the –SH and the N-terminal –NH_2, allowing specific fluorescence detection of the protein.[18] However the application of these dyes to proteomic studies still has some limitations: organic dyes often suffer from photobleaching; they usually have a large size (~1 nm in diameter), sometimes limiting their access to target amino acids located inside the protein because of steric restrictions; and dye molecular rigidity might be destroyed, leading to fluorescence signal suppression.[19]

Common probes include large fusion proteins such as the green fluorescent protein (GFP) or b-lactamase.[20] Although their potential has been convincingly demonstrated, possible problems, including their degradation and high background signal, might arise from the use of such large fusion proteins. In addition, large fluorescent proteins could interfere with the function of the targeted protein.[21]

Recent approaches for quantitative proteomics are based on isotopically (^{2}H, ^{13}C, ^{15}N, or ^{18}O) labeled derivates of proteins or peptides producing a mass shift in molecular MS between light and heavy labeled compounds. Stable isotope labeling by amino acids in cell culture (SILAC)[22] or the introduction of ^{18}O using $H_2{}^{18}O$ in the enzymatic digestion[23], are examples of metabolic and enzymatic labeling, respectively. The most extended methodologies are the chemical labeling with ICAT (isotope coded affinity tag)[24], CDIT (culture-derived isotope tags)[25], iTRAQ (isobaric tag for relative and absolute quantitation)[26], or PROTEIN-AQUA (protein absolute quantitation)[27].

Although the development of these techniques for protein quantitation on a large scale is increasing, the comparison and validation among different laboratories of the wide data obtained by different methods is very difficult.[28] The quantitation needs efficient labeling of the detected peptides or proteins and also relies on the accuracy of the mass measurement and the chromatographic reproducibility. PROTEIN-AQUA using a synthetic isotopically labeled standard of each target peptide is limited to a small number of analytes due to the high cost of the standard preparation.[29]

In the last decades, the outlook about protein quantitation has changed noticeably with the incorporation in this field of the screening of multiple heteroatoms naturally present, or introduced as labels in biological samples.

In recent years the analysis of naturally covalently incorporated heteroelements such as sulphur or phosphorous by inductively coupled plasma-mass spectrometry ICP–MS has an increasing interest.[28] However, isobaric interferences, the high first ionization energies of these heteroelements, and the resulting high limits of detection often lead to unsatisfactory results.[30]

Proteins frequently contain one or more essential coordinated metal(loid) ions in their catalytic or functional centres.[31] In addition, metal ions involved in allosteric regulations of proteins may be bound to other sites. Metals are typically coordinated to histidine (N), Cys (S) or carboxyl functions (O).[32] Even though trace metals play a vital role in living systems and their application as tags for selected bio-molecules has been demonstrated in a number of papers, their use as tags is limited since they are often only weakly associated with their ligands. This makes them susceptible to changes in the tag stoichiometry especially during the complex sample preparation procedures, which may result in inaccurate quantitative results.[29]

Peptides and proteins that do not contain naturally detectable elements can be chemical derivatized with metals, or radionuclides, in order to make them "visible" and quantifiable and to allow sensitive and specific detection of the analytes. In order to quantify proteins on the basis of their metal content, specific factors must be considered: (i) the metal-protein stoichiometry has to be known, and (ii) the thermodynamic and kinetic stability of the protein has to be guaranteed.

The aim of this review is to focus on the determination and quantitation of proteins containing a naturally occurring heteroelement (such as Se, Fe, etc.) or after labeling with a metal(loid)s (such as lanthanides, Ru, Hg, I, etc.) using element-specific hyphenated techniques.

Hyphenated techniques for proteins / peptides quantitation.

Quantitation and bio-speciation studies require the separation step to distinguish each single species. Among the steps required for proteome analysis (sample preparation, separation of the proteins or the digested peptides, identification of the proteins and data processing of the huge information generated) separation is the most challenging due to the high complexity of protein/peptide samples.

The increasing progress in separation techniques for purification and isolation coupled to the ultrasensitive elemental detectors are the basis of hyphenated analytical methods (Figure 3).

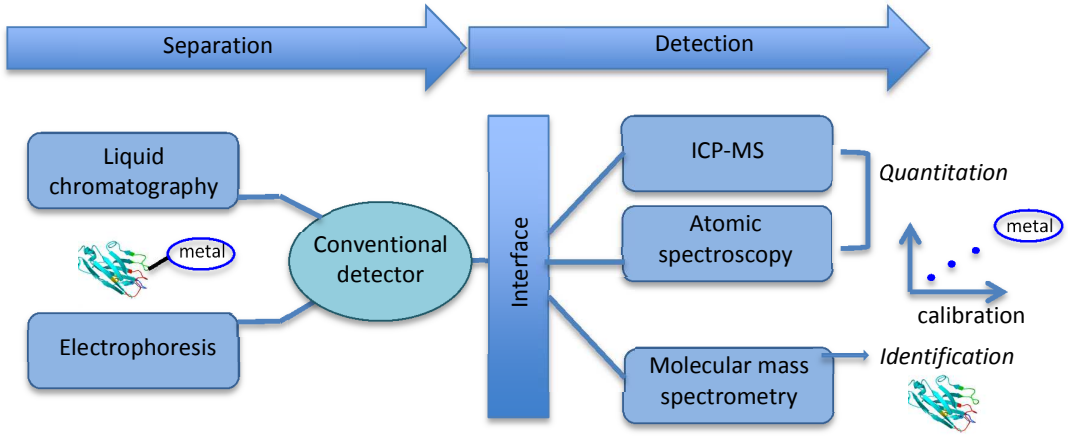


Fig. 3 Schematic workflow showing the information obtainable by hyphenated techniques for the analysis of heteroelement containing/tagged proteins.

For the analysis of metalloproteins and metal-tag proteins the configuration mainly considered is the on-line hyphenation of a separation technique (high-performance liquid chromatography, HPLC, or capillary electrophoresis, CE) with an element (moiety, species)-specific detector (in general atomic spectrometry or ICP-MS).

Different modes of HPLC (reversed phase, ion exchange, size exclusion, affinity, hydrophobic interaction) and CE (capillary zone, isoelectric focusing, isotachophoresis, affinity, micellar) or a

combination of both techniques in hyphenated multidimensional formats can be used for the fractionation and/or separation of peptides. The choice of the hyphenated technique depends on the characteristic of the system under investigation and on the quantity of sample available. When the target species have similar physico-chemical properties, the separation component of the hyphenated system becomes very important. For complex biological matrices, it may even be necessary to combine in series two or more separation steps. The choice of the detector becomes crucial when the concentration of analyte species in the sample is very small and low limits of detection are required.

Electrophoretic techniques

Electrophoresis was first introduced in 1930 by Arne Tiselius, a Swedish chemist.[33] Electrophoresis is generally employed to characterize a biological system and to select specific protein bands for sequencing and identification. This technique involves the separation of charged species under the influence of an applied electric field. In proteins, the charged species can be produced by dissociation of carboxylic groups or protonation of amino groups, or by uniform coating of proteins with an anionic surfactant, such as sodium dodecylsulfate (SDS). As a result, SDS imparts the same free-solution mobility to all proteins, regardless of their identity, so their separation is controlled only by molecular weight. The charged species moves in a (semi)liquid medium, which serves as a conducting medium for the generated electric current and it is supported by an inert substance (paper or a semi-solid gel), where the migration velocity is an important factor. Polyacrylamide is the most common gel support matrix and it is obtained from the polymerization of monomeric acrylamide (polyacrylamide gel electrophoresis, PAGE).

Gel electrophoresis with its various formats, such as PAGE, isoelectric focusing (IEF) and immunoelectrophoresis, offers a number of attractive features for the characterization of metalloproteins. Protein separation by gel electrophoresis can be performed in one- or two-dimensions. One-dimension SDS-PAGE may not guarantee the complete dissociation of

multimeric proteins into their subunits and it may give rise to several labeled bands originating from the same compound, so an orthogonal separation mechanism (2-D) is required. Two-D separations do not require treatment with SDS to modify the sample and the analytes are separated in two stages on the basis of different parameters (e.g. size, charge or hydrophobicity). Proteins are separated in the gradient gel according to the mass-to-charge ratio in the first dimension. A second dimension can be added by isoelectric focusing using pH gradients and separating proteins by their isoelectric points.

The amount of proteins concentrated in the tiny gel volumes is very small and hardly accessible to standard analytical chemical methods. The first approaches for metal detection in gel are autoradiography, Instrumental Neutron Activation Analysis (INAA), particle induced x-ray emission (PIXE), but currently laser ablation-ICP-MS is the most common.

Another important approach involving electrophoresis is capillary electrophoresis (CE), which has been applied to protein analyses in the last two decades and has become an important separation tool for chemists and life scientists. Capillary electrophoresis is a high speed and high-resolution separation technique, which requires exceptionally small sample volumes (0.1–10 nL, in contrast to gel electrophoresis, which requires samples in the μL range). CE can be easily hyphenated with different detection techniques.

Whereas in CE the separation of small peptides often is relatively straightforward and well understood, it appears that no single strategy is applicable for large peptides and proteins. As might be expected, this is due largely to the wide diversity and complexity associated with these biomolecules. Thus, different strategies often work for different protein separation problems, hence requiring different CE separation modes.

Capillaries can be filled with a replaceable or fixed solid gel (capillary gel electrophoresis) or with a replaceable running buffer (capillary zone electrophoresis, CZE).[34] CZE has been suggested as a new tool for separation and quantitation of proteins from serum, urine, cerebrospinal fluid, synovial fluid and saliva. CZE combines the separation principles of conventional electrophoresis with the

advanced instrumental design of high-performance liquid chromatography and capillary technology.[34] The sample is introduced using pressure into a buffer-filled fused silica capillary (internal diameter 20 to 200 μm and lengths of 10-100 cm), either electrokinetically or hydrodynamically. For separation, both ends of the capillary are placed into a buffer solution that contains the electrodes and a high voltage is applied to the system. The applied voltage induces the migration through the capillary of the analytes and through the detector window.[34] The walls of untreated fused silica capillaries are negatively charged in contact with aqueous solution due to the ionisation of surface silanol groups (pI=1.5). The negatively charged silica surface attracts cations from the buffer, creating an electrical double layer. When a voltage is applied across the capillary, cations in the diffuse portion of the double layer migrate in the direction of the cathode, carrying water with them. The final result of the protein separation is affected by capillary length and diameter, buffer composition and pH, sample injection mode, capillary thermostating (Joule heat), separation temperature, electroosmotic flow, solute concentration effects, wall-solute interactions and applied field.

CZE has been suggested as an alternative for the conventional agarose gel electrophoresis in separating human serum proteins since it allows fast protein separation with good resolution, using only small amounts of sample. The main problem of the protein separation in body fluids with CZE is the effects of sample matrix composition, because the migration time of the same proteins varies significantly depending on the nature of the matrix.[35] Electropherograms are consequently difficult to compare and the peak identification is uncertain.

Olesik et al. designed the first interface between CZE and ICP-MS.[36] After this various interfaces have been described.[37,38,39]

Although initial separations of proteomic samples have traditionally been accomplished by electrophoretic techniques, chromatographic separations of intact proteins are becoming attractive alternatives. Electrophoresis limitations are due to the difficulty of the automation of 2-D electrophoresis, low sensitivity, bias against categories of proteins (e.g., membrane proteins) and

low dynamic range.[40] A good overview of methods and protocols for proteins gel electrophoresis has been published in a book.[41,42]

High-performance liquid chromatography

HPLC had a remarkable development in the past two decades. Liquid chromatography has been, traditionally, the basis of most methods for the separation of proteins. Size-exclusion (SE, ion exchange (IE) and reversed-phase (RP) chromatography are the principal HPLC separation techniques used for protein analysis. The separation mechanism of SE chromatography is based on differences in size and tridimensional configuration of proteins.[43] Differences in the global charge of proteins at a certain pH allow the use of ion exchange (IE) chromatography in both cationic and anionic modes [44,45], while RP chromatography separates proteins on the basis of their different hydrophobicity given by the different polarity of the 20 essential amino acids.[46] Moreover, the presence of specific prosthetic groups permits the separation of protein isoforms with affinity chromatography.[47,48]

Size-exclusion chromatography

The application of a hyphenated technique to the analysis of metal-binding proteins requires that column stationary or mobile phases do not compete with ligands, displacing them from the analyte-metal complex. From this point of view SEC is an excellent chromatographic option for the separation of metal-binding proteins, because this chromatography technique operates in mild conditions. The most widely used types of packing materials are cross-linked dextrans (Sephadex), crosslinked agarose (Sepharose), cross-linked polyacrylamide (Biogel), cross-linked allyldextran (Sephacryl), controlled pore glass beads and silica.

However, silica and organic polymer stationary phases tend to absorb proteins through ionic and hydrophobic interactions, respectively, giving non-ideal SEC behaviour.[43,49] So, while the choice of buffer does not affect resolution, salts (e.g. 25-150 mM NaCl) are usually used to reduce the

electrostatic interactions between proteins and the stationary phase, thus suppressing the residual silanol activity of the column packing. The selected buffer conditions should also avoid inactivation or precipitation, and maintain the stability of biomolecules and target proteins activity and it should be compatible with the detection technique. In the particular case of metal-binding proteins, weak alkaline eluents are recommended to avoid dissociation of metals.[43]

SE-HPLC shows a good compatibility with ICP and atomic spectrometry both in terms of flow rates (0.7-1 mL min^{-1}) and mobile phase composition. Up to 30 m mol L^{-1} Tris–HCl was found to be well tolerated ICP-MS applications whereas 20 m mol L^{-1} formate or acetate buffer in 10% methanol is acceptable for ESI MS.

SE-HPLC has the following advantages: i) good separation of proteins from small molecules with a minimal volume of eluate, ii) the use of aqueous eluent phase (that preserves the biological activity of proteins and is tolerated in flame atomic spectroscopy), iii) minimal interaction between proteins and the stationary phase.[50]

However, the number of theoretical plates in SEC is small and in most cases only 6–8 peaks can be obtained because SEC can resolve only more than a 1.5–2-fold difference in molecular weight. Furthermore, the coupling with ICP-MS is difficult because of the presence of salt in the eluent. The lacks of resolution of this technique is a frequently encountered problems, e.g. in the separation of serum selenoproteins [51] or in the separation of human albumin and transferrin.[52]

Ion-exchange chromatography

Ion-exchange chromatography is based on the reversible interaction between a charged molecule and an oppositely charged chromatography medium. Several side-chain groups of the amino acid residues in proteins (e.g. lysine or glutamic acid) as well as the N-terminal amino and C-terminal carboxyl groups are involved in proteolytic equilibria. The choice of the optimal ion exchanger and separation conditions allows the separation with high resolution of biomolecules with even small

differences in net surface charge. In biological field, this technique has been widely used for the fractionation of metallothioneins and serum proteins.[43]

Two common weak exchangers used for protein separation are carboxymethyl (that at neutral pH is ionized as $-CH_2OCH_2COO^-$ so it is a weak cation exchanger) and diethylaminoethyl group (positively charged at neutral pH, so it is a weak anion exchanger). Two strong exchangers are quarternary amine, which have a non-titratable positive charge, and the sulphonyl group ($-SO_3^-$).

The Sepharose types are particularly useful for the separation of high molecular weight proteins.

Both the immobilized charged groups and the backbone structures of the stationary phase are important in the separation of proteins by IEC, because they may interact with proteins, giving unspecific binding. Cellulose (carboxymethylcellulose and diethylaminoethyl-cellulose) is the most traditional material but also dextran, agarose, silica and polymeric materials have been used as backbone structures.

Elution is usually performed by increasing salt concentration or changing pH in a gradient, or stepwise. The most common salt is NaCl, but other salts can also be used. The concentration of buffers used in anion-exchange (AE) chromatography of proteins often exceeds 0.1 mol L^{-1}, and their use may result in variations of ICP-MS sensitivity because of the clogging of the nebulizer, sampler and skimmer cones, while the percentage of organic solvents usually is not problematic for plasma stabilization. Cation exchange could be more feasible for the coupling with ICP-MS because several millimolar pyridine-formate buffer [53] or citric acid is sufficient to achieve an optimal separation.[54]

Reverse phase chromatography

Reverse phase (RP)-HPLC is based on proteins solubility and hydrophobicity. All peptides and proteins carry a mix of hydrophilic and hydrophobic amino acids, but those with high net hydrophobicity are able to participate in hydrophobic interactions with the stationary phase. The stationary phase is packed with silica containing covalently bounded silyl ethers with non-polar

alkyl groups, typically C_8 or C_{18}, which create a hydrophobic stationary phase. However, as big proteins are more hydrophobic, it is convenient to use stationary phases with short alkyl chains (C_2, C_4) to avoid losses of protein due to their irreversible binding to the solid phase.[49]

Conversely, the mobile phase contains relatively polar organic solvents such as methanol, butanol, isopropanol or acetonitrile. The use of ion-pairing reagents in the mobile phase (ion-interaction chromatography) permits to extend the application of RP-HPLC to ionic analytes.

As polar solvents often induce protein denaturation and loss of metals eventually bounded, RPC in general is used for the analysis of small and stable proteins.

The main disadvantages of RP-HPLC are long chromatographic runs (40–60 min) and the need of modifying the normal working configuration of ICP-MS detector. The introduction of an organic solvent in percentages > 20–30% methanol and 10% acetonitrile at 1 ml/min into the ICP-MS affects negatively the ICP stability, leading to a decrease in signal intensity, and to the deposition of carbon on the cones. This issue can be solved by removing solvent vapor, using a cooled spray chamber or a membrane desolvator accompanied by the addition of oxygen to the plasma gas and the use of platinum cones. Moreover, as the organic solvents modify the plasma ionization conditions, their concentration has a significant effect on the ICP-MS signal intensity. The use of capillary HPLC (4 μl min^{-1} flow rate) and nano HPLC (200 nL min^{-1}flow rate) is fundamental to control organics-rich mobile phases. The introduction of HPLC eluent at low flow rates allows the introduction up to 100% organic solvent without cooling the spray chamber or the need of oxygen addition.[55]

Affinity and hydrophobic interaction chromatography are also employed for protein separation and they can be hyphenated with atomic spectrometric techniques, but not with mass spectrometric detector because of the high concentration of salts typically used in the eluent phase.

An application of affinity chromatography is the immobilized metal ion affinity chromatography (IMAC), a highly versatile separation method based on interfacial interactions between biopolymers

in solution and metal ions fixed to a solid support, usually a hydrophilic cross-linked polymer. IMAC is commonly used for fractionation of metalloproteins dependent on their differential binding affinities of the surface exposed amino acids (imidazole, Trp and Cys) towards immobilized metal ion. Metal depleted samples are loaded on an IMAC column/chip saturated with the metal of interest, and proteins with affinity to the metal are recovered and can be analyzed by any of the classical proteomics methods. However, IMAC provides information on the presence of metal-binding sites in proteins but it does not detect eventual endogenous metals. The other drawback of the IMAC technique is that metalloproteins with a high metal affinity site do not interact with column/chip stationary phase and are not detected as the metal sites are already occupied.[56]

Metals specific detectors

Speciation analysis performed by hyphenated techniques is fundamental in analytical science. The added value provided by speciation analysis compared to classical elemental analysis is not only of academic interest, but it is the key to answer important biological questions.

Flame atomic absorption spectroscopy (AAS), atomic fluorescence spectroscopy (AFS), inductively coupled plasma (ICP)-optical emission spectroscopy (OES) and ICP-MS are the major element-specific detectors used in chromatography (Figure 4).

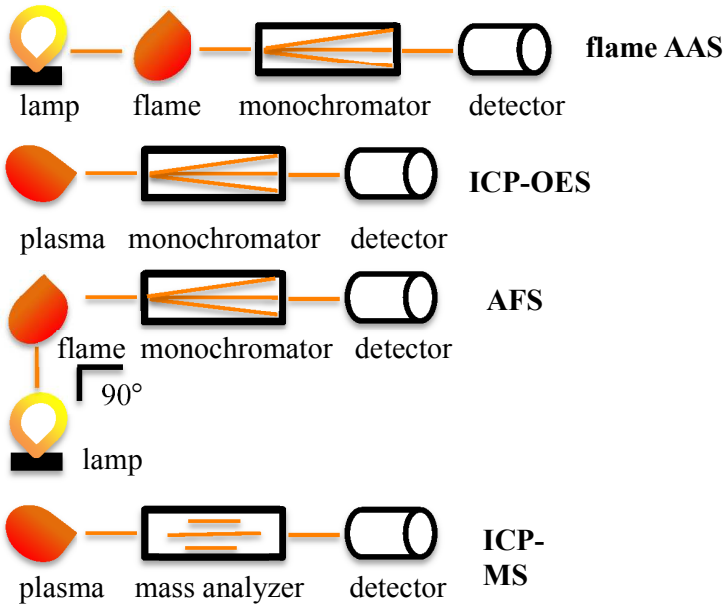


Fig. 4. Schematics of the main element-specific detectors.

The main issues related to the interfacing of a separative technique with a detector are: i) the concentrations of the mobile phase eluting from the separative system (salts and organic solvents) and ii) the efficiency of the sample transfer, which includes the optimization of flow rates, peak broadening and dead volume.

AAS

Flame atomic absorption spectrometry is the atomic spectrometric technique most widely used for trace element determination. This is due to its easy set up and low running costs, its robustness and few interference issues in the determination of trace elements. AAS is not a truly multi-element technique, but some instruments guarantee the simultaneous analysis of about four elements, which is satisfactory for a number of practical applications. Flame AAS can be straightforwardly coupled with HPLC, and it is compatible both with its flow rates and mobile phase composition (including organic solvent). The sensitivity of flame atomic absorption spectrometry measurements can be

improved significantly by increasing the efficiency of aerosol generation/transport and prolonging the residence time of the free analyte atoms in optical path. For this reason, several advanced interfaces based on thermospray have been proposed. In the thermospray interface the liquid is transported into the flame furnace by a low or high-pressure pump through a very hot and simple ceramic capillary tip.[57]

HPLC-AAS was the first hyphenated technique employed for the determination of metal–protein complexes. The major fields of applications include the detection of complexes with metals that give intense response in AAS (Cd, Zn, Cu) or of species that can be converted on-line into volatile hydrides (As, Se, Cd)[58, 59].

AFS

Atomic fluorescence spectrometry represents a suitable alternative to the other atomic and mass spectrometric techniques. AFS is more sensitive than AAS and has a sensitivity similar to ICP-MS (LOD < 1 μg L^{-1}) and dynamic linear range between mg L^{-1} to μg L^{-1} for arsenic, selenium and mercury analysis.[60] Further advantages are its simplicity and lower acquisition and running costs.

AFS spectrometers are commonly based on the use of non-dispersive instruments, equipped with a discharge hollow cathode lamps as excitation radiation source and often with chemical vapour generation systems. Volatile species of As, Se and Hg, for example, obtained after hydride or vapor generation, are stripped from the solution and delivered by an argon flow to a gas–liquid separator and then atomized and detected in an argon–hydrogen diffusion flame.

For those chemical species that do not readily form volatile species, such as organometallic species, additional online derivatization steps are needed (e.g. photo-oxidation, pyrolysis or microwave digestion) before hydride or vapor generation.

Quenching reactions and interferences are the drawbacks of atomic fluorescence spectrometry. Quenching occurs when excited atoms collide with other molecules in the atomisation sources. An

additional disadvantage of ‘‘generic’’ AFS is source scatter and atomizer emission that cause spectral interferences. These are minimal when hydride or vapor generation are used.[60]

ICP-OES

ICP-OES is a powerful analytical tool for the detection of elements. Compared to AAS techniques, ICP-OES enjoys a higher atomization temperature, a more inert environment, and the natural ability to provide simultaneous determinations for up to 70 elements. This makes the ICP less susceptible to matrix interferences, and better able to correct for them when they occur.[61]

ICP-OES offers detection limits at the 1 ng ml^{-1} concentration level (continuous infusion), which translates into 10–100 ng ml^{-1} for a transient signal of an analyte eluted from the column.[62] Because of the absence of cones (in the plasma radial configuration) or for the larger orifices than the cones used in ICP-MS, ICP-OES tolerates complex matrices in terms of salt concentration and, because of higher rf power, organic solvents. Instruments equipped with a polychromator offer the advantage of multi-element analysis.

ICP-MS

ICP-MS provides excellent analytical characteristics for elemental detection in clinical biomarkers containing heteroatoms, including: (1) elevated sensitivity (detection limits between ng g^{-1} and pg g^{-1}) and specificity to the heteroatom; (2) multielemental capabilities to simultaneously monitor different metals and heteroatoms associated to a protein; (3) direct isotopic information and quantitations (by isotope dilution analysis); (4) versatility and easy coupling to separation techniques with the aim of monitoring the metal or metalloid associated to a certain protein; (5) minimal matrix effects; (6) capability of up to 8 magnitudes of linear dynamic range.[28]

It is not surprisingly that liquid chromatography and ICP-MS is the most common hyphenated system employed for speciation analysis. About 1/3 of all publications are related to LC-ICP-MS.

The possibility of interfacing HPLC to ICP-MS is strongly dependent on the type of nebulizer employed as sample introduction device. In the simplest form the interface is a conventional pneumatic nebulizer, i.e. a concentric nebulizer (using 1 ml /min for regular bore HPLC flow) connecting the outlet of the column to the liquid sample inlet using an inert polymeric or stainless steel tubing. The length of the tubing has to be minimized to avoid peak broadening. The concentric nebulizer is not so different from the nebulizer described by Gouy at the end of the nineteenth century[63], but, to overcome the limitations of this interface (i.e. the low transfer efficiency ranging between 1-5%, losses in the spray chamber and, thus, lower sensitivity), a significant number of alternative designs have been published.

The match of the optimum column flow with the optimum nebulizer flow is critical to achieve both efficient separation and sample nebulization. Any nebulizer has a range of flows over which it produces the highest proportion of fine droplets in the aerosol. This is critical since fine droplets are more efficiently transported through the spray chamber, atomized and ionized in the plasma. Typical HPLC flows ranging from 100 μL/min to 1 mL/min are compatible with conventional concentric nebulizers, either in glass, quartz, or fluoropolymer. At significantly higher flows, part of the sample has to be split off prior to the nebulizer. In general, ICP-MS requires more diluted buffers and tolerates lower concentrations of organic solvents with respect to ICP-OES.[64]

Conventional nebulizers operate at typical sample flow rates of 0.5–2 mL min^{-1}. This makes it necessary to have a sample volume available for the analysis ranging from about 1 to 10 mL. The simplest proposed solutions for analysis of micro-samples has been to decrease the liquid flow rate down to 10–300 μL min^{-1}. However, because with conventional pneumatic nebulizers working with these conditions leads to a dramatic loss of sensitivity and an increase in the washout times, new nebulizers have been purposely developed. The development of micro-nebulizers (e.g. direct injection nebulizer, DIN, hydraulic high pressure nebulizers, HHPN, high efficiency nebulizer, HEN, Micro Mist nebulizer, MMN, PFA nebulizer) has increased the use of narrow bore columns

minimizing the mobile phase introduced into the ICP-MS. So-called micronebulizers are optimized to work at a solution delivery rate below 200–300 mL min^{-1}, usually in the range 20–100 μL min^{-1}. The direct injection nebulizer (DIN) interface is a microconcentric pneumatic nebulizer without spray chamber, which nebulizes the sample directly into the central channel of the torch. This interface offers several advantages, such as low dead volume, minimization of post-column peak broadening and fast sample washout with minimal memory effects.[65,46]

In the hydraulic high pressure nebulizers (HHPN) interface the liquid to be nebulized is pressed through a very fine nozzle of Pt/Ir (20 μm inner diameter) resulting in an aerosol jet with a diameter of a few tenths of a millimetre, which is converted into an aerosol cloud on a converter ball. With the HHPN interface the sensitivity is enhanced by one order of magnitude and the tolerance to high salt concentrations is higher than the pneumatic nebulizer.[66]

Laser ablation (LA) coupled to ICP-MS is an apparently cheap and competitive alternative detection technique coupled to gel electrophoresis, which is attractive for the scanning of gels with heteroatom-containing proteins. This technique pioneered by Neilsen et al. [67] consists of the ablation of the analyte with a laser beam guided over the gel within an electrophoretic lane. The ablated analytes are swept into the ICP by a continuous stream of argon, and MS analyses the ions. As a result, an electropherogram is obtained in which the quantity of a given element is a function of its position in the gel. Quantitation by LA-ICP-MS is a fast and robust technology, since the signal is theoretically directly proportional to the quantity of the analyte element in the gel and eliminates the quantitation problems related to the recovery of the protein from the gel.

Although imaging LA-ICP-MS methods have been established for the distribution of metals and non-metals in sections of biological tissue, quantitative measurements that use LA are not enough accurate so far as much the hyphenation of HPLC with ICP-MS. The technique is prone to elemental fractionation and other matrix effects so that accurate quantitation still remains difficult. Several approaches for quantitation have been proposed in several works in recent years.[68]

In his pioneering work Neilsen [67] proposed the use of element-doped gels as standards for external calibration, but, despite a good calibration precision (6% RSD), this approach did not take into account the possibility of inhomogeneous distribution of the analytes within the gel.

Also, the use of liquid standards has been suggested, but the different characteristics of a nebulised solution and the laser-generated aerosol in ICP leads to another significant source of uncertainty.

Another approach frequently used in LA is to use the ion signal of a matrix element as internal standard. However, the internal standard and the analyte have to enter the ICP in the same form, which might be unknown.

For those elements that have at least two or more stable isotopes, accuracy and precision of the quantitation can be considerably improved by isotope dilution analysis (IDA) coupled with ICP-MS. The two principal approaches of IDA include speciated IDA (in which a species-specific spike is used) and non-speciated IDA (when the isotopic spike ignores the speciation of the analyte compounds).[69]

In the speciated IDA an isotopically labeled analyte species is added to the sample before any sample treatments and/or chromatographic separations incomplete recoveries and matrix effects can be corrected. The use of this approach is limited by the availability of labeled analyte molecules and the equilibration of the spike with the analyte species.

For most of biomolecules, the isotopically labeled calibration standards are unavailable and the only possibility allowing the improvement of precision and accuracy is the continuous introduction of an isotopically enriched, species-unspecific spike solution after treatment and/or separation step. However, quantitation by this external calibration gives rise to problems as a result of matrix-induced differences in detector sensitivity between standards and samples.

Species-specific isotope dilution analysis (SS-IDA), that allows the correction of multiple matrix effects, has been proposed for the first time by Kingston in 1990s [70] and has been applied to proteomic studies by Deitrich et al.. They use this approach for the absolute quantitation of superoxide dismutase (SOD) by GE-LA-ICP-MS using ^{65}Cu and ^{68}Zn isotopically enriched SOD as

a spike. Although unsatisfactory LOD and recoveries were achieved, this work demonstrated the potentiality of the method for protein quantitation and pointed out some important issues such as the cross contamination in 1D-PAGE gels or the stability of the metal– protein interactions, two factors that make the isotope dilution unusable.[71]

The use of ICP-MS with IDA as a quantitation methodology has been successfully applied for the accurate determination of other metalloproteins such as transferrin [72] and haemoglobin [73] in biological fluids. In all these cases, the concentration calculations are based on the determination of the metal associated to the protein by IDA after chromatographic separation and assessing the preservation of the metal: protein stoichiometry during the sample handling and chromatography.

A thorough discussion of the IDA method is beyond the scope of this article. Further details are reported in the review of Bettmer.[74]

Table 1 shows the principal advantages and disadvantages of the techniques described here for the quantitation of metals.

Table 1. Typical features for the major metal detection techniques.

Metal detection technique	Advantages	Drawbacks	Sensitivity	Dynamic range
FAAS	Easy to use Fast Cheap Very compact instrument Good performance Robust interface	Moderate detection limits Element limitations 1-10 elements per determination No screening ability Burner-nebulizer system is a relatively inefficient sampling device	100 – 1 μg L^{-1}	10^3
ICP-OES	Easy to use Multi-element Few chemical interferences Robust interface Good screening abilities Solid and organic samples	Moderate/low detection limits Possible spectral interferences Some element limitations	100 – 0.1 μg L^{-1} (radial) 10 – 0.01 μg L^{-1} (axial)	10^6
AFS	High degree of element specificity Relatively free from interferences Separation and pre-concentration of the analytes with vapor/hydride generation Lower acquisition and running costs	Quenching Interferences Source scatter Atomizer emission	0.1 – 0.01 μg L^{-1}	10^3 – 10^7 depending on the source
ICP-MS	Excellent detection limits Multi-element Wide dynamic range Isotopic measurements Fast semiquantitative screening LA-ICP-MS hyphenation (solids)	Some method development skill required Expensive Some spectral interferences Limited to <0.2% dissolved solids	1 – 0.0001 μg L^{-1}	10^8

Although ICP-MS detection is used for quantitation of biological analytes characterized by the presence of natural or added metal-containing fractions, molecular mass spectrometry (MS) is still the main analytical tool used in proteomics for the large-scale identification of proteins. The coupling of chromatographic separation with molecular mass spectrometry has opened the possibility of high-throughput peptide mapping, protein sequencing, and the determination of post-translational modifications of proteins. The availability of different fragmentation approach in MS/MS experiments, such as CID (collision-induced dissociation), ECD (electron capture dissociation) or ETD (electron transfer dissociation), provided highly specific structural information.[75]

ESI-MS is the most popular method for protein identification because of its powerful MS/MS ability and the easy coupling with liquid chromatography. On the other hand, MALDI mass spectrometry offers higher tolerance toward sample contaminants (such as buffers, salts and surfactants), higher speed of analysis and lower sample consumption for each analysis. On-line coupling of MALDI with liquid separations is relatively challenging as this system requires the continuous delivery of separation effluent to the MALDI interface and the simultaneous co-crystallization of the analyte and matrix. The off-line coupling of MALDI to LC is easier and involves the collection of the eluted fractions from the separation column and their deposition on the MALDI target.[76]

In the case of labeled proteins, it must be taken into accounts that the labeling may affect the mass and the charge of the proteins. As a consequence, smaller peptides might appear at higher m/z values in ESI-MS, while larger peptides, especially with multiple labels, become too heavy and less suitable for protonation, falling out of the measuring range.[77] On the other hand, labeled protein with metals that have three or more isotopes leads to very characteristic cluster that allow, especially with high resolution mass spectrometry, to identify with high accuracy the protein/peptide.

Molecular mass spectrometry techniques for proteome analysis has been reviewed in an excellent work by Aebersold and Mann.[78]

Proteins quantitation by detection of endogenous metal(loid)s.

Approximately one-half of all known protein crystal structures in the protein data bank (PDB, http://www.rcsb.org) contains metal ion cofactors, which play vital roles in charge balance, structure, and function.[79] Examination of the PDB shows that Zn is the most abundant, while Fe, Mg and Ca are also frequently observed, associated with proteins as ferritin (Fe, Cu, Zn), β-amylase (Cu), alcohol dehydrogenase (Zn), carbonic anhydrase (Cu, Zn) and others.[80]

Proteins contain several functional groups in the side-chains of amino acids that are particularly well suited for metal coordination. They include cysteine ($–CH_2SH$) and methionine ($–CH_2CH_2SCH_3$) that bind metals with sulphur affinity (Cd, Cu, Zn), and histidine, whose nitrogen atoms is available for coordination after deprotonation (e.g., Cu, Zn in superoxide dismutase). Peptide-complexed metal ions are known to perform a wide variety of essential specific functions (regulatory, storage, catalytic, transport) associated with life processes.[81]

Selenium.

Although selenium is not a metal, it is a heteroatom and the quantitation of selenoproteins is an important challenge in the bioanalytical field.

The human selenoproteome consists of 17 selenoprotein families, some with multiple genes with similar functions. The major Se-containing proteins are selenoprotein P (SelP), sometimes used as a biochemical marker of selenium status, selenoenzymes such as several glutathione peroxidases (GPx), selenoalbumin (SeAlb), thioredoxin reductases (TrxR) and iodothyronine deiodinases (DIO).[82]

The quantitative concentrations of specific selenium-tagged proteins provide significant information concerning physiological changes, and the relationship between the level of specific selenium-tagged proteins and diseases (as hypertension, coronary heart disease, cancer, asthma and diabetes [83]) has been widely recognized.[84]

For this reason it is imperative to establish robust, accurate and straightforward analytical approaches suitable for the routine speciation analysis and quantitation of selenoproteins in human serum and plasma. This topic still remains a challenge in physiological research and clinical diagnosis.[85]

The principal analytical approach developed for the identification and determination of selenoproteins is based on affinity and size exclusion chromatography.[43]

The main column packing materials for affinity chromatography are based on a heparin–Sepharose or Blue 2-Sepharose, a group of specific adsorbents widely used for serum proteins. Akesson and Martensson showed that heparin interacts with some Se-containing proteins[86] and, after this, they separated plasma selenoproteins into heparin-binding and non-heparin binding fractions.[87] Following this, Deagen et al. succeeded in separating plasma Se-containing proteins into three components using in tandem two affinity columns, a heparin-Sepharose and a reactive blue 2-Sepharose column.[88]

The main drawback of affinity-HPLC coupled with ICP-MS arise from the poor retention of GPx and hence its co-elution with not retained species, such as Cl^- and Br^- (present at high levels in serum), which lead to serious spectral interferences, such as $^{40}Ar^{37}Cl$ on ^{77}Se, $^{79}Br^{1}H$ on ^{80}Se and $^{81}Br^{1}H$ on ^{82}Se. The control of these interferences can be performed offline by serum clean-up using anion exchange solid phase extraction (SPE) and multi-affinity media, or on-line by resolving selenoproteins from Br/Cl by two dimensional HPLC separation employing anion exchange – affinity HPLC (AE-AF-HPLC) before ICP-MS detection.[89] However, these approaches introduce additional steps in the analytical process hence increasing the time of analysis and the uncertainty

sources, and would be preferable to eliminate the polyatomic interferences with instruments equipped with a reaction cell or using a high-resolution ICP-MS.

Size exclusion-HPLC is also employed for the analysis of selenoproteins. However, it lacks of an adequate resolution, it cannot allow the separation of the major serum selenoproteins, and the large dilution factor limits the sensitivity of this technique.

Jitaru et al. [89] quantified selenoproteins in human serum using microbore affinity-HPLC hyphenated to ICP-sector field-MS coupled with on-line (post column) isotope dilution. They compared the method with external calibration by using Se-L-cystine (SeCys) standards and assessed the method accuracy for the determination of total Se-protein by the analysis of a human serum reference material certified for total Se content. This method enables the determination of selenoproteins in 5 µL of human serum.

Shigeta et al. [90] reported a method based on micro-affinity chromatography coupled with low flow ICP-MS, which enabled the separation and analysis of selenoproteins in sub-µl samples.

Table 2 shows the most recent chromatographic and detection conditions proposed for the separation of the main Se-containing proteins in plasma.

Table 2. Analytical methods for the species-selective analysis of selenoproteins/peptides by hyphenated techniques with element-selective detection.

Analyte	Sample type	Separation technique	Detector	Ref.
SeCys GPx SelP SelAlb	Human serum	Affinity HPLC	ICP - sector field-MS in high resolution mode	89
GPx SelP SelAlb	Human serum reference material (BCR-637)	Affinity HPLC	ICP-quadrupole-MS	91
SelP isoforms	Human serum reference material (SRM 1950)	SDS-PAGE Nano reversed phase HPLC	ICP-MS ESI-linear triple quadrupole-MS	92
GPx SelP SelAlb Two unknown selenospecies	Human plasma	Anion exchange HPLC	ICP-dynamic reaction cell-quadrupole-MS	85
GPx SelP SelAlb	Human plasma standard reference material (SRM 1950)	Affinity HPLC Nano-reversed phase HPLC	LA-ICP MS ESI-LTQ ion trap-MS	93
SelP GPx3	Human plasma candidate standard reference material (SRM 1950)	SDS-PAGE Electroblotting onto PVDF membrane Nano reversed phase HPLC	LA-ICP-MS ESI-LTQ ion trap-MS	94
GPx	Selenium-yeast candidate reference material	SDS-PAGE	Electrothermal vaporization-ICP MS	95
Selenomethionine	Yeast extracts	Gas chromatography	ICP-sector field-MS	96
Selenomethionine Selenocysteine	Human serum	Size exclusion HPLC Capillary reversed phase HPLC	ICP-octapole reaction cell-MS	97
SelP	Sub-μL samples of human plasma	Size exclusion HPLC	Low flow ICP-MS	90

Analyte	Sample type	Separation technique	Detector	Ref.
Mixture with more than 30 selenopeptides	Selenized yeast extract	Affinity HPLC Capillary reversed phase HPLC	ICP-collision cell-MS	55
GPx SelP SelAlb	Human plasma	Size exclusion HPLC Capillary reversed phase HPLC SDS-PAGE	ICP-MS	98
GPx SelP SelAlb	Human serum samples and reference materials	Double affinity followed by size exclusion HPLC	ICP-MS	83
Selenomethionyl calmodulin	Protein obtained by heterologous expression in *Escherichia coli*	Reversed phase nano HPLC	ICP-octapole reaction cell-MS ESI-quadrupole/time of flight-MS	99
SelP	Human and mouse plasma	Affinity HPLC Size exclusion HPLC	ICP-MS	100
GPx SelP SelAlb	Human serum	Anion exchange HPLC Affinity HPLC	ICP-octapole reaction cell-MS	101
GPx Formate dehydrogenase selenoprotein	Bacterial cultures of *Desulfococcus multivorans* and *Escherichia coli*	SDS-PAGE Size exclusion HPLC	LA-ICP-MS ICP-MS	102
GPx3 SelP SelAlb	Human serum form patients with colorectal cancer	Anion exchange HPLC Double affinity HPLC	ICP-quadrupole-MS	103
Selenomethionine	Selenium-enriched yeast	Gas chromatography	MS	104
Se-rich glutenins	Wheat	Isoelectric focusing separation 1-D SDS-PAGE IEF/SDS-PAGE 2D gel electrophoresis Reversed phase HPLC	LA-ICP-MS ICP-MS ESI-linear triple quadrupole/Orbitrap-MS	105

Iron.

Iron is an essential element for life, playing a vital catalytic and structural role in numerous metalloproteins. Iron is also toxic to cells in its free form and in excessive amounts.[106] Under physiological conditions, indeed, ferrous ion is highly insoluble and rapidly auto-oxidizes to ferric iron, catalyzing the formation of highly damaging oxygen radicals able to attack cellular membranes, proteins and DNA.[107]

Under physiological conditions the majority of iron is bound to proteins. The main iron proteins in humans are globins, hemoglobin and myoglobin, followed by ferritins, and then by a variety of heme and iron−sulfur proteins where iron cofactors are directly bound to protein, e.g., in ribonucleotide reductases.

The ICP-MS allows a very sensitive and isotope-specific analysis of Fe-proteins, without using radioactive tracers. It is known that accuracy and precision for the determination of the four isotopes of iron (^{54}Fe 5.8%, ^{56}Fe 91.7%, ^{57}Fe 2.14%, ^{58}Fe 0.31%) by ICP-MS with a conventional quadrupole analyzer is limited by polyatomic interferences coming from the argon, the atmospheric gases and the biological material.[108] However, the use of double focusing sector field, ICP-(SF)-MS, multicollector, MC-ICP-MS, or collision/reaction cell, ICP-(ORS)-MS makes easier the elimination of such interferences and provides robust, high sensitivity and specific iron detection.[106]

Quantitation of Fe-proteins is mainly conducted using post-column isotope dilution – ICP-MS after their separation by ion exchange or size exclusion HPLC.

Table 3 shows the most recent chromatographic and detection conditions proposed for the quantitation of the main Fe-containing proteins in various types of samples.

Table 3. Analytical methods for the species-selective analysis of iron-containing proteins by hyphenated techniques with element-selective detection.

Analyte	Sample type	Separation technique	Detector	Ref.
Myoglobin Ferritin	Raw and cooked beef steak	Size exclusion HPLC	ICP-double-focusing sector field-MS	109
Transferrin	Serum samples from human and harbour seals	Anion exchange HPLC	ICP-octapole reaction cell-MS	110
Transferrin isoforms	Human serum from healthy individuals and alcoholic patients	Anion exchange HPLC	ICP-octapole reaction cell-MS ESI-quadrupole/time of flight-MS	72
Nine transferrin glycoforms	Blood samples of harbour seals	Anion exchange HPLC	ICP-octapole reaction cell-MS	111
Five transferrin isoforms	Human serum	Capillary zone electrophoresis or anion exchange HPLC	UV ICP-octapole reaction cell-MS	112
Myoglobin Holo-transferrin	Proteins standard solutions	Size exclusion HPLC Reversed phase HPLC	ICP-OES Particle beam/hollow cathode-OES	113
β_2-transferrin	Cerebrospinal fluid	Anion exchange HPLC	ICP-octapole reaction cell-MS ESI-quadrupole/time of flight-MS	114
Cytochrome C Haemoglobin Transferrin Ferritin	Proteins standard solutions	SDS-PAGE Anodal native PAGE Cathodal native PAGE	ICP-MS	115
Ferritin	Edible plant seeds	Anion exchange HPLC	Sector field-MS	116
Glycated and non-glycated haemoglobin	Human blood from healthy individuals and diabetic patients	Cation exchange HPLC	ICP-octapole reaction cell-MS ESI-quadrupole/time of flight-MS	117

Copper.

The determination of the free/protein-bound copper ratio is an important subject of research. The knowledge of the copper distribution in biological samples helps understanding the copper metabolism and this contributes to the diagnosis and follow up of the copper related diseases (Wilson and Menkes disease).[118] In Wilson disease, a mutation in the gene ATP7B leads to a dysfunction of ceruloplasmin (Cp), which is the major Cu binding protein.[119] Clinically, serum Cp concentration diminishes and the so-called "free Cu" increases becoming toxic due to Cu deposits in target organs (liver, brain, kidney, and eyes). If not treated, irreversible damages can occur. Quantitation of ceruloplasmin, transcuprein and superoxide dismutase is mainly conducted using ICP-MS after separation in size exclusion columns packed with Sephadex or silica TSKGel. Table 4 shows the most recent chromatographic and detection conditions proposed for the quantitation of the major copper-containing proteins in various types of samples.

Table 4. Analytical methods for the species-selective analysis of copper-containing proteins by hyphenated techniques with element-selective detection.

Analyte	Sample type	Separation technique	Detector	Ref.
Superoxide dismutase	Tissue samples from bovine liver	Non denaturing 1-D PAGE	LA-ICP-MS	71
Four native and recombinant copper proteins	Cell extracts from *Escherichia. coli* and *Synechocystis*	Capillary reversed phase HPLC Size exclusion HPLC Anion exchange HPLC	ICP-dynamic reaction cell-MS ESI-time of flight-MS	120
Ceruloplasmin	Human serum from four different diseases and a set of normal controls	Size exclusion HPLC Reversed phase HPLC	ICP-octapole reaction cell-MS ESI-ion trap-MS	121
Transcuprein Ceruloplasmin	Human plasma from healthy subjects and an untreated Wilson disease patient	Size exclusion HPLC	ICP-dynamic reaction cell-MS	122
Albumin-copper Ceruloplasmin	Human serum	Size exclusion HPLC	ICP-quadrupole-MS	123

Metallothioneins.

Metallothioneins (MTs) are a group of non-enzymatic low molecular mass (6–7 kDa), cysteine-rich metal-binding proteins. The interest in the determination and characterization of MT-isoforms derive from their multifunctional physiological role in homeostatic control, storage, transport and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg) trace metals.[124] Furthermore, the characterization of MT-isoforms is important in the study of metal-mediated gene expression mechanism, because they are the product of genetic polymorphism characteristic of MT genes in animals and humans.[125]

Conventional methods used by biochemists for the analysis of MTs include metal-saturation assays, immunochemical methods such as radio immunoassays or ELISAs and electrochemical techniques such as differential pulse polarography (DPP).[126] However, these techniques lack in selectivity for the different MT isoforms, may suffer of interferences, and are unable to provide information on metal compositions.[127] As a result of genetic polymorphism, indeed, a number of isoforms and sub-isoforms of MTs, similar in hydrophobicity but slightly different in total electric charge, can be isolated.

HPLC and CE are capable to separate different MT isoforms; however when these techniques are coupled with UV detection, they suffer of a relatively poor sensitivity and they do not offer elemental specificity for unequivocal indication of the different forms of the protein bound to a given metal. Thus, in the last decades, the detection of MTs has been addressed using the coupling of HPLC and CE with element-specific detectors like atomic spectroscopy and ICP-MS.[128-130]

MT isoforms may differ only in few amino acids and therefore their separation requires a high-resolution technique that is able to separate compounds with very small differences in charge or hydrophobicity.[131] CE has a great potential in the separation of MT isoforms and sub-isoforms. Moreover, the small sample volume required (20–30 μL) makes CE an ideal technique to analyse biological material.

The critical point of CE-ICP-MS coupling is the interface, for two main reasons: the different MT isoforms and sub-isoforms appear very close to each other in the time scale (so a minimum ‘‘suction’’ effect in the nebulizer would degrade the separation achieved), and the metal content present in the different forms of MTs in living organisms is extremely low, so the interface should not compromise the high sensitivity required.[132]

In their work, Wolf et al. quantified MT-3 in complex biological samples (tissue cytosol) reducing the amount of sample matrix prior to the CZE-separation step with a precipitation step in acetonitrile of the high molecular weight proteins. The remaining matrix material caused a shift in the migration time of the different components, but it was possible to obtain comparable electropherograms by correcting the migration times mathematically using several internal standards.[35]

An advantage of HPLC compared to CE is its higher sensitivity due to the larger injection volume: a few μL up to 1 mL in HPLC vs. a few nL in CE.

In their work, Alvarez-Llamas et al. tested two different interfaces for CE-ICP-MS coupling, based on two commercially available microflow nebulizers (HEN and MicroMist). They found that the interface design was critical in order to keep the separation profile as obtained with UV detection. However, comparing both interfaces, similar performances in terms of sensitivity, linearity of response and resolution were observed.[132]

In another work, Alvarez-Llamas et al. developed an alternative CE–ICP–MS interface based on chemical volatile species generation (VSG) for the specific detection of Cd bound to MTs, as an alternative to conventional sample introduction systems via nebulisation. They observed an eight times improvement in peak height for Cd detection by VSG as compared to a classic microflow nebulizer. However, in order to make on-line VSG a suitable alternative interface, further studies are necessary to improve the analytical performance of the method (such as to decrease the high background noise derived from the VSG interface).[133]

Size-exclusion HPLC coupled to ICP-MS or atomic spectroscopy is a valuable tool for the detection of MTs in real matrices. SE-HPLC advantages are the good separation of proteins from small molecules with a minimal volume of eluate, the use of aqueous eluent phase (that preserve the biological activity of proteins) and the minimal interaction between proteins and the stationary phase.[50] However, the coupling with ICP-MS is disadvantageous because of the presence of salt eluent and SE-HPLC has poor resolution: MT-1 and MT-2 isoforms cannot be separated by SE-HPLC whereas the MT-1 peak is clearly resolved from the MT-2 peak with ion-exchange-HPLC and RP-HPLC in modified silica columns (typically C_4, C_8, or C_{18}).[127] In anion-exchange HPLC MT isoforms can be separated because of their negative charge. MT-1 and MT-2 can be separed, but the sub-isoforms of each class cannot be distinguished because the differences in electric charge are too small.[127]

Table 5 summarizes the most recent separation and detection conditions proposed for the quantitation of MTs isoforms in biological samples.

Table 5. Analytical methods for the species-selective analysis of metallothioneins by hyphenated techniques with element-selective detection.

Analyte	Sample type	Separation technique	Detector	Ref.
Cd MTs	Standard solutions of rabbit liver Cd-MTs	Capillary electrophoresis	Volatile species generation – ICP-quadrupole-MS	133
Zn, Cu and Cd MTs isoforms	Rat liver tissue	Capillary zone electrophoresis	ICP-sector-field double-focusing-MS ESI-MS	134
Up to five Zn, Cu and Cd MT isoforms	Cytosolic extracts of carp *Carassius auratus gibelio*	Size-exclusion HPLC Anion-exchange HPLC	ICP-time-of flight-MS	135
Zn, Pb, Cu and Cd MTs isoforms	Hepatic cytosols of Cd exposed carp *Cyprinus carpio*	Reversed phase HPLC	ICP-time-of flight-MS ESI-time of flight-MS	136
Zn and Cu MTs	Human peripheral blood mononuclear cells	High resolution size exclusion HPLC	ICP MS	137
MT-3 isoforms	Human brain cytosols	Capillary zone electrophoresis	ICP-sector field-MS	35
Zn and Cd MT-1 and MT-2	Standard solutions of rabbit liver Cd and Zn MT1	Capillary zone electrophoresis	UV ICP-quadrupole-MS ICP-double-focusing-MS	132
Zn, Cu and Cd MTs	Cytosolic extracts of bream *Abramis brama L.*	Capillary electrophoresis	ICP-octapole reaction cell-MS	138
Al, Ba, Cu, Fe, Mn, Sr and Zn MTs	Raft mussels (*Mytilus Galloprovincialis*) cytosols	Anion exchange HPLC	UV ICP-OES	139
Hg, Cd, Cu and Zn MTs	White-sided dolphin (*Lagenorhynchus acutus*) liver homogenate	Hydrophilic interaction HPLC	ICP-MS ESI-hybrid linear/orbital trap-MS	140
MTs sub isoforms	Kidney pig cell line exposed to CdS nanoparticles	Microbore reversed-phase HPLC	ICP-MS ESI-LTQ/Orbitrap-MS	141
Zn and Cd MT-1 and MT-2	Mussel cytosolic extracts	Size exclusion HPLC Anion exchange HPLC	ICP-quadrupole-MS	142
MT-1 and MT-2 isoforms	Rabbit liver cytosol and human	Fast liquid HPLC Size exclusion HPLC	ICP OES	143

Analyte	Sample type	Separation technique	Detector	Ref.
	cirrhotic livers	Anion exchange HPLC		
Cd, Zn and Cu MT-1 and MT-2	Cytosolic extracts of eels (*Anguilla anguilla*) exposed to Cd	Size exclusion followed by anionic exchange fast protein HPLC	ICP-quadrupole-MS ICP-double focusing-MS	144
Cd MTs	Cd-treated and untreated rat livers	Anion exchange HPLC	Flame AAS	145
MTs isoforms	Mouse hepa cells	2-D gel filtration and anion exchange HPLC	ICP-MS	146
MTs isoforms	Alzheimer's disease and control brains	Size exclusion HPLC	UV ICP-MS	147
Cd MTs	Cytosolic extracts of eels (*Anguilla anguilla*)	Vesicle mediated HPLC	Hydride generation-ICP MS	148
Zn, Cu and Cd MTs isoforms	Preparation of rabbit-liver MT Purified rabbit-liver MT-1	Capillary electrophoresis	ICP-sector field-MS	124
MT-1 and MT-2 isoforms	Rabbit liver MT-1, MT-2 and MT preparations	Capillary zone electrophoresis	UV ICP-MS ESI-triple quadrupole-MS	149

Some authors compared the separation capability of capillary LC and CE for MTs separation and quantitation, coupling both separative techniques with ICP-MS detectors. The results of these studies, although very similar, supported the use of capillary LC instead of CE [150] but different opinions have been expressed.[151]

Current strategies for proteins and peptides quantitation by metal labeling.

Most peptides and proteins are invisible to metal-specific detectors, and, in order to make them detectable by ICP-MS, ICP-OES, AAS or AFS, a proper elemental tag must be employed.[152]

The quantitation of proteins and peptides using a tag requires:

- the formation of a stable bond between the tag and proteins;
- a quantitative, reproducible and specific reaction;
- mild reaction conditions if the biological activity of the protein must be retained;
- the knowledge of the stoichiometry of the complex.

In the present review we report four types of common labeling agents: inorganic and organic mercury, iodination tags, metallocene-based reagents and lanthanide-based reagents. The advantages and drawbacks of the various labeling agent described in the subsequent paragraphs are reported in Table 6.

Table 6. Typical features for the most common heteroatom-labeling techniques.

Labeling element	Advantages	Drawbacks	Target sites
Mercury	Highly stable complexes Low blank levels Relatively fast reaction	Toxicity High ionization potential Persistent memory effects	Cysteine
Iodine	Fast labeling reactions Cheap and simple reagent	Not specific labeling Possible oxidative side reactions High background Low sensitivity in ICP-MS	Tyrosine Histidine
Ferrocene	Turn highly polar analytes into less polar Low ppt detection limits Cheap	Isobaric interferences in normal ICP-MS	Cysteine Amino groups
Lanthanides	Low ionization potential Limited interferences Low blank levels	High polarity of protein-complexes Two-step reaction	Cysteine Amino groups

Inorganic and organic mercury.

The interaction between mercury and biological thiols (low molecular thiols, i.e. cysteine or glutathione, and proteins) have been extensively studied since the 90s,[153] but only in the last decade the quantitation of proteins and peptides has become significant.

The combination of the high affinity of inorganic and organic mercury (Hg^{II} and RHg^{+}) for the sulfhydryl group (-SH) in the 1–13 pH range and the presence of cysteine in about 70% of proteins of proteome [154] makes possible the use of mercury for analytical purposes.[155]

The reaction of mercury with –SH group has been extensively investigated: it belongs to the soft–soft interactions, it is exothermic and thermodynamically favorable, with average bond energy of 217 kJ mol^{-1} for Hg-S.[153,156,157,158] In the protein labeled with organic mercurial probe, Hg is associated to C atom (in the organic moiety) at an average distance of 2.03 ± 0.02 Å and to S atom (in the –SH) at an average distance of 2.34 ± 0.03 Å, clearly indicating the formation of a Hg–S covalent bond.[159, 160] The standard entropy change is also very favorable for the labeled protein and the final complex is characterized by a large stability constant (e.g. for ethylmercury the stability

constant varies from $10^{16.3}$ to $10^{16.7}$).[161] This makes the labeled proteins stable adducts during chromatographic separations.[162]

Bramanti et al.[163] employed $HgCl_2$ to study the behavior of Hg(II) and Hg(II)–thiol complexes with chemical vapor generation (CVG)-AFS detector in different reducing media. $HgCl_2$ is highly soluble in aqueous solution under physiological conditions and is highly specific for -SH groups, reacting readily without requiring any incubation time or excess reagent and interfering with the protein molecular structures less than larger hydrophobic compounds (organic compounds of mercury, fluorescent labels, etc.).[163] However, inorganic mercury has the drawback of adsorbing to many chromatographic stationary phases [164] and of forming several mercury-thiol complexes with different stoichiometry, where $Hg(SR)_2$ and $Hg_2(SR)_2$ are the most commonly observed.[155]

Mono-functional organic mercurial probes ($MFOHg^+$) like alkyl and phenylmercury compounds of the type RHg^+ do not present the latter inconvenient and they specifically react at room temperature with active sulfhydryl groups forming stable, soluble and covalently bounded complexes of defined 1:1 stoichiometry (-S-Hg-R).[157]

Several studies show the advantages of using organic mercurial compounds, such as metylmercury ($MeHg^+$), ethylmercury ($EtHg^+$) and 4(hydroxymercuric)benzoic acid (*p*HMB) (Figure 5).[157,164]

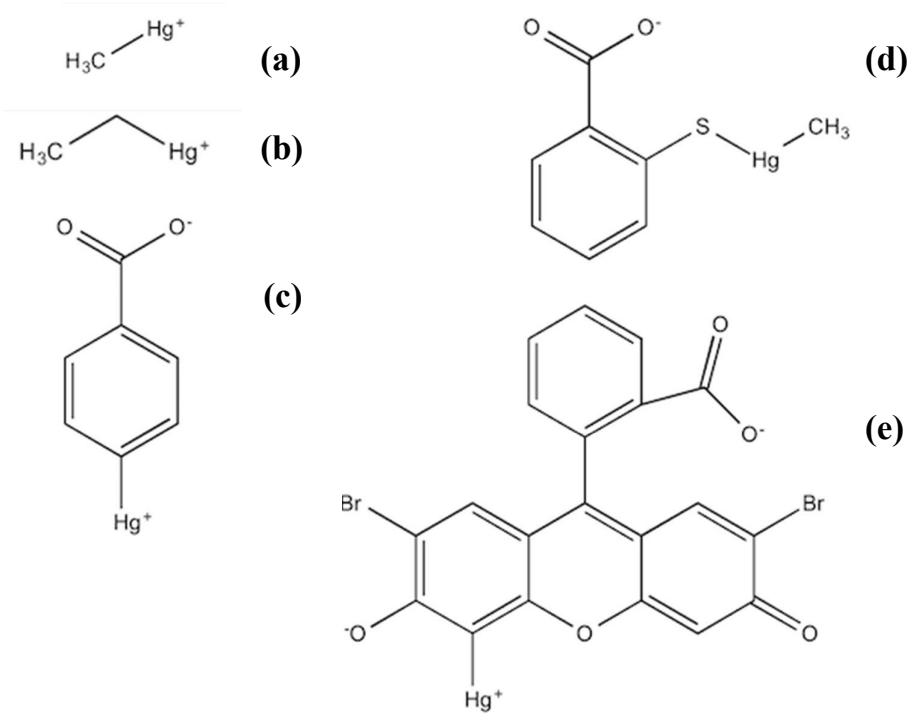


Fig 5. Structures of the organic mercurial probe used to tag cysteine-containing proteins. (a) methylmercury; (b) ethylmercury; (c) *p*HMB; (d) methylmercurithiosalicylate; (e) 2,7-dibromo-4hydroxymercurifluoresceine disodium.

Over the last 10 years Bramanti et al., extensively studied the interaction between *p*HMB and –SH groups for analytical and diagnostic purposes in proteins, [163-167] low molecular weight thiols,[168-170] mercaptans,[170] metallothioneins[165] and nitrosothiols [168, 169, 171] by means of liquid chromatography coupled to CVG-AFS, a sensitive, selective and relatively inexpensive technique for mercury determination.

Xu et al. [162] using size exclusion HPLC-ICP-MS studied the size-dependent effects of monofunctional organic Hg ions ($MFOHg^+$), including $MeHg^+Cl^-$ (4.84 Å), $EtHg^+Cl^-$ (6.06 Å), *p*HMB (9.65 Å), and 2,7-dibromo-4hydroxymercurifluoresceine disodium (Merbromin, 12.03 Å) on the labeling efficiency toward the sulfhydryl in intact proteins taking β-lactoglobulin as a model. Kinetic studies showed that the labeling reaction rate constants of $MFOHg^+$ are in the order

CH_3Hg^+ > $CH_3CH_2Hg^+$ > *p*HMB > Merbromin, which is in agreement with the increased trend in their size, suggesting that the smallest CH_3Hg^+ is the most effective agent for β-lactoglobulin labeling.

Considering the toxicity of CH_3Hg^+, Xu et al. searched for a CH_3Hg^+-equivalent tag, and synthesized methylmercurithiosalicylate (CH_3Hg-THI) and ^{204}Hg-enriched methylmercurithiosalicylate ($CH_3{}^{204}Hg$-THI) for protein labeling.[172] The labeling strategies have been applied to the separation and detection of glutathione, β-lactoglobulin and ovalbumin as model peptide/proteins by SEC-ICP-MS and the absolute quantitation was conducted with isotope labeling strategies.

Kutscher and Bettmer [173] developed a procedure for the absolute and relative quantitation of insulin as a model protein based on the synthesis of ^{199}Hg-enriched *p*HMB. Their approach was divided into two different steps: the first was based on the differential isotope labeling to compare two different samples for their relative quantitation using MALDI-MS followed by the deconvolution of the isotope pattern. The approach was extended to the absolute protein quantitation, by characterizing isotopically labeled insulin by ICP-MS and by adding it to the sample as an internal standard. Proteins labeled with either [^{199}Hg]*p*HMB or [^{nat}Hg]*p*HMB, can be easily distinguished by the observed isotope pattern provided by MALDI-MS. The main advantage of this approach is that the isotopically labeled protein used as internal standard can be independently quantified by ICP-MS on the basis of the reverse isotope dilution analysis of mercury (a common and accurate quantitation method for isotopically labeled species), whereas molecular mass spectrometry allows the detection and quantitation of [^{nat}Hg]*p*HMB labeled protein.

Cold vapour generation coupled with atomic spectrometry is traditionally the technique most widely used for mercury determination.[174, 175, 176]

Among the atomic spectrometric techniques, CVG-AFS is the most sensitive, selective, and low-cost technique for mercury detection reaching detection limits (LOD) ≤ 0.1 ng/L.[177] Cold vapor-AFS has also the advantage of being free of interference from any other vapor or hydride forming

elements. However, the direct introduction of organic mercury into the detector lowers the CVG-AFS performance, so this technique usually requires the use of decomposition systems for the conversion of organomercury species to Hg^{II}, before their introduction into the AFS detector. Thus, online decomposition systems are mandatory to obtain higher sensitivity and reproducible results using atomic spectrometric detectors. Decomposition systems include (i) chemical oxidants (the more common include $KBr/KBrO_3$[178,179], $K_2S_2O_8$ in presence of copper sulphate[180] and $K_2Cr_2O_7$[181]) and (ii) UV irradiation assisted[182] or not [183,184] by microwaves (MW).

The latter has introduced a novel "green strategy" in the analytical determination of mercury, leading to the digestion of mercury species without the use of toxic and carcinogenic chemicals. The mixture Br^-/BrO_3^-, for example, has the advantage of being performed at room temperature but BrO_3^- is a reagent classified as carcinogenic. Furthermore, bromine is a fluorescence quencher and the generated excess has to be reduced into bromide during the subsequent reducing step by hydrazine, a compound classified as carcinogenic, flammable, toxic by inhalation, in contact with skin and if swallowed, and very toxic to aquatic organisms.[185] Falter and co-workers adopted UV irradiation to decompose organic mercury.[186] Bendicho et al. have reviewed in an excellent work the photo-oxidation and photoreduction of mercury and other elements.[187]

Tang et al. [188] proposed UV/HCOOH-induced Hg CVG as an effective interface between HPLC and CVG, instead of K_2SO_8-KBH_4/NaOH-HCl and/or $KBrO_3$/KBr-KBH_4/NaOH-HCl systems as oxidizing/reducing system for the simultaneous determination of low molecular mass thiols tagged with *p*HMB. Other authors proposed acidic $K_2S_2O_8$ solution combined with microwave (MW) digestion[189] or MW digestion in acidic conditions.[190]

Recently, Angeli et al.[185] have proposed a novel HPLC-MW/UV combined reactor coupled to CVG-AFS detection system for the determination of *p*HMB-tagged thiols. The use of a fully integrated MW-UV photochemical reactor[190],[191] allowed to obtain the on-line digestion of *p*HMB and thiols- *p*HMB complexes to Hg(II). Hg(II) was reduced to Hg^0 in a knitted reaction coil with $NaBH_4$ solution, and detected by AFS. The integrated photochemical reactor is able to measure and

control the MW power working on the sample during experiments and overcome the large amount of drawbacks given by reactors placed in a microwave oven, or in a waveguide applicator working at 2450 MHz [191] [192], or by an immersed electrodeless MW/UV lamp.[185]

In the last years ICP-MS has become an attractive tool for the determination of mercury, as shown by the growing number of papers that use this technique as detector for mercury. Unfortunately, the relatively high ionization potential of mercury and persistent memory effects seriously limit the attractiveness of mercury compounds for routine analysis with ICP-MS.

Table 7 summarizes the most recent separation and detection conditions proposed for the quantitation of mercury-tagged proteins in biological samples

Table 7. Analytical methods for the species-selective analysis of proteins tagged with mercury by hyphenated techniques with element-selective detection.

Sample type	Labeling tag	Separation technique	Detector	Ref.
Ovalbumin β-lactoglobulin	• CH_3Hg^+ • $CH_3CH_2Hg^+$ • *p*HMB • 2,7-dibromo-4-hydroxymercurifluorescein	Size exclusion HPLC Reversed phase HPLC	ICP-dynamic reaction cell-quadrupole-MS ESI-ion trap-MS MALDI-time of flight-MS UV Fluorescence	193
Glutathione Ovalbumin β-lactoglobulin	• CH_3Hg-thiosalicylate • $CH_3{}^{204}Hg$-thiosalicylate	Reversed phase HPLC Size exclusion HPLC	ICP-MS UV ESI-ion trap-MS ESI-time of flight-MS	172
Ovalbumin	*p*HMB	Reversed phase μHPLC	ICP-MS MALDI-time of flight-MS ESI-time of flight-MS	194
Insulin	*p*HMB	Reversed phase μHPLC	ICP-MS MALDI-time of flight-MS ESI-time of flight-MS	173
Bovine pancreatic ribonuclease A Lysozyme Insulin	CH_3Hg^+	Reversed phase HPLC	ICP-dynamic reaction cell-MS ESI-ion trap-MS	195
Glutathione Phytochelatins, Lysozyme β-lactoglobulin	• CH_3Hg^+ • $CH_3CH_2Hg^+$ • *p*HMB	Reversed phase HPLC	ESI-ion trap-MS	19
Glyceraldehyde-3-phosphate dehydrogenase Aldolase Pyruvate kinase Triose phosphate isomerase Phosphoglucose isomerase	*p*HMB	Hydrophobic interaction HPLC	CVG-AFS	164

Sample type	Labeling tag	Separation technique	Detector	Ref.
MTs from rabbit liver	*p*HMB	Reversed phase HPLC	CVG-AFS	165
Phytochelatins Extracts of cell cultures from *Phaeodactylum tricornutum*	*p*HMB	Size exclusion HPLC Reversed phase HPLC	UV CVG-AFS MALDI-time of flight-MS	196
Cysteine Glutathione Homocysteine	*p*HMB	Reversed phase HPLC	CVG-AFS	185
Cysteinyl-glycine S-nitrosoglutathione in human blood	*p*HMB	Reversed phase HPLC	CVG-AFS	169
Human serum albumin Bovine serum albumin Rat serum albumin Horse serum albumin Sheep serum albumin Ovalbumin β-lactoglobulin	*p*HMB	Reversed phase HPLC	CVG-AFS	197

Iodination tags.

Among the ICP-MS-detectable halogens, only iodine has been used as labeling agent for protein derivatization, because the determination of other halogens is affected by low ionization efficiency.[198] Iodinization proceeds with electrophilic substitution of iodine to the aromatic side chains of histidine and tyrosine (about 50%) (Figure 6), so this labeling it is not specific for only one functional group in a protein. Nevertheless, a recent work that uses the more complex iodinization-reagent bis(pyridine)iodonium tetrafluoroborate demonstrated the complete and specific derivatization only of tyrosine residues in standard peptides.[199]

Iodinization is a long known method and has been applied in particular for the incorporation of radioactive ^{125}I or ^{127}I and detection by ICP-MS. This type of labeling offers some advantages, such as fast labeling reactions (2–15 min) and the use of a cheap and simple reagent like sodium iodide after its oxidation to I^+.[200] The reaction is possible at two different sites: at the orthoposition of tyrosine and at the 2, 5 positions of the imidazole-ring of histidine.[201]

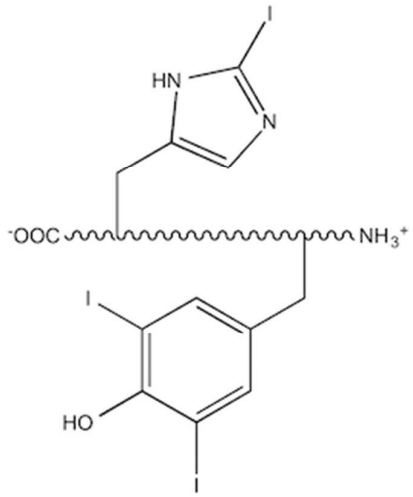


Fig 6. Schematic protein chain with tyrosine and histidine derivatized with iodine.

The iodination process should provide both labelling efficiency and, when required, the preservation of the protein activity. A possible drawback is the occurrence of oxidative side reactions such as the oxidation of methionine and tryptophan residues during the iodination

process.[202] For this reason, specific reagents and procedures have been designed with the aim of minimizing these negative side effects on protein function and structure. For example, iodination by means of chloramine T (N-chloro 4-methylbenzenesulfonamide, sodium salt) and in particular of immobilized chloramine T has been claimed to be a protein structure preserving method.[203]

An alternative approach to label proteins with iodine is indirect labelling using iodine-containing compounds that can be coupled to proteins *via* their functional groups, thus avoiding the direct contact of proteins with iodine species. For this purpose, iodinated Bolton-Hunter reagent, N-succinimidyl-3-(4-hydroxyphenyl)- propionate, is used, which binds to the amino group of lysine side chains (Figure 7).[204] Besides this, the well known reagent N-succinimidyl-3-iodobenzoate as well as N-succinimidyl 4-guanidinomethyl-3-iodobenzoate were successfully applied.[205,206]

Pereira Navaza and his co-workers [199] reported the labelling of tyrosine residues by bis(pyridine)iodonium tetrafluoroborate (IPy_2BF_4) (Figure 7) for quantitative detection of polypeptides using β-casein, a well-characterized protein, as a model. Two iodine atoms are specifically bioconjugated to the meta-positions of the aromatic ring of every tyrosine residue. Characterization studies performed by capillary HPLC with parallel ICP-MS and ESI-MS/MS detection clearly demonstrated that the tyrosine residues present in the peptide are completely diiodinated. They optimized the proposed method for tyrosine labeling and then they performed the validation by applying the method to the absolute quantitation of β-casein after tryptic digestion and of three standard peptides present in a reference material.

Jakubowski et al. explored the use of immobilized chloramine T (IODO-Beads™) to label intact proteins with the iodine isotope ^{127}I, followed by protein electrophoresis and electro-blotting and detection by LA-ICP-MS.[207] Unfortunately, laser ablation requires harsh conditions and the results obtained demonstrated that the labeling process on the separated spotted proteins was neither quantitative nor site-specific. Additionally, oxidation of methionine residues were observed, which implies the risk of affecting the functionality of proteins.

Waeting et al. in their work compared a mild protein iodination by KI_3 with the IODO-Beads method, demonstrating, by labeling single proteins, whole proteome and antibodies, that the labeling with KI_3 is fast, cheap and efficient for ICP-MS based analytics.[208]

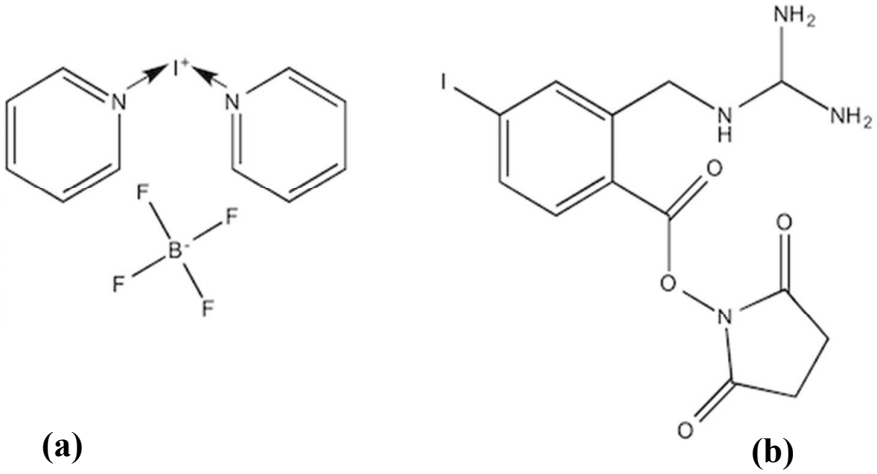


Fig 7. Structures of two iodination tags. (a): IPy_2BF_4 [199]; (b): N-succinimidyl 4-guanidinomethyl-3-iodobenzoate[204].

The sensitivity of iodine in ICP-MS detection and a considerable background of natural iodine contained in some biological samples are general drawbacks of iodine labelling. [209] The sensitivity (100–1000 ng/L) is 3–4 orders of magnitude lower than that observed for lanthanides because of iodine high ionization energy (10.45 eV). The energy transferred by the plasma is sufficient to excitate only a minor fraction of halogens atoms and ions, which leads to only about 29% of ionized iodine in argon plasma,[209] and consequently higher LODs are typically reached.

Metallocene-based reagents.

Metallocenes are compound with general formula M $(C_5H_5)_2$, containing two cyclopentadienyl anions bound to a transition-metal center (M) usually in the +2 oxidation state.

Ferrocene, an iron(II) complexe of low polarity, is the best known and representative metallocene compound.[210,211] A unique property of metallocenes is the possibility of introducing substituents on one or both the cyclopentadienyl rings, although it retains the properties of a simple one-electron redox couple.[212]

Their detection by ICP-MS under normal conditions suffers the formation of the isobaric $[^{40}ArO]^+$ ion (m/z 56 cannot be discriminated from the respective main isotope of iron ^{56}Fe at low resolution), which leads to moderate LODs.[213] Hence, a resolution over 2500 would be needed to separate ^{56}Fe from $[^{40}ArO]^+$, which is achievable by sector-field (SF) instrument. On the other hand quadrupole instruments equipped with a hexapole or octapole reaction or collision cell can almost entirely eliminate the argon interferences. Combined with a reaction or collision cell, the ICP-quadrupole MS can give low ppt detection limits for iron in bulk analysis, which are about the same or slightly higher than those obtained with ICP-SF-MS instrument.[214] Works based on reversed phase/size exclusion-HPLC-ICP-MS measurements have been published for the analysis of ferrocene-derivatized lysozyme, β-lactoglobulin A and insulin.[215]

The ability of different metallocenes to react with amino acid side chains of proteins was mentioned for the first time in 1972 by Giese et al.[216] However, the derivatization of functional groups in proteins with metallocene derivatives was published for the first time by Peterlik, who analysed the reaction of ferrocenesulfonyl chloride with ovalbumin using the X-ray structure analysis. In that work an average of 8.6 out of the total 20 lysines in the protein structure were derivatized.[217]

Many ferrocene-based derivatizing agents have been proposed and used in combination with liquid chromatography and electrochemical detection (i.e. amperometry or voltammetry). Eckert and Koller synthesised several ferrocenes for the derivatization of the N-terminus and lysine residues in peptides and proteins and tested them in the reaction with bovine serum albumin followed by LC-electrochemical detection analysis.[218] AAS as well as ICP-OES or ICP-MS were also proposed as detection techniques for ferrocene derivates .[212]

Bomke et al. [215] applied for the first time the ferrocene-based reagent succinimidylferrocenyl propionate (SFP) as dual labelling reagent for amino and thiolic groups present in peptides and proteins. The previously reduced thiolic groups were functionalised with ferrocenecarboxylic acid(2-maleimidoyl)ethylamide (FMEA) at pH 7, and subsequently the amino groups were derivatized with SFP at pH 9 (Figure 8). The derivatized biomolecules were analysed using reversed phase HPLC coupled with ESI-MS and ICP-MS.

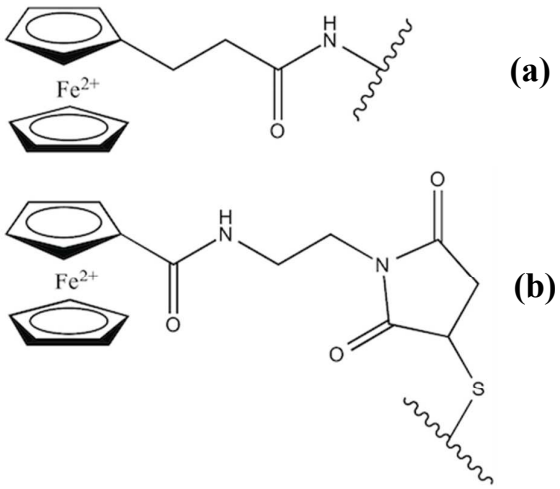


Fig 8. Amino (a) and thiolic (b) groups derivatized respectively with SFP and FMEA.

All 6 lysine residues and the N-terminus present in the basic protein lysozyme were quantitatively derivatized by SFP. However, as the reaction proceeded, also the basic side chain of histidine and arginine reacted with the ferrocene-based reagent. With acidic proteins, as insulin and β-lactoglobulin A, a distribution of different labelling degrees was achieved but in both cases no underivatized proteins remained.

They subsequently applied the dual labeling approach to the tripeptide glutathione and insulin. This new approach of the multiple labelling leads to a strong increase of quantifiable information and, independently on the reagents used for the labelling process, it is a promising tool for bioanalysis in

the future. Using this strategy the discrimination between amino and thiol groups on the same peptide by ICP-MS is not possible.

The FMEA reagent has also been employed by Braütigam et al. to derivatize several phytochelatins and thiolic species (PC_{2-4}, CysGSH, $CysPC_{2-4}$, $CysPC_2$desGly, $CysPC_2$Glu and $CysPC_2$Ala) from algal extracts. PCs are peptides with the general structure $(GluCys)_nGly$, and their identification and quantification is essential for physiological studies. After the derivatization, the phytochelatins have been identified with HPLC-MS/MS and quantify by ICP-MS. However, they did not observe a constant Fe signal in ICP-MS by gradient elution and they did not obtain a baseline separation of the derivatized PC by isocratic separation. Thus, a species independent Fe determination by LC/ICP-MS was not possible and the quantification was performed with the help of standard compounds. Besides the identification of canonic phytochelatins, they confirmed the presence of PC3desGly, which was only proposed before. [219]

Tanaka et al. [220] developed an on-chip type cation-exchange chromatography system with electrochemical detection of HbA_{1c}, which is one of the most important marker protein in diabetes, using ferrocene-conjugated antihuman hemoglobin (Hb) monoclonal antibody (FcAb). Ferrocene-conjugated anti-human haemoglobin monoclonal antibody, which can react with all Hbs, was used as an electrochemical probe, and an optimized 15 minutes procedure allowed the separation of HbA_{1c} from other Hbs in blood samples.

Table 8 summarizes the analytical methods for the species-selective analysis of proteins tagged with iodine and ferrocene by hyphenated techniques with element-selective detection

Table 8. Analytical methods for the species-selective analysis of proteins tagged with iodine and ferrocene by hyphenated techniques with element-selective detection.

Sample type	Labeling tag	Separation technique	Detector	Ref.
Tyrosine Three peptides from reference material (NIST 8327) Tryptic digests of β-casein	Bis(pyridine)iodonium tetrafluoroborate	Reversed phase capillary HPLC	ICP-collision cell-MS ESI-quadrupole/time of flight-MS	199
Cytochromes P450	Monoclonal antibody labeled with iodine	SDS-PAGE Semidry immunoblot	LA-ICP-MS	221
Lysozyme Bovine serum albumin Cytochrome c β-casein	Potassium triiodide IODO-Beads	SDS-PAGE Semidry blotting Western blotting Reversed phase HPLC	LA-ICP-sector field-MS ESI-linear triple quadrupole/ Fourier transform- MS	208
Porcine gastric mucosa pepsin Lysozyme Bovine serum albumin	Sodium iodide	SDS-PAGE Semidry blotting	LA-ICP-sector field-MS nanoESI- Fourier transform ion cyclotron resonance-MS	201
Lysozyme β-lactoglobulin A Insulin	• Succinimidylferrocenyl propionate (for amino groups) • Ferrocenecarboxylic acid(2-maleimidoyl)ethylamide (for thiolic groups)	Reversed phase HPLC	ICP-octapole reaction cell- MS ESI-quadrupole/ion trap-MS	215
Haemoglobin A_{1c}	Ferrocene-conjugated anti-human haemoglobin monoclonal antibody	On-chip type cation-exchange chromatography Cation exchange HPLC	Electrochemical detector	220
Lysozyme β-lactoglobulin A Insulin	N-(2-Ferroceneethyl)maleimide	Reversed phase HPLC	Cyclic voltammetry hyphenated with a single-quadrupole-MS	222
α-lactalbumin β-lactoglobulin B β-lactoglobulin A	N-(2-ferrocene-ethyl)maleimide Ferrocenecarboxylic acid-(2-maleimidoyl)ethylamide	Reversed phase HPLC	Cyclic voltammetry hyphenated with ESI-quadrupole/ion trap-MS	210
Phytochelatins from algae extracts	Ferrocenecarboxylic acid (2-maleimidoyl)ethylamide	Reversed phase HPLC	ESI-time of flight-MS ESI-Triple quadrupole/ion trap-	219

Sample type	Labeling tag	Separation technique	Detector	Ref.
			MS ICP-MS	

Metal-coded affinity tag.

Other interesting labeling strategies use bi-functional chelating agents loaded with different lanthanide (Me^{3+}) ions and a second functional group for specific covalent interaction with the target biomolecule (cysteine residues or amino groups in the case of NHS-ester derivatives). The lanthanide series ranges from Ce to Lu (where La and Y are often included because of their similar chemistry) and they differ primarily in their ionic radii, which show a decrease along the series (lanthanide contraction).[223] By using different lanthanides within the chelate complex, different proteomic states or samples can be assessed.

The strategy of protein labeling based on rare earth metal is an excellent method for protein quantitation because of its unique advantages. First, the elemental labeling for protein quantitation can be applied not only for biological mass spectrometry, but also combined with ICP-MS, enabling the absolute determination of proteins and peptides via the measurement of the incorporated lanthanide ion without the need for a structurally related standard. Second, the rare earth metal chelated tags are inexpensive and can be easily obtained, compared with the stable isotope-labeling agents.[224]

As detector for rare-earth elements, ICP-MS has major detection capabilities due to (i) the low ionization potential of these elements, (ii) their high mass, so doubly charged species of other elements does not interfere with them and (iii) the low blank values due to their low natural abundance in biological samples.[225] Absolute quantitation of rare earth labeled peptides and proteins can be achieved by external calibration using salt standards, as element signals in ICP-MS are largely matrix independent and they have up to 12 decades of linear dynamic range.[226]

Derivatives of the diethylenetriaminepentaacetate (DTPA) and tetraazacyclododecane (DOTA) macrocycles have been extensively used as chelating agents to label proteins, peptides, and antibodies. A common derivatizing agent is the commercial available bifunctional chelating agent maleimido-mono-amide-DOTA (or MMA-DOTA), which forms an extremely stable complex with

lanthanide ions, while the functional maleimide group binds covalently to the –SH group in the proteins with high specificity and efficiency under mild conditions.[227]

In addition to the MMA group for specific thiol labelling,[7,228] other commonly reactive groups used as chelating agents are the isothiocyanates (SCN) for the labeling of amino groups.[207]

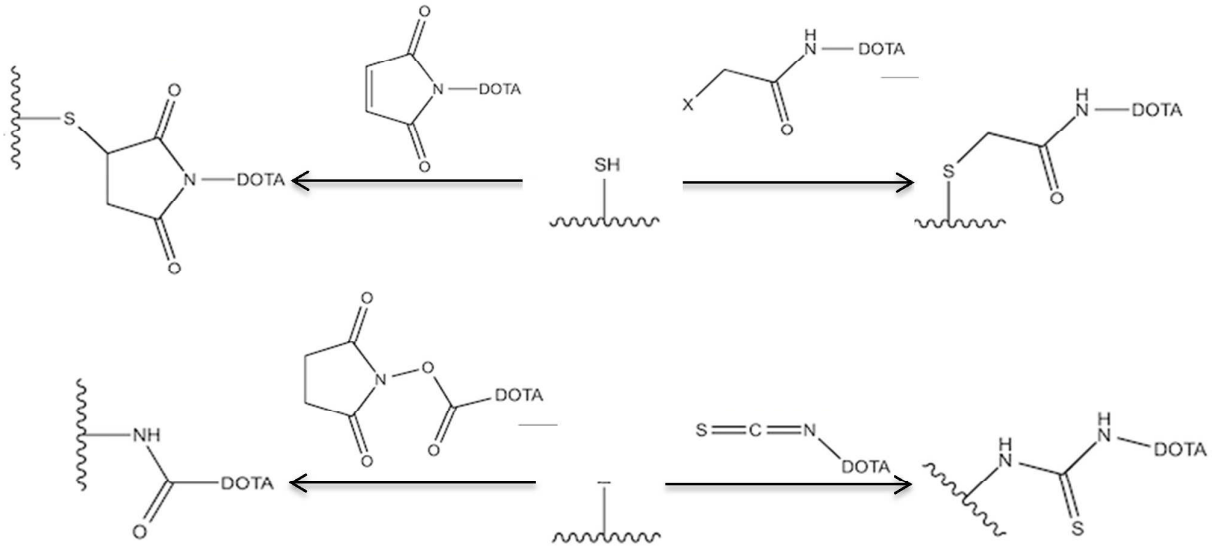


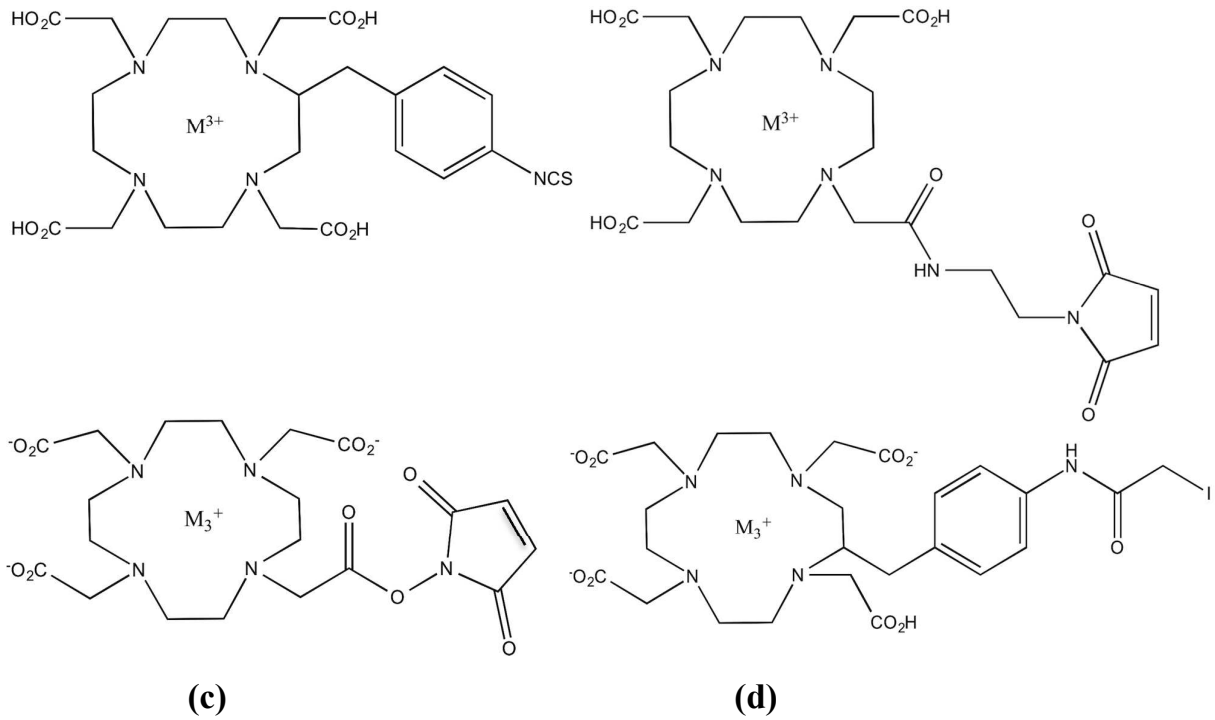
Fig 9. The reactions involving thiolic (a) and amino (b) group with different MeCAT reagents. Thiolic groups react with maleinimides and halogen acetamides, while amino groups react with NHS and isothiocyanate functionalities.

In the case of DOTA and 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA), the complex coordination is maintained via free electron pairs of heteroelements as well as via carboxylate groups and is mainly influenced by pH, temperature and concentration.[229]

DOTA-rare earth chelates have exceptional properties if used as affinity tags. Unlike biotin, they have no natural analogues that might interfere with affinity purification. They are highly polar and water-soluble. Many of the rare earth elements are naturally monoisotopic, providing a variety of simple choices for preparing mass tags.[230] The polydental macrocyclic DOTA and the noncyclic

open form DTPA generate extremely stable metal complexes with stability constants (log *k*) up to 25.4.[231]

In 2004 Meares and his colleagues,[230] based on DOTA labeling, developed a method for the relative and absolute quantitation of peptides and proteins called metals-coded affinity tag (MeCAT) based on the cysteine-specific chemical labels by tags containing different element-coded metal chelates with similar chemical nature. The MeCAT approach together with flow injection analysis-ICP-MS was applied for eye lens proteomics quantitation.[228]



(c) **(d)**

Fig 10. Structures of some of the MeCAT reagents used in the articles cited by the present review. MeCAT reagents consist of three parts: a macrocycle for metal chelating, a spacer with connects the macrocycle, and a functional group for specific labeling of amino acid side chains. (a): isothiocyanate-DOTA; (b) and (c): MMA-DOTA; (d): iodoacetamide-DOTA.

With respect to DOTA, the bicyclic anhydride of DTPA used to introduce the lanthanides is an inexpensive and easily obtained labeling agent that reacts with primary amines (amino terminus and

internal Lys) present in the proteins.[232] DTPA-based tags allow the choice of different metals and can be bound to amino groups for peptide and protein labeling with two-step reactions: once the protein is derivatized with DTPA, the complex with rare earth metal of interest is obtained by adding the metal to the solution.[233]

Liu et al. [224] demonstrated the labeling of peptides by using yttrium and terbium–DTPA complexes. Furthermore, lanthanide ions such as Eu, Tb, and Ho were implemented in a DOTA-acid succinimide ester (SCN–DOTA) complex to label bovine serum albumin and hen egg white lysozyme.[224]

Commercially available fluorescent probes (DELFIA™) containing the lanthanides Eu, Tb, and Sm were employed for DOTA labeling of different antibodies with a specific metal and detection by ICP-MS.[234] Recently, the first ICP-MS-based multiplex profiling of glycoproteins was published, in which lectins conjugated to lanthanide-chelating compounds were used.[235]

The integration of elemental labeling in quantitative bio-analysis requires fundamental experiments concerning the yield of complexation stability of protein-metal complexes during the analysis. McDevitt et al. [236] had compared the yield of binding of attinium (^{225}Ac) in chelates based on DTPA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-propionic acid (DOTPA), 1,4,8,11-tetra-azacyclotetradecane-1,4,8,11-tetrapropionic acid (TETPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-methylenephosphonic acid (DOTMP), a-(5-isothiocyanato-2-methoxyphenyl)-1,4,7,10-tetraazacyclodo-decane-1,4,7,10-tetraacetic acid (MeO-DOTA-NCS) and 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (p-SCNBn-DOTA). In a second step, they assessed also the yield in the binding of the chelate to an antibody (IgG). Among the chelates investigated, only the compounds based on DOTA showed the highest labelling yield of the antibody and the best recovery during sample preparation.

Lewis et al. described the use of a sulfo-SHN (N-hydroxysuccinimide) linker attached to DOTA.[237] This group used a 100-fold excess of sulfo-NHS-DOTA with respect to the protein of interest and

performed the labelling with radioactive isotopes (^{111}In and ^{90}Y). After the optimisation of the labelling procedure, the maximal number of labels detected per protein molecule was not more than 3.8 for an antibody and about 9 for cytochrome c, demonstrating that this value strongly depends on the protein structure. In all cases this value was by far below the theoretical number of binding sites.[207]

Kretschy et al. [229] investigated the complex stability of the chelating moieties DOTA, NOTA and DTPA in combination with 11 different lanthanides under typical chromatographic conditions. Measurements were carried out via LC-ICP-quadrupole-MS using a novel mixed mode separation method. The influence of chromatographic separation, pH and temperature on complex stability constants was assessed, and they found that, for all investigated complexes, the stability was significantly decreased by the chromatographic conditions. Ln^{3+}-DOTA and Ln^{3+}-NOTA complexes provided high stability at 5 °C and 37 °C over a time of 12 hours, whereas Ln^{3+}-DTPA complexes showed significant degradation at 37 °C. Moreover, although Ln^{3+}-DOTA complexes exhibited the highest stability constant values, during the chromatographic separation they show an additional signal suggesting a positively charged intermediate product.

Zhang et al. developed a strategy for dual labelling of peptides based on an elemental tag and a fluorescent tag.[238] MMA-DOTA loaded with Eu was used to conjugate the peptide *via* the specific reaction between -SH and MMA, and with a typical fluorescent tag (fluorescein isothiocycanate, FITC) for the subsequent conjugation of the peptide *via* the reaction between –N=C=S and $–NH_2$. The peptide is determined using both ^{153}Eu isotope dilution ICP-MS and capillary electrophoresis-laser induced fluorescence (CE-LIF). The LODs of the three tested model peptides obtained using HPLC-IDA-ICP-MS were two orders of magnitude lower than those found using (CE-LIF), suggesting that HPLC-IDA-ICP-MS is the election platform for the quantitation of peptides.

El-Khatib et al. recently developed a strategy in which thiolic and amino groups in peptides were targeted with different reagents. Amino groups were labeled with DOTA-NHS and thiolic groups using DOTA with iodoacetamide functionality. They showed that both labeling sites could be

addressed quantitatively using different metals and thereby could be distinguished in ICP-MS. Alternatively, an increase in sensitivity *per* protein or peptide can be achieved when the same metal for the different reagents is used.[239]

An alternative approach is based on the tagging of antibodies with rare earth elements-chelates, which react with antigens with an extremely high degree of specificity even in complex matrices. Once the labeling procedure is optimized, the activity of the metal-tagged antibodies can be preserved.

Terenghi and his co-workers[240] developed a method for the multiplexed determination of five protein cancer biomarkers as complexes with antibodies tagged with different rare earth elements, separated in size exclusion-HPLC and detected by ICP-MS (Figure 10). Their aim was to optimize the conditions to determine simultaneously target proteins directly in the sample matrix without any sample pretreatment. Size exclusion-HPLC allowed the online separation of the protein-antibody complexes from the unreacted antibodies and the degradation products of the labeling reagent. Despite the coelution of the immunocomplexes of different proteins, as well of the free antibodies, the detection and determination of each protein-antibody complex occurs on the basis of each metal-specific chromatogram.

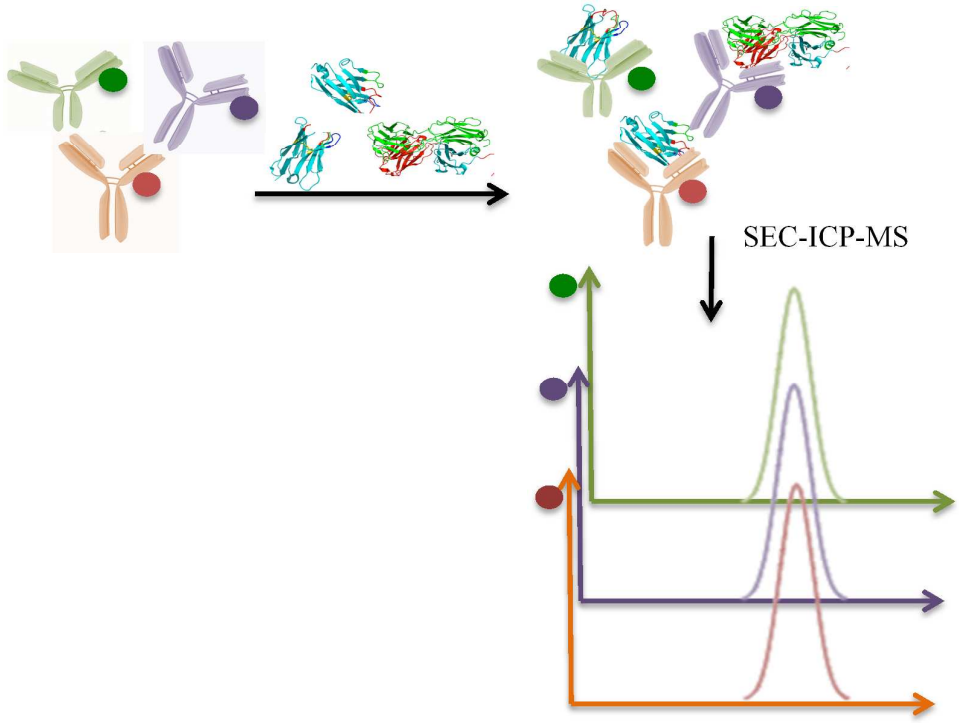


Fig. 11. Schematic workflow showing the antibody-labeling strategies for protein quantitation. Different antibodies are labeled with different MeCAT reagents and react with the respective antigens. The labeled proteins are subsequently analyzed *via* SEC-ICP-MS.[240]

Muller et al. [241] investigated the maleimide-based modification procedure via size exclusion-HPLC coupled with ICP-MS and LC-time-of-flight-MS, by analyzing the antibody structure after modification with MeCATs. The maleimide-based tagging of antibodies is a procedure quiet complex. The drawback of this linking chemistry is, indeed, the pretreatment of the antibody, which needs to be reduced to generate free sulfhydryl groups to react with the maleimide linker of the metal tag. As the tagged antibody usually still shows antigen selectivity in the immune reaction, the assumption is that the hinge region of the antibody is preferentially reduced, leading to two identical parts of the antibody with intact antigen-binding sides.[242] They found that maleimide-modified antibodies show an excellent specificity and sensitivity during the immune reaction. The functional efficiency of the maleimide-tagged antibodies even after the reduction of the interchain

disulfide bridges with TCEP, can be explained by the fact that the antibody structure is preserved by its hydrophobic interactions. Nevertheless, each antibody needs to be validated after tagging to prove its specificity against the target antigen. The complexity of the metal-tagged antibody prevents the development of a quantitation concept, because a calculation of an exact tagging degree, which is the prerequisite for calculating the amount of antibody molecules in the sample, is not possible. Thus, the absolute quantitation of element-tagged antibodies by ICP-MS requires the development of new strategies.[241]

The validation of the analytical methods based on MeCAT is an important topic because peptide quantitation is an upcoming issue in in pre-clinical studies of drug development, as many peptides are recognized as promising drugs. Koellensperger et al. [243] loading DOTA not with a lanthanide but with indium, compared the quantitation of labeled peptides by LC-ICP-MS with the results obtained using LC-ESI-MS measurement without labeling, in the same chromatographic conditions. The analysis of aqueous standards using the two methods showed comparable results in terms of sensitivity and limit of detection, whereas in cell culture experiments the measurement of the cytoplasm samples revealed severe matrix effects in the case of LC-ESI-MS, which made impossible quantitative measurements. On the contrary, LC-ICP-MS quantitation of peptides in combination with elemental labeling showed the advantage of a matrix-independent signal intensity.

The work of Tanner and Nolan about the development of ICP-MS-linked metal-tagged immunophenotyping deserves some mention. This is a rather hot topic in medical research, as it has great potential for highly multiplexed proteomic analysis.[244,245,246] They found that the sensitivity of ICP-MS linked immunophenotyping employing commercially available tags is comparable with that of fluorescence activated flow cytometry analysis. However flow cytometry, the common optical methods for detection of intracellular and extracellular proteins within single cells, is not suitable for multiplex analysis. In a work of Tanner et al.[247], the data obtained provide that lanthanides labeling coupled with ICP-MS detection can be used in the multiplexed molecular

analysis of human leukemia cell lines, detecting proteins on the cell surface as well as intracellularly in permeabilized cells. A critical further application of ICP-MS to cell biology would be to combine this detection methodology with single cell analysis in a novel flow-based ICP-MS instrument.

Despite the excellent detectability of lanthanide ions by ICP-MS and the high stability of the reagents avoiding metal loss or metal exchange, the high polarity of complexes and their derivatives makes their separation on reversed-phase (RP) columns impossible.[30] Unfortunately, without a satisfactory separation of the derivatized biomolecules, an absolute quantitation cannot be expected. Moreover, the rare earth element labeling needs a two-step reaction and the reaction efficiency of each step would affect the quantitative results.

Table 9 summarizes the analytical methods for the species-selective analysis of proteins tagged with MeCAT by hyphenated techniques with element-selective detection.

Table 9. Analytical methods for the species-selective analysis of proteins tagged with MeCAT by hyphenated techniques with element-selective detection.

Sample type	Labeling tag	Separation technique	Detector	Ref.
Vasopressin GGYGGC Somatostatin	• MMA-DOTA loaded with Eu(III) Fluorescein isothiocycanate	Reversed phase HPLC Capillary electrophoresis	UV ESI-ion trap-MS ICP-MS CE-LIF	238
Sus scrofa eye lens proteins α-lactalbumin Bovine serum albumin	DOTA loaded with Lu(III), Ho(III), Tb(III), Tm(III)	1-D SDS-PAGE 2-D SDS-PAGE Reversed phase nanoHPCL	ICP-quadrupole-MS ICP-high resolution sector field-MS MALDI-time of flight-MS ESI-time of flight-MS	228
Insulin Insulin chain A Insulin chain B	DTPA loaded with Lu(III)	Reversed phase nanoHPCL	ICP-MS ESI-quadrupole/time of flight-MS	232
Lysozyme Bovine serum albumin	MeCAT-Eu (Proteome Factory AG, Berlin, Germany)	Reversed phase HPCL	ICP-MS	7
Lysozyme Insulin Ribonuclease A	MMA-DOTA loaded with Eu(III)	Reversed phase HPCL	ICP-MS ESI-ion trap-MS	227
Bradykinin Substance P	DTPA loaded with Eu(III)	Reversed phase HPCL	ICP-quadrupole-MS UV ESI-MS/MS	248
$B\beta_{15-42}$	DOTA loaded with In(III)	Reversed phase HPCL	ICP-dynamic reaction cell-MS ESI-time of flight-MS	243
Bovine serum albumin Lysozyme	DOTA loaded with lanthanides	SDS-PAGE Semidry blotting	LA-ICP-MS nanoESI-ion cyclotron resonance Fourier transform -MS	207
Bradykinin	DOTA-NHS-ester loaded with Eu(III)	Reversed phase HPLC Gas chromatography	ICP-quadrupole-MS ESI-ion trap-MS	249

Sample type	Labeling tag	Separation technique	Detector	Ref.
Aprotinin::β-galactosidase fusion protein	MeCAT reagent loaded with Ho(III) and Lu(III)	2-D SDS-PAGE Reversed phase nanoHPLC	MS GC-MS ICP-MS ESI-quadrupole/time of flight-MS	250
Bovine serum albumin *Escherichia coli* cell lysate	MeCAT reagent loaded with Tb(III), Ho(III), Tm(III), Lu(III)	Reversed phase nanoHPLC	ESI- linear triple quadrupole/ion cyclotron resonance Fourier transform -MS	231
Bovine serum albumin Human serum albumin Cysteine-containing synthetic standard peptides	MeCAT reagent and DOTA-NHS ester loaded with lanthanides	Reversed phase nanoHPLC	ESI-ion cyclotron resonance Fourier transform -MS ICP-MS	239
Bovine serum albumin Ovalbumin β -casein Proteinaceous binders (animal glue, egg yolk, egg white, whole egg, casein)	DTPA loaded with Eu(III)	Reversed phase HPLC	ICP-MS MALDI-time of flight-MS	251
Vasopressin Oxytocin RNase A Somatostatin Cytochrome C Lysozyme	azido-DOTA loaded with Eu(III)	Reversed phase HPLC	ESI-ion trap-MS ICP-MS	252
Bovine serum albumin Chymotrypsin Elastase Carbonic anhydrase α-lactalbumin	MeCAT- iodoacetamide reagent loaded with Eu(III), Tb(III), Lu(III), Tm(III)	SDS-PAGE Reversed phase nanoHPLC Reversed phase HPLC	ICP-MS ESI-linear triple quadrupole/ion cyclotron resonance Fourier	226

Sample type	Labeling tag	Separation technique	Detector	Ref.
Lysozyme Human serum albumin Transferrin Human serum samples	MeCAT- iodoacetamide reagent loaded with Yb(III)	SDS-PAGE	transform-MS LA-ICP-MS	253
Lysozyme Bovine serum albumin Transferrin	MeCAT reagent loaded with Eu(III), Ho(III), Lu(III), Tm(III)	2-D strong cation exchange and reversed-phase HPLC	ICP-MS ESI- linear triple quadrupole/ion cyclotron resonance Fourier transform -MS	254
β-lactoglobulin Bovine serum albumin	MeCAT- iodoacetamide reagent loaded with Ho(III)	Reversed phase nanoHPLC	ESI- linear triple quadrupole/ion cyclotron resonance Fourier transform -MS MALDI-time of flight-MS	255
Synthetic model peptides	DOTA-NHS ester loaded with Tm(III) and Tb(III)	Reversed phase HPLC	ESI- linear triple quadrupole/ion cyclotron resonance Fourier transform -MS Nanospray source-quadrupole/ion trap-MS	256
Bovine serum albumin α-lactalbumin β-lactoglobin Myoglobin (nonapo form) Lysozyme Bovine apotransferrin Bovine insulin	DTPA loaded with Y(III) and Tb(III)	Reversed phase HPLC	MALDI-time of flight-MS ESI-quadrupole/time of flight-MS	224
RNase A Cytochrome c Lysozyme	DTPAA loaded with Ce(III) and Sm(III)	Cation exchange HPLC	ICP-MS	233
Synthetic model peptides Lysozyme	DOTA-NHS-ester loaded with Ho(III), Tm(III), Lu(III), Er(III)	Nano ion pairing reversed-phase HPLC	ICP-MS MALDI-time of flight/time	225

Sample type	Labeling tag	Separation technique	Detector	Ref.
			of flight-MS	
Myoglobin Transferrin Thyroglobulin	Ru-NHS ester	Size exclusion HPLC	ICP-quadrupole-MS ICP-sector field-MS	257
Angiotensin I Angiotensin II Bradykinin MARCKS peptide clip r-fetoprotein	DOTA-NHS-ester loaded with Tb(III), Tm(III) and Ho(III)	Reversed phase HPLC	ICP-collision cell-MS MALDI-MS ESI-quadrupole/ion trap-MS	258
Human chorionic gonadotropin Carcinoembryonic antigen Ovarian tumor antigen Gastrointestinal tumor antigen	Antibodies labeled with Pr(III), Eu(III), Gd(III), Ho(III), and Tb(III)	Size exclusion HPLC	UV ICP-MS	240

Concluding remarks.

The necessity to understand fundamental biological processes in living organism has led to an accelerated development of accurate, relative or absolute methods for quantitation of peptides and proteins related to the metabolism or to certain pathological conditions (e.g. HbA1c as a marker of diabetes, or transferrin glycosylation in the recognition of alcoholism). The detection of endogenous metal(loid)s or metal-tag covalently bound to proteins has been recognized as a powerful complementary approach to modern techniques as ESI or MALDI MS (which misses the comparability of different analytes due to different ionization behaviour) or the classical ELISA and Western blot test.

The absolute quantitation of proteins is also fundamental especially for the pharmaceutical industry. As a matter of fact, the yield of a purification procedure obtained with the common enzymatic assays measures activities rather than actual amounts, and immunological assays rely on antibodies, which are difficult to assess for specificity and activity. Thus, metal coded tagging may open an alternative approach to protein quantitation when antibody-based approaches reach their limits.

The relative quantitation with respect to a comparative proteome analysis has recognized to be important, but the challenge for the future is the ability to perform absolute protein quantitation for a large numbers of proteins. It is also necessary to validate the proposed methods to demonstrate their applicability and robustness for their application to real samples.

Hyphenated techniques are an attractive tool for a rapid, sensitive and comprehensive characterization and quantitative determination of metal–protein complexes in biological samples.

The development and improvement of ICP-MS based methods, free of interferences will be fundamental for the absolute protein quantitation based on endogenous elements, such as phosphorous and sulphur.

The development of quantitative MS-based proteomics strategies is still a great challenge due to various limitations, as the low concentration level of the biomarkers, the lack of available standards

and instrumental limitations. Moreover, a disadvantage of absolute metal-binding proteins quantitation is frequently the lack of internationally recognized certified reference materials of known purity and international conventional measurement procedures. The acceptance of elemental labels for biological studies and their entry into biochemical and clinical laboratories will strongly depend on these two key points, i.e. the development of reliable procedures and the commercial availability of standards and certified reference materials that would permit the validation of hyphenated techniques against the classical methods.

List of abbreviations

AE Anion-exchange

AFS Atomic fluorescence spectroscopy

ASP Aspartic acid

CDIT Culture-derived isotope tags

CE Capillary electrophoresis

CE-LIF Capillary electrophoresis-laser induced fluorescence

CID Collision-induced dissociation

Cp Ceruloplasmin

CVG Chemical vapor generation

CYS Cysteine

CZE Capillary zone electrophoresis

DOTA Tetraazacyclododecane

DTPA Diethylenetriaminepentaacetate

DTT Dithiothreitol

ECD Electron capture dissociation

ELISA Enzyme-linked immunosorbent assay

ESI Electrospray ionization

ETD Electron transfer dissociation

EtHg Ethylmercury

FAAS Flame atomic absorption spectroscopy

FMEA Ferrocenecarboxylic acid(2-maleimidoyl)ethylamide

GLU Glutamic acid

GPx Glutathione peroxidases

HPLC High-performance liquid chromatography

ICAT Isotope coded affinity tag

ICP-MS Inductively coupled plasma-mass spectrometry

ICP-OES Inductively coupled plasma-optical emission spectroscopy

IDA Isotope dilution analysis

IE ion exchange

IMAC ion mobility affinity chromatography

IEF Isoelectric focusing

IPy_2BF_4 bis(pyridine)iodonium tetrafluoroborate

iTRAQ Isobaric tag for relative and absolute quantitation

LA Laser ablation

LYS Lysine

LOD Limit of detection

MALDI Matrix-assisted laser desorption ionization

MeCAT Metals-coded affinity tag

MeHg Metylmercury

MFOHg Mono-functional organic mercurial probes

MMA Maleimido-mono-amide

MS Mass spectrometry

MT Metallothionein

MW Microwaves

NOTA 1,4,7-triazacyclononane-N,N',N''-triacetic acid

PAGE Polyacrylamide gel electrophoresis

PDB Protein data bank

***p*HMB** 4(hydroxymercuric)benzoic acid

PROTEIN-AQUA Protein absolute quantitation

RP Reversed-phase

SCN Isothiocyanates

SDS Sodium dodecylsulfate

SE Size-exclusion

SeAlb Selenoalbumin

SeCys Selenium-L-cystine

SF Sector field

SFP Succinimidylferrocenyl propionate

SILAC Stable isotope labeling by amino acids in cell culture

SPE Solid phase extraction

TCEP 3,3',3''-phosphanetriyltripropanoic acid

TrxR Thioredoxin reductases

VSG Volatile species generation

References.

1. S. Lehmann, A. Hoofnagle, D. Hochstrasser, C. Brede, M. Glueckmann, J. A. Cocho, U. Ceglarek, C. Lenz, J. Vialaret, A. Scherl, C. Hirtz and I. W. G. C. Quantita, *Clinical Chemistry and Laboratory Medicine*, 2013, 51, 919-935.
2. D. S. Kirkpatrick, S. A. Gerber and S. P. Gygi, *Methods*, 2005, 35, 265-273.
3. B. L. Ackermann and M. J. Berna, *Expert Review of Proteomics*, 2007, 4, 175-186.
4. S. Pan, R. Aebersold, R. Chen, J. Rush, D. R. Goodlett, M. W. McIntosh, J. Zhang and T. A. Brentnall, *Journal of Proteome Research*, 2009, 8, 787-797.
5. R. Aebersold and D. R. Goodlett, *Chemical Reviews*, 2001, 101, 269-295.
6. M. Bantscheff, S. Lemeer, M. M. Savitski and B. Kuster, *Analytical and Bioanalytical Chemistry*, 2012, 404, 939-965.
7. D. Esteban-Fernandez, C. Scheler and M. W. Linscheid, *Analytical and Bioanalytical Chemistry*, 2011, 401, 657-666.
8. S. P. Mirza and M. Olivier, *Physiological Genomics*, 2008, 33, 3-11.
9. A. Leitner and W. Lindner, *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 2004, 813, 1-26.
10. A. Miseta and P. Csutora, *Molecular Biology and Evolution*, 2000, 17, 1232-1239.
11. A. Leitner and W. Lindner, *Journal of Chromatography B*, 2004, 813, 1-26.
12. K. Amarnath, V. Amarnath, K. Amarnath, H. L. Valentine and W. M. Valentine, *Talanta*, 2003, 60, 1229-1238.
13. A. Pastore, R. Massoud, C. Motti, A. Lo Russo, G. Fucci, C. Cortese and G. Federici, *Clinical Chemistry*, 1998, 44, 825-832.
14. J. Russell and D. L. Rabenstein, *Analytical Biochemistry*, 1996, 242, 136-144.
15. N. Ercal, P. Yang and N. Aykin, *Journal of Chromatography B*, 2001, 753, 287-292.
16. T. Oe, T. Ohyagi and A. Naganuma, *Journal of Chromatography B*, 1998, 708, 285-289.

17. H. Wang, S. C. Liang, Z. M. Zhang and H. S. Zhang, *Analytica Chimica Acta*, 2004, 512, 281-286.
18. A. Karlstrom and P. A. Nygren, *Analytical Biochemistry*, 2001, 295, 22-30.
19. Y. Guo, L. Chen, L. Yang and Q. Wang, *Journal of the American Society for Mass Spectrometry*, 2008, 19, 1108-1113.
20. B. P. Duckworth, Z. Zhang, A. Hosokawa and M. D. Distefano, *Chembiochem*, 2007, 8, 98-105.
21. S. P. Perfetto, P. K. Chattopadhyay and M. Roederer, *Nature Reviews Immunology*, 2004, 4, 648-U645.
22. S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey and M. Mann, *Molecular & Cellular Proteomics*, 2002, 1, 376-386.
23. O. A. Mirgorodskaya, Y. P. Kozmin, M. I. Titov, R. Korner, C. P. Sonksen and P. Roepstorff, *Rapid Communications in Mass Spectrometry*, 2000, 14, 1226-1232.
24. S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold, *Nature Biotechnology*, 1999, 17, 994-999.
25. Y. Ishihama, T. Sato, T. Tabata, N. Miyamoto, K. Sagane, T. Nagasu and Y. Oda, *Nature Biotechnology*, 2005, 23, 617-621.
26. P. L. Ross, Y. L. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson and D. J. Pappin, *Molecular & Cellular Proteomics*, 2004, 3, 1154-1169.
27. S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner and S. P. Gygi, *Proceedings of the National Academy of Sciences of the United States of America*, 2003, 100, 6940-6945.
28. M. Wang, W.-Y. Feng, Y.-L. Zhao and Z.-F. Chai, *Mass Spectrometry Reviews*, 2010, 29, 326-348.
29. A. Prange and D. Proefrock, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 432-459.

30. S. Bomke, M. Sperling and U. Karst, *Analytical and Bioanalytical Chemistry*, 2010, 397, 3483-3494.
31. K. J. Waldron and N. J. Robinson, *Nature Reviews Microbiology*, 2009, 7, 25-35.
32. D. F. Shriver and P. W. Atkins, in *Inorganic Chemistry*, Oxford University Press, 1999.
33. M. Melvin, ed. D. Kealy, John Wiley & Sons, London, 1987.
34. Y. M. C. v. D.-V. Henskens, M.P., *Ned Tijdschr Klin Chem*, 2000, 25, 219-229.
35. C. Wolf, D. Schaumloffel, A. N. Richarz, A. Prange and P. Bratter, *Analyst*, 2003, 128, 576-580.
36. J. W. Olesik, J. A. Kinzer and S. V. Olesik, *Analytical Chemistry*, 1995, 67, 1-12.
37. R. M. Barnes, *Fresenius Journal of Analytical Chemistry*, 1998, 361, 246-251.
38. J. W. Olesik, J. A. Kinzer, E. J. Grunwald, K. K. Thaxton and S. V. Olesik, *Spectrochimica Acta Part B-Atomic Spectroscopy*, 1998, 53, 239-251.
39. S. R. Haider, B. L. Sharp and H. J. Reid, *Trac-Trends in Analytical Chemistry*, 2011, 30, 1793-1808.
40. M. Bantscheff, M. Schirle, G. Sweetman, J. Rick and B. Kuster, *Analytical and Bioanalytical Chemistry*, 2007, 389, 1017-1031.
41. D. B. Friedman, S. Hoving and R. Westermeier, in *Guide to Protein Purification, Second Edition*, eds. R. R. Burgess and M. P. Deutscher, 2009, vol. 463, pp. 515-540.
42. D. E. Garfin, in *Guide to Protein Purification, Second Edition*, eds. R. R. Burgess and M. P. Deutscher, 2009, vol. 463, pp. 497-513.
43. J. Szpunar, *Analyst*, 2000, 125, 963-988.
44. J. W. I. Brunnekreeft and H. H. M. Eidhof, *Clinical Chemistry*, 1993, 39, 2514-2518.
45. J. S. Rohrer and N. Avdalovic, *Protein Expression and Purification*, 1996, 7, 39-44.
46. H. Chassaigne and J. Szpunar, *Analusis*, 1998, 26, M48-M51.
47. R. L. Garlick, J. S. Mazer, P. J. Higgins and H. F. Bunn, *Journal of Clinical Investigation*, 1983, 71, 1062-1072.

48. U. Gless, Y. Schmitt and J. D. Krusejarres, *Fresenius Journal of Analytical Chemistry*, 1992, 343, 88-89.

49. M. Kastner, in *Protein Liquid Chromatography*, ed. Elsevier, Amsterdam, 2000, vol. 61.

50. D. A. Skoog, in *Chapter 28*, ed. T. Brooks/Cole, 2006, ch. 28.

51. H. Koyama, K. Omura, A. Ejima, Y. Kasanuma, C. Watanabe and H. Satoh, *Analytical Biochemistry*, 1999, 267, 84-91.

52. J. Szpunar, A. Makarov, T. Pieper, B. K. Keppler and R. Lobinski, *Analytica Chimica Acta*, 1999, 387, 135-144.

53. W. Goessler, D. Kuehnelt, C. Schlagenhaufen, Z. Slejkovec and K. J. Irgolic, *Journal of Analytical Atomic Spectrometry*, 1998, 13, 183-187.

54. Y. Inoue, Y. Date, T. Sakai, N. Shimizu, K. Yoshida, H. Chen, K. Kuroda and G. Endo, *Applied Organometallic Chemistry*, 1999, 13, 81-88.

55. D. Schaumloffel, J. R. Encinar and R. Lobinski, *Analytical Chemistry*, 2003, 75, 6837-6842.

56. W. Shi and M. R. Chance, *Current Opinion in Chemical Biology*, 2011, 15, 144-148.

57. J. Davies and H. Berndt, *Analytica Chimica Acta*, 2003, 479, 215-223.

58. H. G. Infante, M. L. F. Sanchez and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 1999, 14, 1343-1348.

59. K. T. Suzuki, *Analytical Biochemistry*, 1980, 102, 31-34.

60. D. Sanchez-Rodas, W. T. Corns, B. Chen and P. B. Stockwell, *Journal of Analytical Atomic Spectrometry*, 2010, 25, 933-946.

61. X. J. Hou, B., in *Encyclopedia of Analytical Chemistry*, ed. R. A. Meyers, John Wiley&Sons Ltd, 2000, pp. 9468-9485.

62. A. Mazzucotelli and P. Rivaro, *Microchemical Journal*, 1995, 51, 231-237.

63. G. L. Gouy, *Annales de chimie et de physique*, 1879, 18, 5-101.

64. J. Szpunar, H. Chassaigne, O. Donard, J. Bettmer and R. Lobinski, *Applications of Inductively Coupled Plasma Mass Spectrometry*, RSC, 1997.

65. S. C. K. Shum and R. S. Houk, *Analytical Chemistry*, 1993, 65, 2972-2976.

66. H. Berndt, *Z. Anal. Chem.*, 1988, 331, 321-323.

67. J. L. Neilsen, A. Abildtrup, J. Christensen, P. Watson, A. Cox and C. W. McLeod, *Spectrochimica Acta Part B-Atomic Spectroscopy*, 1998, 53, 339-345.

68. D. J. Kutscher, M. B. Fricker, B. Hattendorf, J. Bettmer and D. Guenther, *Analytical and Bioanalytical Chemistry*, 2011, 401, 2691-2698.

69. T. V. O'Halloran and V. C. Culotta, *Journal of Biological Chemistry*, 2000, 275, 25057-25060.

70. J. D. Fassett and H. M. Kingston, *Analytical Chemistry*, 1985, 57, 2474-2478.

71. C. L. Deitrich, S. Braukmann, A. Raab, C. Munro, B. Pioselli, E. M. Krupp, J. E. Thomas-Oates and J. Feldmann, *Analytical and Bioanalytical Chemistry*, 2010, 397, 3515-3524.

72. M. Estela del Castillo Busto, M. Montes-Bayon and A. Sanz-Medel, *Analytical Chemistry*, 2006, 78, 8218-8226.

73. E. del Castillo, M. Montes-Bayon, E. Anon and A. Sanz-Medel, *Journal of Proteomics*, 2011, 74, 35-43.

74. J. Bettmer, *Analytical and Bioanalytical Chemistry*, 2010, 397, 3495-3502.

75. T. E. Thingholm, O. N. Jensen and M. R. Larsen, *Proteomics*, 2009, 9, 1451-1468.

76. L. Hu, M. Ye, X. Jiang, S. Feng and H. Zou, *Analytica Chimica Acta*, 2007, 598, 193-204.

77. G. Schwarz, S. Beck, M. G. Weller and M. W. Linscheid, *Journal of Mass Spectrometry*, 2012, 47, 885-889.

78. R. Aebersold and M. Mann, *Nature*, 2003, 422, 198-207.

79. E. I. Solomon, R. K. Szilagyi, S. D. George and L. Basumallick, *Chemical Reviews*, 2004, 104, 419-458.

80. W. Shi, M. Punta, J. Bohon, J. M. Sauder, R. D'Mello, M. Sullivan, J. Toomey, D. Abel, M. Lippi, A. Passerini, P. Frasconi, S. K. Burley, B. Rost and M. R. Chance, *Genome Research*, 2011, 21, 898-907.

81. J. L. Szpunar, R., in *Hyphenated Techniques in Speciation Analysis*, ed. R. M. Smith, Royal Society of Chemistry, 2003.

82. I. L. Heras, M. Palomo and Y. Madrid, *Analytical and Bioanalytical Chemistry*, 2011, 400, 1717-1727.

83. S. Letsiou, Y. Lu, T. Nomikos, S. Antonopoulou, D. Panagiotakos, C. Pitsavos, C. Stefanadis and S. A. Pergantis, *Proteomics*, 2010, 10, 3447-3457.

84. X. Du, H. Li, Z. Wang, S. Qiu, Q. Liu and J. Ni, *Metallomics*, 2013, 5, 861-870.

85. M. Xu, L. Yang and Q. Wang, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 1545-1549.

86. B. M. Akesson, B., *Journal of Inorganic Biochemistry*, 1988, 33, 257-261.

87. B. Akesson and B. Martensson, *International Journal for Vitamin and Nutrition Research*, 1991, 61, 72-76.

88. J. T. Deagen, J. A. Butler, B. A. Zachara and P. D. Whanger, *Analytical Biochemistry*, 1993, 208, 176-181.

89. P. Jitaru, M. Roman, G. Cozzi, P. Fisicaro, P. Cescon and C. Barbante, *Microchimica Acta*, 2009, 166, 319-327.

90. K. Shigeta, K. Sato and N. Furuta, *Journal of Analytical Atomic Spectrometry*, 2007, 22, 911-916.

91. P. Jitaru, M. Prete, G. Cozzi, C. Turetta, W. Cairns, R. Seraglia, P. Traldi, P. Cescon and C. Barbante, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 402-406.

92. G. Ballihaut, L. E. Kilpatrick, E. L. Kilpatrick and W. C. Davis, *Metallomics*, 2012, 4, 533-538.

93. G. Ballihaut, L. E. Kilpatrick and W. C. Davis, *Analytical Chemistry*, 2011, 83, 8667-8674.

94. G. Ballihaut, L. E. Kilpatrick, E. L. Kilpatrick and W. C. Davis, *Journal of Analytical Atomic Spectrometry*, 2011, 26, 383-394.

95. C. C. Chery, H. Chassaigne, L. Verbeeck, R. Cornelis, F. Vanhaecke and L. Moens, *Journal of Analytical Atomic Spectrometry*, 2002, 17, 576-580.

96. L. Yang, Z. Mester and R. E. Sturgeon, *Analytical Chemistry*, 2004, 76, 5149-5156.

97. J. R. Encinar, D. Schaumloffel, Y. Ogra and R. Lobinski, *Analytical Chemistry*, 2004, 76, 6635-6642.

98. O. Palacios, J. R. Encinar, D. Schaumloffel and R. Lobinski, *Analytical and Bioanalytical Chemistry*, 2006, 384, 1276-1283.

99. P. Giusti, D. Schaumloffel, J. R. Encinar and J. Szpunar, *Journal of Analytical Atomic Spectrometry*, 2005, 20, 1101-1107.

100. Y. Suzuki, T. Sakai and N. Furuta, *Analytical Sciences*, 2012, 28, 221-224.

101. L. H. Reyes, J. M. Marchante-Gayon, J. I. G. Alonso and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 2003, 18, 1210-1216.

102. G. Ballihaut, F. Claverie, C. Pecheyran, S. Mounicou, R. Grimaud and R. Lobinski, *Analytical Chemistry*, 2007, 79, 6874-6880.

103. M. Roman, P. Jitaru, M. Agostini, G. Cozzi, S. Pucciarelli, D. Nitti, C. Bedin and C. Barbante, *Microchemical Journal*, 2012, 105, 124-132.

104. L. Yang, P. Maxwell and Z. Mester, *Analytical Methods*, 2013, 5, 525-529.

105. J. Bianga, E. Govasmark and J. Szpunar, *Analytical Chemistry*, 2013, 85, 2037-2043.

106. M. E. del Castillo Busto, M. Montes-Bayon and A. Sanz-Medel, *Analytica Chimica Acta*, 2009, 634, 1-14.

107. T. M. Brewer and R. K. Marcus, *Analytical Chemistry*, 2007, 79, 2402-2411.

108. P. A. Krayenbuehl, T. Walczyk, R. Schoenberg, F. von Blanckenburg and G. Schulthess, *Blood*, 2005, 105, 3812-3816.

109. C. F. Harrington, S. Elahi, S. A. Merson and P. Ponnampalavanar, *Analytical Chemistry*, 2001, 73, 4422-4427.

110. M. Grebe, D. Proefrock, A. Kakuschke, J. A. C. Broekaert and A. Prangea, *Metallomics*, 2011, 3, 176-185.

111. M. Grebe, D. Proefrock, A. Kakuschke, M. E. del Castillo Busto, M. Montes-Bayon, A. Sanz-Medel, J. A. C. Broekaert and A. Prange, *Journal of Analytical Atomic Spectrometry*, 2012, 27, 440-448.

112. S. A. Rodriguez, E. B. Gonzalez, G. A. Llamas, M. Montes-Bayon and A. Sanz-Medel, *Analytical and Bioanalytical Chemistry*, 2005, 383, 390-397.

113. T. M. Brewer and R. K. Marcus, *Journal of Analytical Atomic Spectrometry*, 2007, 22, 1067-1075.

114. E. del Castillo Busto, M. Montes-Bayon, J. I. Garcia Alonso, J. A. Caruso and A. Sanz-Medel, *Analyst*, 2010, 135, 1538-1540.

115. M. G. Anorbe, R. Messerschmidt, I. Feldmann and N. Jakubowski, *Journal of Analytical Atomic Spectrometry*, 2007, 22, 917-924.

116. M. Hoppler, C. Zeder and T. Walczyk, *Analytical Chemistry*, 2009, 81, 7368-7372.

117. M. E. del Castillo Busto, M. Montes-Bayon, E. Anon and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 758-764.

118. P. C. Bull, G. R. Thomas, J. M. Rommens, J. R. Forbes and D. W. Cox, *Nature Genetics*, 1993, 5, 327-337.

119. S. Lutsenko, A. Bhattacharjee and A. L. Hubbard, *Metallomics*, 2010, 2, 596-608.

120. S. Hann, C. Obinger, G. Stingeder, M. Paumann, P. G. Furtmueller and G. Koellensperger, *Journal of Analytical Atomic Spectrometry*, 2006, 21, 1224-1231.

121. V. Lopez-Avila, O. Sharpe and W. H. Robinson, *Analytical and Bioanalytical Chemistry*, 2006, 386, 180-187.

122. S. El Balkhi, J. Poupon, J.-M. Trocello, F. Massicot, F. Woimant and O. Laprevote, *Analytical Chemistry*, 2010, 82, 6904-6910.

123. K. Inagaki, N. Mikuriya, S. Morita, H. Haraguchi, Y. Nakahara, M. Hattori, T. Kinosita and H. Saito, *Analyst*, 2000, 125, 197-203.

124. D. Schaumloffel, A. Prange, G. Marx, K. G. Heumann and P. Bratter, *Analytical and Bioanalytical Chemistry*, 2002, 372, 155-163.

125. M. J. Stillman, C. F. Shaw and K. T. Suzuki, *Metallothionein: Synthesis, structure, and properties of metallothioneins, phytochelatins, and metal-thiolate complexes*, VHC, 1992.

126. J. Petrlova, S. Krizkova, O. Zitka, J. Hubalek, R. Prusa, V. Adam, J. Wang, M. Beklova, B. Sures and R. Kizek, *Sensors and Actuators B-Chemical*, 2007, 127, 112-119.

127. A. Prange and D. Schaumloffel, *Analytical and Bioanalytical Chemistry*, 2002, 373, 441-453.

128. H. Chassaigne and R. Lobinski, *Fresenius Journal of Analytical Chemistry*, 1998, 361, 267-273.

129. H. Chassaigne, S. Mounicou, C. Casiot, R. Lobinski and M. Potin-Gautier, *Analusis*, 2000, 28, 357-360.

130. K. Polec, S. Mounicou, H. Chassaigne and R. Lobinski, *Cellular and Molecular Biology*, 2000, 46, 221-235.

131. D. E. K. Sutherland and M. J. Stillman, *Metallomics*, 2014, DOI: 10.1039/c3mt00216k.

132. G. Alvarez-Llamas, M. R. F. de la Campa, M. L. F. Sanchez and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 2002, 17, 655-661.

133. G. Alvarez-Llamas, M. R. de la Campa and A. Sanz-Medel, *Analytica Chimica Acta*, 2005, 546, 236-243.

134. K. Polec-Pawlak, D. Schaumloffel, J. Szpunar, A. Prange and R. Lobinski, *Journal of Analytical Atomic Spectrometry*, 2002, 17, 908-912.

135. H. G. Infante, K. Van Campenhout, R. Blust and F. C. Adams, *Journal of Chromatography A*, 2006, 1121, 184-190.

136. H. G. Infante, F. Cuyckens, K. Van Campenhout, R. Blust, M. Claeys, L. Van Vaeck and F. C. Adams, *Journal of Analytical Atomic Spectrometry*, 2004, 19, 159-166.
137. M. Malavolta, F. Piacenza, L. Costarelli, R. Giacconi, E. Muti, C. Cipriano, S. Tesei, S. Spezia and E. Mocchegiani, *Journal of Analytical Atomic Spectrometry*, 2007, 22, 1193-1198.
138. D. Proefrock, P. Leonhard and A. Prange, *Analytical and Bioanalytical Chemistry*, 2003, 377, 132-139.
139. S. Santiago-Rivas, A. Moreda-Pineiro, A. Bermejo-Barrera and P. Bermejo-Barrera, *Talanta*, 2007, 71, 1580-1586.
140. Z. Pedrero, L. Ouerdane, S. Mounicou, R. Lobinski, M. Monperrus and D. Amouroux, *Metallomics*, 2012, 4, 473-479.
141. S. Mounicou, L. Ouerdane, B. L'Azou, I. Passagne, C. Ohayon-Courtes, J. Szpunar and R. Lobinski, *Analytical Chemistry*, 2010, 82, 6947-6957.
142. C. Ferrarello, M. R. F. de la Campa, H. G. Infante, M. L. F. Sanchez and A. Sanz-Medel, *Analusis*, 2000, 28, 351-357.
143. C. Wolf, U. Rosick and P. Bratter, *Fresenius Journal of Analytical Chemistry*, 2000, 368, 839-843.
144. A. Rodriguez-Cea, M. D. F. de la Campa, E. B. Gonzalez, B. A. Fernandez and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 2003, 18, 1357-1364.
145. K. Nostelbacher, M. Kirchgessner and G. I. Stangl, *Journal of Chromatography B*, 2000, 744, 273-282.
146. T. Miyayama, Y. Ogra and K. T. Suzuki, *Journal of Analytical Atomic Spectrometry*, 2007, 22, 179-182.
147. A. N. Richarz and P. Bratter, *Analytical and Bioanalytical Chemistry*, 2002, 372, 412-417.
148. H. Goenaga Infante, M. L. Fernandez Sanchez and A. Sanz-Medel, *Journal of Analytical Atomic Spectrometry*, 2000, 15, 519-524.

149. S. Mounicou, K. Polec, H. Chassaigne, M. Potin-Gautier and R. Lobinski, *Journal of Analytical Atomic Spectrometry*, 2000, 15, 635-642.

150. M. Montes-Bayon, D. Profrock, A. Sanz-Medel and A. Prange, *Journal of Chromatography A*, 2006, 1114, 138-144.

151. A. Prange and D. Profrock, *Analytical and Bioanalytical Chemistry*, 2005, 383, 372-389.

152. A. Sanz-Medel, M. Montes-Bayón, J. Bettmer, M. Luisa Fernández-Sanchez and J. Ruiz Encinar, *TrAC Trends in Analytical Chemistry*, 2012, 40, 52-63.

153. K. Takatera and T. Watanabe, *Analytical Chemistry*, 1993, 65, 3644-3646.

154. I. B. Bošnjak, V.; Šegvić-Bubić, T.; Bielen, A., *Protein Engineering, Design and Selection*, 2014, in press, doi:10.1093/protein/gzt063.

155. E. D. U. Bramanti, A.; Lampugnani, L.; Zamboni, R.; Raspi, G., *J. Anal. At. Spectrom.*, 1999, 14, 179-185.

156. E. J. Zaluzec, D. A. Gage and J. T. Watson, *Journal of the American Society for Mass Spectrometry*, 1994, 5, 359-366.

157. Y. F. Guo, L. Q. Chen, L. M. Yang and Q. Q. Wang, *Journal of the American Society for Mass Spectrometry*, 2008, 19, 1108-1113.

158. D. J. Kutscher, M. E. D. Busto, N. Zinn, A. Sanz-Medel and J. Bettmer, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 1359-1364.

159. J. Qian, U. Skyllberg, W. Frech, W. F. Bleam, P. R. Bloom and P. E. Petit, *Geochimica Et Cosmochimica Acta*, 2002, 66, 3873-3885.

160. D. T. Jiang, S. M. Heald, T. K. Sham and M. J. Stillman, *Journal of the American Chemical Society*, 1994, 116, 11004-11013.

161. G. Schwarzenbach, *Helvetica Chimica Acta*, 1965, 48, 28-46.

162. M. Xu, X. W. Yan, Q. Q. Xie, L. M. Yang and Q. Q. Wang, *Analytical Chemistry*, 2010, 82, 1616-1620.

163. E. Bramanti, A. D'Ulivo, L. Lampugnani, R. Zamboni and G. Raspi, *Analytical Biochemistry*, 1999, 274, 163-173.

164. E. Bramanti, S. Lucchesini, A. D'Ulivo, L. Lampugnani, R. Zamboni, M. C. Spinetti and G. Raspi, *Journal of Analytical Atomic Spectrometry*, 2001, 16, 166-171.

165. E. Bramanti, C. Lomonte, A. Galli, M. Onor, R. Zamboni, G. Raspi and A. D'Ulivo, *Journal of Chromatography A*, 2004, 1054, 285-291.

166. E. Bramanti, C. Lomonte, M. Onor, R. Zamboni, G. Raspi and A. D'Ulivo, *Analytical and Bioanalytical Chemistry*, 2004, 380, 310-318.

167. E. Bramanti, C. Sortino, C. Lomonte, M. Onor, R. Zamboni, G. Raspi and A. D'Ulivo, *Talanta*, 2004, 63, 383-389.

168. E. Bramanti, V. Angeli, Z. Mester and A. D'Ulivo, *Talanta*, 2009, 79, 554-555.

169. E. Bramanti, V. Angeli, Z. Mester, A. Pompella, A. Paolicchi and A. D'Ulivo, *Talanta*, 2010, 81, 1295-1299.

170. E. Bramanti, L. D'Ulivo, C. Lomonte, M. Onor, R. Zamboni, G. Raspi and A. D'Ulivo, *Analytica Chimica Acta*, 2006, 579, 38-46.

171. E. Bramanti, R. Cavallaro, M. Onor, R. Zamboni and A. D'Ulivo, *Talanta*, 2008, 74, 936-943.

172. M. Xu, X. Yan, Q. Xie, L. Yang and Q. Wang, *Analytical Chemistry*, 2010, 82, 1616-1620.

173. D. J. Kutscher and J. Bettmer, *Analytical Chemistry*, 2009, 81, 9172-9177.

174. C. F. Harrington, *Trends Anal. Chem*, 2000, 19, 167.

175. J. E. S.-M. Sanchez Ur´ya, A., *Talanta*, 1998, 47, 509.

176. A. M. M. Carro, M.C., *Journal of Chromatography A*, 2000, 882, 283.

177. H. T. Morita, H.; Shimomura, S., *Spectrochim. Acta, Part B*, 1995, 50, 69.

178. S. R. ı.-S. E. Ramalhosa, E. Pereira, C. Vale, A. Duarte, *Analytica Chimica Acta*, 2001, 448, 135.

179. W. T. C. P.B. Stockwell, D.W. Bryce, *Pittcon - Abstracts*, 2000, 1840.

180. R. D. W. H. Hintelmann, *Appl. Organomet. Chem.*, 1993, 7, 173.

181. J. A. C. B. C. Schickling, *Appl. Organomet. Chem.*, 1995, 9, 29.

182. G.-B. J. L.-N. Liang, J.-F. Liu, J.-T. Hu, *Analytica Chimica Acta*, 2003, 447, 131.

183. S. R. ı.-S. E. Ramalhosa, E. Pereira, C. Vale, A. Duarte, *Journal of Analytical Atomic Spectrometry*, 2001, 16, 643.

184. H. F. S. o. R. Falter, Fresenius, *J. Anal. Chem.*, 1995, 353, 34.

185. V. Angeli, C. Ferrari, I. Longo, M. Onor, A. D'Ulivo and E. Bramanti, *Analytical Chemistry*, 2011, 83, 338-343.

186. R. S. Falter, H. F., *Journal of Chromatography A*, 1994, 675, 253-256.

187. C. Bendicho, F. Pena, M. Costas, S. Gil and I. Lavilla, *Trac-Trends in Analytical Chemistry*, 2010, 29, 681-691.

188. L. Tang, F. Chen, L. M. Yang and Q. Q. Wang, *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 2009, 877, 3428-3433.

189. L. N. Liang, G. B. Jiang, J. F. Liu and J. T. Hu, *Analytica Chimica Acta*, 2003, 477, 131-137.

190. A. Morales-Rubio, M. L. Mena and C. W. McLeod, *Analytica Chimica Acta*, 1995, 308, 364-370.

191. I. Longo and A. S. Ricci, *Journal of Microwave Power and Electromagnetic Energy*, 2007, 41, 4-19.

192. C. Ferrari, I. Longo, E. Tombari and E. Bramanti, *Journal of Photochemistry and Photobiology a-Chemistry*, 2009, 204, 115-121.

193. M. Xu, L. Yang and Q. Wang, *Chemistry-a European Journal*, 2012, 18, 13989-13993.

194. D. J. Kutscher, M. E. del Castillo Busto, N. Zinn, A. Sanz-Medel and J. Bettmer, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 1359-1364.

195. Y. Guo, M. Xu, L. Yang and Q. Wang, *Journal of Analytical Atomic Spectrometry*, 2009, 24, 1184-1187.

196. E. Bramanti, D. Toncelli, E. Morelli, L. Lampugnani, R. Zamboni, K. E. Miller, J. Zemetra and A. D'Ulivo, *Journal of Chromatography A*, 2006, 1133, 195-203.

197. B. Campanella, J. Gonzalez Rivera, C. Ferrari, S. Biagi, M. Onor, A. D'Ulivo and E. Bramanti, *Analytical Chemistry*, 2013, 85, 12152-12157.

198. X. D. Bu, T. B. Wang and G. Hall, *Journal of Analytical Atomic Spectrometry*, 2003, 18, 1443-1451.

199. A. Pereira Navaza, J. Ruiz Encinar, A. Ballesteros, J. M. Gonzalez and A. Sanz-Medel, *Analytical chemistry*, 2009, 81, 5390-5399.

200. A. Tholey and D. Schaumloeffel, *Trac-Trends in Analytical Chemistry*, 2010, 29, 399-408.

201. N. Jakubowski, J. Messerschmidt, M. G. Anorbe, L. Waentig, H. Hayen and P. H. Roos, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 1487-1496.

202. T. J. Tsomides and H. N. Eisen, *Analytical Biochemistry*, 1993, 210, 129-135.

203. M. A. K. Markwell, *Analytical Biochemistry*, 1982, 125, 427-432.

204. F. Frantzen, D. E. Heggli and E. Sundrehagen, *Biotechnology and Applied Biochemistry*, 1995, 22, 161-167.

205. G. Vaidyanathan and M. R. Zalutsky, *Nature Protocols*, 2006, 1, 707-713.

206. G. Vaidyanathan, E. Jestin, T. Olafsen, A. M. Wu and M. R. Zalutsky, *Nuclear Medicine and Biology*, 2009, 36, 671-680.

207. N. Jakubowski, L. Waentig, H. Hayen, A. Venkatachalam, A. von Bohlen, P. H. Roos and A. Manz, *Journal of Analytical Atomic Spectrometry*, 2008, 23, 1497-1507.

208. L. Waentig, N. Jakubowski, H. Hayen and P. H. Roos, *Journal of Analytical Atomic Spectrometry*, 2011, 26, 1610-1618.

209. A. A. Oliveira, L. C. Trevizan and J. A. Nobrega, *Applied Spectroscopy Reviews*, 2010, 45, 447-473.

210. B. Seiwert, H. Hayen and U. Karst, *Journal of the American Society for Mass Spectrometry*, 2008, 19, 1-7.

211. D. R. van Staveren and N. Metzler-Nolte, *Chemical Reviews*, 2004, 104, 5931-5985.

212. B. Seiwert and U. Karst, *Analytical and Bioanalytical Chemistry*, 2008, 390, 181-200.

213. C. Ingle, N. Langford, L. Harvey, J. R. Dainty, C. Armah, S. Fairweather-Tait, B. Sharp, H. Crews, M. Rose and J. Lewis, *Journal of Analytical Atomic Spectrometry*, 2002, 17, 1498-1501.

214. M. Moberg, E. M. Nilsson, S. J. M. Holmstrom, U. S. Lundstrom, J. Pettersson and K. E. Markides, *Analytical Chemistry*, 2004, 76, 2618-2622.

215. S. Bomke, T. Pfeifer, B. Meermann, W. Buscher and U. Karst, *Analytical and Bioanalytical Chemistry*, 2010, 397, 3503-3513.

216. R. W. Giese and B. L. Vallee, *Journal of Americal Chemical Society*, 1972, 94, 6199–6200.

217. M. Peterlik, *Monatshefte für Chemie*, 1967, 98, 2133–2134.

218. H. Eckert and M. Koller, *Journal of Liquid Chromatography*, 1990, 13, 3399-3414.

219. A. Brautigam, S. Bomke, T. Pfeifer, U. Karst, G.-J. Krauss and D. Wesenberg, *Metallomics*, 2010, 2, 565-570.

220. T. Tanaka, K. Izawa, M. Okochi, T.-K. Lim, S. Watanabe, M. Harada and T. Matsunaga, *Analytica Chimica Acta*, 2009, 638, 186-190.

221. P. H. Roos, A. Venkatachalam, A. Manz, L. Waentig, C. U. Koehler and N. Jakubowski, *Analytical and Bioanalytical Chemistry*, 2008, 392, 1135-1147.

222. B. Seiwert and U. Karst, *Analytical and Bioanalytical Chemistry*, 2007, 388, 1633-1642.

223. S. Cotton, in *Inorganic chemistry:A Textbook Series*, Wiley; 2nd edition, 2006.

224. H. Liu, Y. Zhang, J. Wang, D. Wang, C. Zhou, Y. Cai and X. Qian, *Analytical Chemistry*, 2006, 78, 6614-6621.

225. A. Holste, A. Tholey, C.-W. Hung and D. Schaumloeffel, *Analytical Chemistry*, 2013, 85, 3064-3070.

226. G. Schwarz, S. Beck, D. Benda and M. W. Linscheid, *Analyst*, 2013, 138, 2449-2455.

227. X. Yan, M. Xu, L. Yang and Q. Wang, *Analytical Chemistry*, 2010, 82, 1261-1269.

228. R. Ahrends, S. Pieper, A. Kuehn, H. Weisshoff, M. Hamester, T. Lindemann, C. Scheler, K. Lehmann, K. Taubner and M. W. Linscheid, *Molecular & Cellular Proteomics*, 2007, 6, 1907-1916.

229. D. Kretschy, G. Koellensperger and S. Hann, *Metallomics*, 2011, 3, 1304-1309.

230. P. A. Whetstone, N. G. Butlin, T. M. Corneillie and C. F. Meares, *Bioconjugate Chemistry*, 2004, 15, 3-6.

231. R. Ahrends, S. Pieper, B. Neumann, C. Scheler and M. W. Linscheid, *Analytical Chemistry*, 2009, 81, 2176-2184.

232. C. Rappel and D. Schaumloeffel, *Analytical Chemistry*, 2009, 81, 385-393.

233. L.-N. Zheng, M. Wang, H.-J. Wang, B. Wang, B. Li, J.-J. Li, Y.-L. Zhao, Z.-F. Chai and W.-Y. Feng, *Journal of Analytical Atomic Spectrometry*, 2011, 26, 1233-1236.

234. Z. A. Quinn, V. I. Baranov, S. D. Tanner and J. L. Wrana, *Journal of Analytical Atomic Spectrometry*, 2002, 17, 892-896.

235. M. D. Lelpold, I. Herrera, O. Ornatsky, V. Baranov and M. Nitz, *Journal of Proteome Research*, 2009, 8, 443-449.

236. M. R. McDevitt, D. S. Ma, J. Simon, R. K. Frank and D. A. Scheinberg, *Applied Radiation and Isotopes*, 2002, 57, 841-847.

237. M. R. Lewis, A. Raubitschek and J. E. Shively, *Bioconjugate Chemistry*, 1994, 5, 565-576.

238. Z. Zhang, X. Yan, M. Xu, L. Yang and Q. Wang, *Journal of Analytical Atomic Spectrometry*, 2011, 26, 1175-1177.

239. A. H. El-Khatib, D. Esteban-Fernandez and M. W. Linscheid, *Analytical and Bioanalytical Chemistry*, 2012, 403, 2255-2267.

240. M. Terenghi, L. Elviri, M. Careri, A. Mangia and R. Lobinski, *Analytical Chemistry*, 2009, 81, 9440-9448.

241. L. Mueller, T. Mairinger, G. Hermann, G. Koellensperger and S. Hann, *Analytical and Bioanalytical Chemistry*, 2014, 406, 163-169.

242. H. Liu and K. May, *Mabs*, 2012, 4, 17-23.

243. G. Koellensperger, M. Groeger, D. Zinkl, P. Petzelbauer and S. Hann, *Journal of Analytical Atomic Spectrometry*, 2009, 24, 97-102.

244. S. D. Tanner, V. I. Baranov, O. I. Ornatsky, D. R. Bandura and T. C. George, *Cancer Immunology Immunotherapy*, 2013, 62, 955-965.

245. L. Wang, F. Abbasi, O. Ornatsky, K. D. Cole, M. Misakian, A. K. Gaigalas, H.-J. He, G. E. Marti, S. Tanner and R. Stebbings, *Cytometry Part A*, 2012, 81A, 567-575.

246. E. W. Newell, N. Sigal, S. C. Bendall, G. P. Nolan and M. M. Davis, *Immunity*, 2012, 36, 142-152.

247. O. Ornatsky, V. Baranov, D. R. Bandura, S. D. Tanner and J. Dick, *Journal of Immunological Methods*, 2006, 308, 68-76.

248. P. Patel, P. Jones, R. Handy, C. Harrington, P. Marshall and E. H. Evans, *Analytical and Bioanalytical Chemistry*, 2008, 390, 61-65.

249. R. Liu, X. Hou, Y. Lv, M. McCooeye, L. Yang and Z. Mester, *Analytical Chemistry*, 2013, 85, 4087-4093.

250. U. Bergmann, R. Ahrends, B. Neumann, C. Scheler and M. W. Linscheid, *Analytical Chemistry*, 2012, 84, 5268-5275.

251. S. Crotti, C. Granzotto, W. R. L. Cairns, P. Cescon and C. Barbante, *Journal of Mass Spectrometry*, 2011, 46, 1297-1303.

252. X. Yan, Y. Luo, Z. Zhang, Z. Li, Q. Luo, L. Yang, B. Zhang, H. Chen, P. Bai and Q. Wang, *Angewandte Chemie-International Edition*, 2012, 51, 3358-3363.

253. D. Esteban-Fernandez, F. S. Bierkandt and M. W. Linscheid, *Journal of Analytical Atomic Spectrometry*, 2012, 27, 1701-1708.

254. D. Esteban-Fernandez, R. Ahrends and M. W. Linscheid, *Journal of Mass Spectrometry*, 2012, 47, 760-768.

255. G. Schwarz, S. Beck, M. G. Weller and M. W. Linscheid, *Analytical and Bioanalytical Chemistry*, 2011, 401, 1203-1209.

256. X. Wang, X. Wang, W. Qin, H. Lin, J. Wang, J. Wei, Y. Zhang and X. Qian, *Analyst*, 2013, 138, 5309-5317.

257. R. Liu, Y. Lv, X. Hou, L. Yang and Z. Mester, *Analytical Chemistry*, 2012, 84, 2769-2775.

258. S. J. Christopher, E. L. Kilpatrick, L. L. Yu, W. C. Davis and B. M. Adair, *Talanta*, 2012, 88, 749-758.