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Review

Trends in metal-binding and metalloprotein analysis

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Abstract

This review describes recent tendencies for metal-binding and metalloprotein analysis, emphasizing metal quantification in proteins through X-ray, atomic absorption, mass spectrometric techniques, and others. Hyphenated techniques such as capillary electrophoresis–synchrotron radiation X-ray fluorescence (CE–SRXRF), laser ablation–inductively coupled plasma-mass spectrometry (LA–ICP-MS), matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI–TOF-MS), etc. are also presented. As protein separation techniques electrophoresis (mainly sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE), capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) are indicated, due to their inherent sensitivity, resolution and/or easy implementation. Latest challenges in metallomics are also commented.

Keywords: Metal-binding protein; Metalloprotein; Separation techniques; Determination techniques

Contents

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1. Introduction

Genomic and proteomic studies and the complete sequence of several genomes have provoked a revolution in different areas (physical, chemical, biochemical, medical, among others) in terms of research objectives and instrumentation development. In the last five years, more than five thousand articles were published concerning proteomic studies. Due to this increased interest, mainly in proteomics, the moment is opportune to also discuss another important field: metallomics. Interesting definitions about this field can be found in some recently published reviews [1–3].

Most scientists are conscious of the importance of metal species in life. These species can be considered signaling agents, can act as catalysts, and can modify gene expression, among others. However, up to now the information obtained about protein and metal analyses has been disconnected and the conclusions about the importance of metals to proteins are often fragmented. Comprehension of the roles involving metals and proteins will certainly depend on the quality of information obtained after metal–protein analysis.

Due to this, the aim of the present review is to didactically demonstrate the trends in metal binding and metalloprotein analysis. To attain this task, this review was divided into four major topics. The first is devoted to proteins, where it is commented about their formation, structure and important parameters, such as ionization constants and isoelectric points. Within this topic, metal-binding proteins and metalloproteins are also described and defined, and the distinction between these two classes of proteins is discussed. The second topic is devoted to protein separation techniques, where electrophoresis is extensively described, as well as other important techniques such as capillary electrophoresis and high-performance liquid chromatography. The third topic is devoted to determination techniques, where several actual techniques utilized for metal determination are presented, as well as many examples of application, mainly from the last five years. Nuclear techniques such as autoradiography and instrumental neutron activation analysis, X-ray techniques such as particle induced X-ray emission, X-ray fluorescence and synchrotron radiation X-ray fluorescence, atomic absorption techniques such as flame atomic absorption spectrometry and electrothermal atomic absorption spectrometry were included in this topic, as well as inductively coupled plasma mass spectrometry. The fourth topic is devoted to ligand identification, where techniques such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and electrospray ionization mass spectrometry are described, with several examples. Finally, some present limitations are also introduced as possible challenges to improve the performance of metalloprotein and metal-binding protein analysis.

1.1. Proteins

Proteins are linear polymers of amino acids, and are found in all living organisms. They are macromolecules of relative molecular weight (MW) ranging from several hundreds to many thousands of Da (1 Da = 1.661×10^{-24} g). About 20 amino acids are commonly found in plant and animal proteins, and these are combined in countless ways to form a great variety of protein molecules. This diversity is needed because of the vast number of different functions that proteins perform in living organisms.

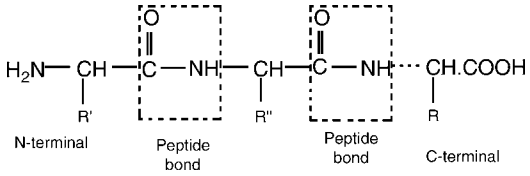

Fig. 1. A typical protein: peptide bonds, N-terminal and C-terminal diagram.

Protein molecules consist of a “backbone”, formed from a large number of peptide bonds, from which protrude the side-chains of the individual amino acid residues. The nature of the component amino acid side-chains is responsible for the varied individual properties of different proteins. An individual amino acid has both carboxylic acid and amino groups, but in protein molecules these are used combined in the formation of the peptide bonds, with the exception of those at the C-terminal and N-terminal of the protein [4], as shown in Fig. 1.

Each amino acid has two ionization constants (expressed as pK_a values), which represent the ionization characteristics of the 1-amino and 1-carboxylic acid groups of the free amino acid. However, some of them have more than two pK_a values, because they have other ionizable groups in their chains. The net charge of a protein molecule in aqueous solution depends on the ionization characteristic of the side-chains of its constituent amino acids and pH of the solution. At low pH values, free carboxylic acid groups are not ionized and the amine groups protonate, so the protein has a net positive charge. On the other hand, at high pH values, free carboxylic acid groups are ionized but the amine groups are not protonated, so the protein has a net negative charge. At intermediate pH values, only some of the free side chain groups are protonated, depending on their individual pK_a values. There is one particular pH at which a protein molecule has no net charge, because it contains an equal number of positively and negatively charged groups. This pH value is named the *isoelectric point* of the protein, and it is designated ‘p*I*’. The p*I* value is characteristic of each individual protein. At pH values lower than its p*I* a protein has a net positive charge, whereas a net negative charge at pH values higher than its p*I*. At any given pH value, different proteins have different net charges because of their characteristic p*I* values [4].

1.2. Metal-binding proteins and metalloproteins

Elements in low concentration, as metal ions, play an important role in biological activity. The investigation of these metallic species in human tissues and organs is indispensable for understanding their essential, toxicological and biochemical impact on biological systems. Most of these metal ions are bound to specific proteins or enzymes, and exert their effects as active or structural centers of proteins [5].

Metal-binding proteins and metalloproteins represent a large portion of the total number of proteins [6]. It has been estimated that around 40% of all proteins and enzymes contain metal ions

in their structures. Metal-binding proteins and metalloproteins are responsible for many metabolic processes, such as biological energy conversion in photosynthesis and respiration, as well as for signaling processes, which govern gene expression and regulation. Metal sites in protein structures also command other process such as catalysts, substrate binding and activation, transportation and storage [7,8]. A metalloprotein is formed when a protein conformation contains the appropriate type and number of ligands. Moreover, another important characteristic is the ligand geometry. Only with correct geometry, do ligands get to encapsulate and activate the metal. Natural and designed proteins demonstrate that a single architecture can be adapted to accommodate a variety of metals [7]. Peptides, protein fragments of different sizes, contain several functional groups in their side-chains, which are suitable for metal coordination. The main constituent amino acids include cysteine and methionine, which bind metals presenting sulfur affinity. In histidine, nitrogen atoms become available for coordination after metal-induced deprotonation [9].

Metalloproteins are considered different from metal-binding proteins. The first are distinguished when the metal binds to proteins with high-affinity interactions, which are not lost during sample handling (isolation and dilution), while in the latter ones the metal–protein interactions are of lower affinity, and are easily broken [10]. Proteins often interact weakly with monovalent ions, such as K^+ and Na^+. However, ions, such as Ca^{2+} and Mg^{2+}, interact moderately with proteins. Transition metal ions, such as Cu^{2+}, Fe^{2+}, Mn^{2+} and Mo^{2+}, due to their features (density, small atomic radius and interaction via electromagnetic and electrostatic forces) have the strongest coordinating interactions, thus being found in the majority of metalloproteins. Table 1 presents some metalloproteins.

One class of metalloprotein of great interest is the metallothioneins (MTs). Mammalian MT proteins present lower molecular weight (around 60 amino acids residues with a molar mass of ca. 6.8 kDa) and are characterized by their high cysteine content (30%). They have high affinities for binding to a very wide range of transition metal ions (mainly elements such as Cd, Cu and Zn) [13].

Efforts and advances have been made to understand the metalloproteins, mainly concerning the structures and functions of the metal sites as well as the biological implications of these interactions. However, it is firstly necessary to localize, identify and quantify the metal-containing macromolecules in organisms. Due to the complex composition and very low level of these species in biological samples, the use of techniques with high resolution for protein separation is necessary, as well as those presenting high detectivity for metal determination [14,15]. In this sense, those techniques such as gel and capillary electrophoresis, and high-performance liquid chromatography, which present high resolution for protein separation, are emphasized and each one are presented.

2. Protein separation

2.1. Electrophoresis

2.1.1. General aspects

The Swedish chemist Arne Tiselius introduced electrophoresis in 1930. Tiselius' interest was in the chemistry of serum proteins and from his investigations the development of specialized apparatus and methodology for electrophoresis was derived [4]. Electrophoresis is relatively simple and highly selective, being applicable to various samples. Its applications are generally to characterize a biological system of interest, and to select specific protein bands for sequencing and identification.

Electrophoresis involves separation of charged species under the influence of an applied electric field. In proteins, the charged species can be produced by dissociation reactions of amino and carboxylic groups or by uniform coating of proteins with an anionic surfactant, such as sodium dodecylsulfate (SDS). This anionic detergent was firstly used to solubilize the proteins. Additionally, it is also used as a reducing agent, thus strongly denaturing the biomolecules, when protein dissulphide bonds are broken. Therefore, the amount of highly charged detergent molecules is enough to overwhelm effectively the intrinsic charges of the polypeptide chain so that the net charge per unit mass becomes approximately constant (This subject will be pointed out in the Section 2.1.2.2). In electrophoresis, the charged species moves in a liquid medium, which is supported by an inert substance (paper or a semi-solid gel), where the migration velocity is an important factor. The liquid serves as a conducting medium for the electric current generated. From the electrophoresis point of view, the most important properties of

Table 1
Examples of some metalloproteins [11,12]

Element	Protein
Selenium	*Glutathione peroxidase*: an enzyme that catalyses the reduction of peroxides and protects the cells from oxidative damage
Chromium	*Transferrin* (plasma protein): transports Cr(III) throughout the body in the blood cells
Copper	*Ceruloplasmin* (human serum protein); *ascorbate oxidase* (plants and bacteria); *plastocyanin* (higher plants and cyanobacteria); *superoxide dismutase*, *tyrosinase*, *cytocrome oxidase* and *hemocuprein* (animals)
Lead	Around 95% of total lead in human blood is bound to erythrocytes. In most erythrocytes, the lead is bound within the cell in *haemoglobin*. In extracellular fluids lead is bound to albumin and some high molecular weight proteins (*globulines*)
Zinc	Zinc is a constituent in more than 200 enzymes and proteins. The principal examples are *insulin* and *carboxypeptidase* A
Manganese	This metal is found in a variety of enzymes such as *pyruvate carboxylase* and *oxalacetate decarboxylase*. This element can be found in proteins such as *glutamine synthetase*, *β-globulin* and *albumin*
Iron	Proteins containing iron are classified in two categories. The first is haeme, where this metal is chelated by porphyyrin (a water-insoluble ligand). This class is constituted by *haemoglobin*, *myoglobin* and *cytochromes*. The second is formed by non-haeme iron. The principal examples are *transferrin*, *ferritin*, *ovotrasferrin*, *casein*, *hemosiderin* and *albumin*

proteins are their size (i.e. their radius, r) and their net charge (Q). So, the proteins will have different migration velocities (v) under electrophoresis conditions and they will be separated from each other by this technique [4]. The equation that relates these two parameters is:

$$v = \frac{Q}{6\pi\eta r}, \quad (1)$$

where v is directly related to Q, and inversely related to 6π (a constant), η (the electrophoretic medium viscosity), and r (the protein radius) [4].

The electric field strength must be maintained constant to keep the sample in solution, and also to provide enough buffering capacity. Increases in the buffer ionic strength lead to conductivity increases, and heating is generated. This excess heat causes an increase in the ionic diffusion rates, and also increases the ionic mobility. At same time, the viscosity of the medium falls while the temperature rises. In contrast, low electric field strength leads to poor separation. This occurs due to the increase of diffusion rates when the running time is too long.

Gel electrophoresis is a powerful separation method for proteins in complex matrices, including culture cells and tissues. Features that contribute to this power are high resolution to separate the mixture, and high detectivity to detect trace amounts.

Polyacrylamide is a very common gel support matrix. This gel matrix is obtained through polymerization of monomeric acrylamide by using *N*,*N*′-methylene-bisacrylamide. The polymerization reaction affects some factors such as gel properties, the polymerization rate and the resulting properties of the gel. When the gel is prepared, factors such as initiator type and concentrations, reagent purities and temperature, as well as oxygen and monomer concentration, must be considered [16].

Gel porosity is another important point to take into account. The pore size in the gel depends on the amount of acrylamide and the degree of cross-linking. The pore size is decreased by increasing the proportion of bisacrylamide. The acrylamide gel concentration is critical when optimal species separation is required. Larger pore sizes are preferable to resolve larger proteins, but they are unlikely to resolve smaller polypeptides. Therefore, the choice of an appropriate acrylamide concentration is fundamental to obtain good resolving range. Gradient pore size gels are usually employed to avoid this problem, where the pore size changes from the top to the bottom of the gel. During gradient gel electrophoresis, proteins migrate until a specific pore size hinders their further progress. Polyacrylamide gradient gels are prepared using a linear gradient maker and a peristaltic pump. Other factors such as pH and buffer also determine the resolving range.

Polyacrylamide gels may be prepared as rods or slabs. In the first case, the gel is polymerized in a cylindrical glass tube with ca. 5 mm i.d. and 70–100 mm length. In a rod gel apparatus, each sample is run separately, making the comparison between different samples under similar running conditions difficult. Due to this fact and other problems, rod gels are currently used only as a step in two-dimensional electrophoresis techniques. On the other hand, slab gels (0.5–1.5 mm thick) are usually preferred. Their most important advantage is that many samples can be electrophoresed at the same time, under identical conditions, thus allowing direct comparison of band patterns in different samples. This fact also implies that less time is required for gel preparation. In addition, the facility for heat dissipation during the electrophoresis, thus reducing protein band distortion, is another advantage. This later characteristic also permits applying higher running voltages, making shorter running times (without loses on resolution range) possible. The slab gels can be developed either vertically or horizontally. There are also two different ways for protein separation, one- or two-dimensions separations. Polyacrylamide gel electrophoresis (PAGE) has been applied for solving separation problems. Therefore, a great number of gels and buffer formulations, as well as separation conditions, have been employed. In addition, some other factors must be considered to obtain proteins separated into a number of distinct zones or bands. The selection of optimum conditions frequently requires a great number of preliminary runs, which examines each influential factor.

2.1.2. *One-dimensional polyacrylamide gel electrophoresis (1D PAGE)*

One-dimensional (1D) polyacrylamide gel electrophoresis is relatively simple. These systems, depending on the protein extraction conditions, can be classified as dissociating and non-dissociating.

2.1.2.1. Non-dissociating systems. When proteins in the native form are studied, non-dissociating systems are employed without changes in biological activities, conformation, etc.

The native proteins are separated on the basis of their size and charge, using isoelectric focusing (IEF). During IEF, a pH gradient is formed, and charged species in the sample (under the electric field) move through the gel until they eventually reach a position in the pH gradient. At this pH, the protein attains the p*I*. IEF has high resolution, is capable of separating macromolecules differing in p*I* by as low as 0.001 pH unit. Due to these factors, in a non-dissociating gel system, information about the p*I* of the proteins under study is essential to adequately choose pH buffers. One IEF limitation is that some proteins (without charge) precipitate at their p*I*, making their recoveries difficult.

Generally, non-dissociating gel electrophoresis presents a poorer estimation of the proteins MW, due to the lack of separation selectivity, thus making necessary a specific detection method.

2.1.2.2. Dissociating systems. In dissociating systems, the proteins are solubilized in the SDS buffer, which is considered the most common dissociating agent. The SDS uniformly surrounds the protein forming a micelle. This interaction between SDS and proteins produces negative charges for all proteins. Their charge magnitudes are related to protein MW. Under these circumstances, when proteins are negatively charged, all of them flow towards to the positive electrode during electrophoresis and the separation is based only on protein MW. An excess of SDS and thiol reagents, when heated at 100 °C, denatures protein mixtures. Due to this fact, proteins are dissociated into individual polypeptides subunits. These polypeptides bind to SDS in

a constant weight ratio, thus forming complexes with identical charge densities. This fact improves protein separation, exclusively according to their sizes.

Smaller proteins move faster through the gel than larger ones. This type of electrophoresis is generally known as SDS-PAGE, due to the combination of SDS protein treatment with polyacrylamide gel electrophoresis. SDS-PAGE, originally described by Laemmli (1970), is a widely used method for determination of polypeptide MW in protein samples [17].

When SDS-PAGE does not give optimal resolution or accurate MW determination, cationic detergents such as cetyltrimethylammonium bromide (CTAB) or *N*-cetylpyridinium chloride (CPC) are then used for PAGE [18].

2.1.2.3. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE). Two dimensional (2D) peptide maps have been accepted and used for many years in studies involving the structure of proteins or their modifications [18]. The literature contains a great variety of 2D electrophoresis procedures, separating molecules on the basis of various parameters, aimed at solving a wide variety of problems. The term "2D macromolecular maps" is usually limited to the separation of intact proteins, glycoproteins or large peptide fragments separated on approximately square gel slabs, nearly always of polyacrylamide gel. 2D maps can be of high or low resolution, and can be prepared under denaturing or non-denaturing conditions [18].

An interesting array of resources and information is available on the Internet related to 2D electrophoresis. A current list can be obtained at the following address: http://www.lecb.ncifcrf.gov/EP/. A careful review of some patterns in databases on the web reveals some discrepancies between databases for identifying protein spots [16].

It is important to distinguish between 2D electrophoresis and two-stage methods. Two-stage methods occur when samples are isolated or subjected to some chemical or enzymic treatment after the first 1D separation step. The treated samples are then reanalyzed in a second 1D stage, which can be performed under the same conditions as the initial separation or not. There are many possible protocols for two-stage separations, depending not only on the procedures used for two-stage separation but also on the treatment given to the samples between the stages. When there is no intermediate sample modification treatment and the two stages separate molecules on the basis of different parameters (e.g. size, charge or hydrophobicity), then it is a typical 2D map.

A frequent approach for preparation of 2D maps involves running the first-dimensional separation in a cylindrical rod gel of 3–6 mm diameter. The rod gel is then placed across the top of a slab gel for the second dimensional separation at right angles to the first. The rod is sealed to the slab with either a little agarose gel or polyacrylamide gel made up in the appropriate buffer. Frequently, however, a gel slab configuration is used for the first dimension separation, as well as the second. In this case, strips of the first dimension gel are cut and inserted across the top of the second dimension slab in much the same way as for rod gels.

IEF electrophoresis usually has two separation modes. One is the traditional carrier ampholyte-based system, and the second is based on the preparation of immobilized pH gradients (IPG). A major advantage of IPG is the availability of pre-cast strips, thus providing an easy-to-use 2D technique while the ampholyte system is utilized primarily when large amounts of proteins are loaded for preparative runs or when basic proteins are separated.

Generally, the second dimension separates proteins on the basis of MW on a SDS gel [16].

2.2. Capillary electrophoresis (CE)

Capillary electrophoresis has been developed and applied to protein analyses in the last two decades and has become an important separation tool for chemists and life scientists. In the last five years more than 1000 examples have described CE as the separation technique for proteins. This fact, of course, shows its importance and utility. Certainly, high speed, high-resolution separations on exceptionally small sample volumes (0.1–10 nL, in contrast to slab electrophoresis, which requires samples in the μL range), and the ease of hyphenation to different determination techniques are responsible for its considerable acceptance. Some specific applications to metal-binding or metalloproteins will be pointed out throughout this review.

CE is similar to high-performance liquid chromatography (HPLC) in many aspects, and most of the words used in chromatography are also found in CE. For example, resolution and efficiency are common for both techniques and are defined in a similar way. However, there are some significant differences: an example, in HPLC, a packed column is used to separate the analytes, while in electrophoresis, an open capillary is used. In HPLC, a pump is used for propelling the constituents through the column; in electrophoresis, the sample constituents move as a result of their mobilities due to an applied potential field and as a result of the induced electroosmotic flow (EOF), if this last is present [19].

Separations in CE are based on the migration velocity ν (already cited in the Section 2.1.1) of an ionic species in an electric field, which is equal to the product of the field strength E and the electrophoretic mobility μ_e:

$$\nu = \mu_e E = \mu_e V/L, \tag{2}$$

where V is the magnitude of the applied potential (V) and L the length over which it is applied. High-applied potentials are desirable to achieve rapid ionic migration and a rapid separation, but it is even more important to achieve high-resolution separations [20].

The EOF occurs when a high potential is applied across a capillary tube in which the solvent migrates toward to the cathode. The cause of the EOF is the electric double layer that develops at the silica/solution surface. Above pH 3, acidic silanol groups at the surface of the capillary dissociate when in contact with an electrolyte solution (buffer). Hydrated cations in the buffer are attracted to the negatively charged silanol groups. One layer is tightly bound by electrostatic forces, and the other is more loosely bound as a diffuse layer. When the electric field is applied, the cations in the diffuse layer are attracted toward the cathode. Since the cations are solvated, they drag the bulk

solvent with them. The electroosmosis leads to bulk solution flows in a flat profile across the tube, and this profile does not contribute significantly to band broadening.

In the presence of electroosmosis, the velocity of an ion is the sum of its migration velocity and the velocity of EOF. Thus,

$$v = (\mu_e + \mu_{eo})E, \quad (3)$$

where μ_{eo} is the EOF mobility.

The EOF is affected by the viscosity and the dielectric constant of the buffer. Besides these factors, EOF is also affected by pH, ionic strength, addition of organic solvents to the buffer to improve resolution, and by the nature of the counter ions associated with the capillary wall. Therefore, the effect of EOF can be altered by careful selection of both the buffer anion and cation, and finally, by the applied voltage.

The separation efficiency and resolution are related to the direction and flow of the EOF that affects the time that a solute resides in the capillary. The EOF flow profile is plug-like, therefore only longitudinal diffusion needs to be considered. Thus, the separation efficiency can be expressed in terms of the number of plates, *N*,

$$N = \frac{\mu V}{2D},$$

where *D* is the diffusion coefficient of the solute, and μ the total mobility.

Resolution in CE is a function of three parameters: selectivity, column efficiency, and migration time. Each of the parameters is influenced by many factors, including applied voltage, electrolyte pH, ionic strength, and treatment of the inner capillary wall. The pH is the dominant factor that can be manipulated to control resolution [19].

CE separations are performed in several ways. These include free solution capillary electrophoresis (FSCE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). These modes are generally hyphenated to different detection techniques, which are described in more detail in Section 3.

2.3. High-performance liquid chromatography (HPLC)

Liquid chromatography (LC) is an analytical technique that is used to separate a mixture "*in solution*" into its individual components. The separation relies on the use of two different "phases" or "immiscible layers", one of which is held stationary while the other moves over it. There are two ways to classify LC methods. The first one is based on the mechanism of retention, where adsorption, partition, size exclusion, affinity, and ion exchange are included. The second classification is based on the separation principle, where the methods of removing the analytes from the column are elution, displacement, and frontal analysis [19].

A column must have both the capacity to retain samples and the ability to separate sample components. The retention factor (k) of a column is a direct measure of the interaction of the sample with the packing material. The efficiency of a column (N) is a number that describes peak broadening as a function of retention and depends on the entire chromatographic system. The separation factor (α) is a measure of the difference in selectivity of the column is defined by retention times (or volumes) between two adjacent peaks, and describes how effectively a chromatographic system separates two compounds. Resolution is the degree of separation between neighboring solute bands or peaks. It is affected by the selectivity, efficiency and capacity of the column.

A variety of chromatographic processes have been developed, and they are based on several different mechanisms. The key chromatographic modes are normal-phase, reversed-phase, ion-exchange, size-exclusion, and affinity chromatography. In addition to these major modes, there are a number of more specialized techniques such as, for example, hydrophobic-interaction, molecular imprint, and chiral separations [19].

There are a number of HPLC applications. Normal-phase chromatography is used for the separation of neutral species on the basis of polarity, reversed-phase is used for the separation of neutral species on the basis of hydrophobicity, ion-exchange is used for separation of ionic solutes on the basis of charge, size-exclusion is used for separation of molecules on the basis of differences in molecular size, and affinity chromatography is used for separation of biomolecules on the basis of the lock-and-key mechanism prevalent in biological systems [19].

Protein isolation and purification may apply ion-exchange, hydrophobic interaction, affinity, reversed-phase and/or gel filtration (exclusion) chromatographic modes. Ion-exchange, hydrophobic interaction and reversed-phase modes are used for peptide analysis and purification, while for nucleic acid analysis ion-exchange, reversed-phase and gel filtration modes are used [19]. Only as an example, the literature reveals more than 3500 publications in the last five years using HPLC as a separation technique for proteins and metalloproteins.

3. Determination techniques

As already commented, with metal-binding proteins and metalloproteins there is the necessity of high power of detection in metal determination techniques after protein separation. In this section, some techniques applied to metal determination in proteins, will be emphasized.

The strategies for on-line coupling techniques include different types of separation, identification, and detection techniques. At the end of 1970s, Van Loon [21] and Suzuki [22] used an approach for acquiring information about selective species at trace and ultra-trace levels. It was based on the coupling of a chromatographic separation technique (later also electrophoretic techniques) with sensitive and element-specific detection (an atomic absorption, emission or mass spectrometer). These couplings became known as hyphenated or coupled or hybrid techniques. In this way, they also became a fundamental tool for the functional characterization of trace elements or otherwise imperceptible metal ions involved in biological studies.

Among the hyphenated techniques for biological systems, some separation techniques are often used, such as high-performance liquid chromatography (HPLC) [11], capillary zone electrophoresis (CZE) [23], and gel electrophoresis (GE).

For detection, atomic absorption spectrometry (AAS) [22], inductively coupled plasma-optical emission spectrometry (ICP OES), inductively coupled plasma mass spectrometry (ICP-MS) [24], autoradiography [25], instrumental neutron activation analysis (INAA) [25], particle induced X-ray emission (PIXE) [15], X-ray fluorescence (XRF) [26], and synchrotron radiation X-ray fluorescence (SRXRF) [5], are commonly used for metal determination. In addition, electro-spray ionization mass spectrometry (ESI-MS) [27], matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI–TOF-MS) [28], laser ablation–inductively coupled plasma-mass spectrometry (LA–ICP-MS) [29], and nuclear magnetic resonance (NMR) [30–32] are used for identification of the protein moieties.

The choice of the hyphenated technique depends primarily on the research objective. The separation component of the coupled system becomes particularly important when target species have close physicochemical properties. It may even be necessary to combine two or more separation mechanisms in series (as 2D SDS-PAGE) to ensure that a unique species arrives at the detector at a given time. The choice of the detector component becomes crucial when the concentration of analyte species in the sample is very small and low limits of detection are required.

Sometimes on-line coupling is not possible. This can be exemplified when the polyacrylamide gel electrophoresis is used and an off-line detection of the metal species is carried out. In this case, the metal species are either directly determined in the gel [29] or determined after extraction (blotting) from the gel [33]. In these extractions, direct elution from the selected portion of the gel placed on a filter [34,35] or into a syringe [36] can be used. Besides these techniques, cavity-type microwave ovens (with acid or oxidant mixtures), is also reported in the literature [37].

The choice for highly sensitive but with discrete atomization technique such as electrothermal atomic absorption spectrometry (ETAAS) [38] or electrothermal vaporization induced coupled plasma mass spectrometry (ETV-ICP-MS) [39] may also be the reason for the off-line coupling method.

3.1. Autoradiography

Autoradiography of radioactive-labeled proteins has been established as a sensitive detection technique for separated proteins [40], presenting similar detectivity as with silver staining for protein detection (from 30 to 100 pg protein mm^{-2}) [41]. An autoradiograph is developed on a photographic film following exposure to a gel containing radioactive-labeled proteins. The radioactive labels most often used are beta-emitting isotopes of fairly high energy, which are linked to proteins. This energy is able to penetrate through the gel and onto the photographic film. The labeling of proteins for detection by autoradiography can be carried out by two different ways. The first one is usually done by introducing the radioactive nuclides to be used as labels on a culture medium that contains cells with the proteins of interest. In this sense, the labels are incorporated into the proteins as they grow in the cell culture. The second one is a direct method of labeling. It involves irradiating the separated proteins on a whole gel and labeling all the elements that are activated by neutron bombardment.

Labeling with radioactive nuclides requires previous knowledge of the elements that are of interest in the samples. This may take considerable planning for their use in cell lines, and often it is not known what elements may be associated with certain proteins. Also, this kind of labeling is often not feasible with human samples [25].

In the last five years at least 84 publications directly relating autoradiography, electrophoresis and proteins can be found in the literature. Specifically for bioinorganic analytical chemistry, this technique has been widely applied to selenoproteins [42–45].

Measurements of the metal-binding protein followed the sector field ICP-MS and autoradiography has been carried out in human and rabbit serum. Rabbits received intraperitoneal injections of “cold” Ga(III) and In(III) in physiological buffer. Human serum was incubated in vitro with radiotracers ^{51}Cr(III), ^{191}Pt(II) or carrier-free ^{48}V(V). The related proteins were separated by IEF and native PAGE. For the autoradiography, the gels were exposed to a phosphorous screen at $-20\,^{\circ}C$, an autoradiogram was obtained by laser densitometry, and subsequently the proteins were detected by silver staining [46].

Song et al. [47] proposed a systematic evaluation of GE combined with autoradiographic image analysis for quantitative protein drug determination in biological fluid and its application in pharmacokinetic studies.

Petrák and Vyoral [48] described a new method to reconstruct the interaction of iron with iron–metalloproteins. Proteins were separated by native gradient PAGE and transferred to polyvinylidene difluoride membrane under native conditions. The immobilized iron–metalloproteins were then labeled with ^{59}Fe using a ‘titrational blotting’ technique and visualized by storage phosphorimaging. This technique enabled separation and detection of iron–metalloproteins under near-physiological conditions and facilitated identification of weak iron–protein complexes.

In different applications of the autoradiographic technique, dinoflagellate cell surface proteins were identified [49], Gromov et al. [50] identified a group of six polypeptides in primary human keratiinocytes (among them, manganese-superoxide dismutase) that are affected by the aging process, and Marxen and Becker [51] investigated the calcium-binding constituents of the organic shell matrix from the freshwater snail *Biomphalaria glabrata*, using ^{45}Ca(II) autoradiography after SDS-electrophoretical separation and a calcium binding assay. Finally, Ireland et al. [52] developed a simple mathematical model of the darkening films in radiography, autoradiography and electrophoresis bands disclosed by enhanced chemiluminescences. A formula to calculate the strength of the source from measurement of film blackening by image analysis was also presented.

3.2. Instrumental neutron activation analysis (INAA)

An alternative and sensitive technique to determine trace elements in the gel stripes obtained after electrophoresis is the neutron activation analysis (INAA).

Three sources of neutrons are employed in neutron activation methods: reactors, radionuclides, and accelerators. All these sources produce highly energetic neutrons (MeV range), which usually pass through a moderating material that reduces their energies to few hundredths of an electron volt. Energy loss to the moderator occurs by *elastic scattering* in which neutrons bounce off nuclei in the moderator material, transferring part of their kinetic energy to each nucleus that they strike. Neutrons presenting energies at about 0.04 eV are called *thermal neutrons*, and the process of moderating high-energy neutrons to thermal conditions is knowledge as *thermalization*. Most activation methods are based upon thermal neutrons, which react efficiently with most elements of analytical interest. Neutron capture is the most important reaction for activation methods. In this case, a neutron is captured by the analyte nucleus to give an isotope with the same atomic number, but with a mass number that is greater by one. The new nuclide is in a highly excited state because it has acquired about 8 MeV of energy from binding the neutron. This excess of energy is released by *prompt gamma ray emission* or emission of one or more nuclear particles, such as neutrons, protons, or alpha particles. The two common types of activation methods are destructive and non-destructive, or instrumental (INAA). In both methods the sample and one or more standards are simultaneously irradiated with neutrons (or other types of radiation). The irradiation time depends upon a variety of factors. It can be previously calculated (from cross section) if the target nucleus is known. Different nuclides require different irradiation times due to different cross sections, case with which the neutron is "accepted". The other factor, defined by neutron source, is flux or number of neutrons "available". After finishing the irradiation, the sample and standards are often allowed to decay ("cool") for a period that varies from a few minutes to several hours or more, and the sample and standards are counted using appropriate detectors [20]. In the destructive mode a chemical step is usually included between irradiation and counting, i.e. what is "counted" is the radionuclide separated from the overall target.

There are several papers determining selenium bonded to proteins, some of them used INAA after electrophoretic separation. Thus, Kyriakopoulos et al. [53] labeled homogenate rat tissue (liver, lung, spleen and prostate) in vivo with ^{75}Se-selenite and separated the proteins by SDS-PAGE. The selenium-containing proteins in the 16 kDa range were found to be mainly by membrane-bound. As Se substitutes S in several amino acids used to synthesizes proteins by living organisms, in 1995 Behne et al. [42] injected ^{75}Se-labelled selenite into live rats on different diets and at different time periods before and after eating. The proteins in body fluids, tissues and sub-cellular fractions were separated by SDS-PAGE or 2D IEF-SDS-PAGE. The selenium content in the diets and in non-labeled samples was determined by instrumental neutron activation analysis via ^{75}Se reactor with thermal neutrons. For most biological materials, the limit of detection was around 0.1 ng of Se [54], but as the amounts in the tissues stemmed almost exclusively from the tracer, the selenium content of the proteins could directly be determined from the ^{75}Se activity, and a limit of detection of 2×10^{-14} g was reached for Se. Several additional proteins or protein sub-units were detected besides the 13 previously described. More than 25 selenium-containing bands could be distinguished after separation by SDS-PAGE.

In 1998, Behne et al. [55] applied the method for the determination of selenocysteine and selenomethionine in selenium-containing proteins. The identification of four specific selenoproteins was achieved by a combination of several biochemical methods (including GE) with tracer techniques and procedures for trace elements (INAA and autoradiography) to obtain information on their characteristics and possible biological functions.

Hammel et al. [56] found high selenium concentrations (7–12 g kg^{-1} dry mass) in the seeds of the selenium-accumulator plant, coco mono (*Lecythis ollaria*). Dialysis and SDS-PAGE were used, combined with neutron activation analysis, to obtain information on the protein-bound part of selenium in extracts of these seeds. For the protein-bound selenium, concentrations of 0.7 g and 2.4 g kg^{-1} of seeds and 40 and 25 g kg^{-1} of extractable protein were determined at pH 4.5 and 7.5, respectively. Stone et al. also related INAA and PAGE for determination and quantification of proteins [57,58]. In 1994, Stone et al. [59] evaluated various nuclear techniques (including neutron activation analysis) for the detection of selenium contained in the selenoprotein glutathione peroxidase. This enzyme had previously been separated by PAGE. Neutron activation analysis was used for quality-control measurements, with 0.08 ng as limit of detection.

To study metalloproteins and protein-bound trace elements in bovine kidneys, Jayawickreme and Chatt [60] used several bioanalytical techniques (including electrofocusing and isotachophoresis) in conjunction with INAA. Dialysis of the homogenate bovine kidney showed that more than 90% of Ca, Cd, Cu, Fe, Mg, Mn, Se, V, and Zn, and about 20% of Br were bound to macromolecules, mainly proteins. An IEF technique showed distribution patterns for Cu, Mn, Mo and Zn bound in proteins. Cu, Mn and Zn were, respectively, associated with two, three, and two distinct proteins, and Mo was fairly evenly associated with four proteins. Although concentrated protein zones were obtained in isotachophoresis, the resolution between the peaks was reduced due to their bands being adjacent to each other.

3.3. Particle induced X-ray emission (PIXE)

The PIXE technique appears especially useful considering its non-destructive characteristic. Micro-PIXE (microscopy-PIXE) and macro-PIXE are now involved in different fields, such as archaeology, art, geology, metallurgy, mineralogy, and others. Their popularity in the life sciences, biochemistry, biology, environmental and medicinal fields is constantly increasing [61].

In a recent review, Bertrand et al. [61] presented a brief description and indicated the advantages of PIXE, such as multi-elementary analysis, detectivity comparable to AAS, easy hyphenation to chromatographic analyzers, less time consumption for sample preparation, easy adaptation to micro-PIXE,

among others. Finally, this review emphasized PIXE for metalloprotein analysis using denaturing and non-denaturing gel electrophoresis.

In PIXE, a collimated beam of energetic charged particles, usually a proton beam of 2–4 MeV energy, bombards the sample, thus removing electrons from the inner shell of the atoms. During the electron-rearrangement process, characteristic X-rays are emitted and measured, usually by a high resolution Si(Li) semiconductor detector. By measuring the energy and the intensity of the emitted X-rays, the atomic number and quantity of atoms in the bombarded volume can be determined. Under usual experimental conditions, all elements from Al to U can simultaneously be measured at mg kg^{-1} range [62].

After their separation by electrophoresis, Szökefalvy-Nagy et al. [62] determined Fe in an iron–sulfur protein (HiPiP) from *Thiocapsa roseopersicina*. In 1999, Szökefalvy-Nagy et al. determined Fe and Ni of hydrogenase from *T. roseopersicina* and *Desulfovibrio gigas*, and showed that these metals were located on different polypeptides that formed the enzyme [15].

To identify and determine the relative amount of protein-bound metal ions in situ, Strivay et al. [63] proposed that two gels could be simultaneously run under the same experimental conditions. The first one was stained with coomassie blue dye and the second remained colorless to avoid any loss of metals bound to protein. They dried the gels using an adequate process, and scanned the colored gel to determine the number of proteins in each band through densitometry, using computer analysis of the digitized gel image. The second gel was carbon coated to avoid charge build-up problems, placed inside a homemade aluminum holder, and introduced into a reactor chamber. The authors described the experimental set-up used, where a rectangular beam (8 mm × 1 mm) was used for covering the protein band. The dried gel was automatically moved by 1 mm-steps, and the characteristic X-rays induced by proton bombardment were detected by a LEGe diode, placed at 135° with respect to the incident beam. They used a calibration method to get a linear relation between the PIXE yield normalized with the counts and the metal concentration. Once the gel was completely analyzed the concentration of different metals (Cu, Fe and Zn) in each protein band could be plotted as a function of their distances on the gel. Through these results, and taking into account the gel volume (1 mm), they proposed to determine the total number of metal atoms in a protein band as well as the number of metal sites in the protein, thus showing that quantitative metal site number determinations in proteins can be obtained by combining electrophoresis and PIXE.

In another application examples, Wittrisch et al. [64] used capillary electrophoresis to observe the hydrolysis behavior of titanocene dichloride, a potential anticancer drug, in aqueous solutions. The hydrolysis products were characterized by PIXE, and the results obtained corresponded to the hydrolysis mechanism described in the literature. Solís et al. [65] also used a PIXE technique, but to detect, in a qualitative way, the presence of Fe in eight protein band sub-units obtained by SDS-PAGE from the isolated complex photosystems (PSI) of the thermophilic cyanobacteria *Synechoccus* sp.

3.4. Synchrotron radiation X-ray fluorescence (SRXRF)

There are a few papers in the literature using SRXRF as a detection technique after electrophoretic separation of proteins. Synchrotron radiation as an X-ray source has superior properties of high intensity (10^3 to 10^6 times stronger than conventional X-ray sources), it is highly collimated and linearly polarized in the electron orbital plane. This technique has many attractive features for trace analysis. A 10^{-12} to 10^{-15} g absolute limit of detection (LOD), and a relative LOD of μg g^{-1} [66] can be achieved, and only small volumes and mass of sample are needed. Since it is considered a non-destructive technique, sample preparation is generally simple and fast. In addition, a multi-elemental application (from Na to the end of the periodic table) can be carried out. It has a short turn-around time and presents the possibility of measuring both surface and volume distributions of elements [66].

Although SRXRF has many attractive features for trace analysis, sometimes its cost is not comparable to other analytical techniques, which present the same (or better) detectivity. However, when a microprobe of synchrotron radiation is concerned, this situation changes completely, since the capability to measure trace element concentrations in the sub-μg g^{-1} range with lateral resolution at the micrometer level is a rather unique feature. A comparison between (micro)-PIXE and (micro)-SRXRF is given by Vis [67]. The difference in radiation damage induced in the samples gives micro-SRXRF a major advantage over micro-PIXE.

As an application of the SRXRF detection technique, Mann et al. [68] coupled on-line SRXRF with capillary electrophoresis (CE–SRXRF) to determine Fe(III), Co(II), Cu(II), and Zn(II) complexes. These metals were selected for their biological and environmental significance. They carefully studied the choice of materials that would be compatible with both the separation and detection requirements. Therefore, as fused silica is largely opaque to X-rays at 10 keV, it was necessary to create a detection window of polyethylene in the fused-silica capillary for X-ray fluorescence detection. They utilized CDTA as the chelating agent because it provides high equilibrium binding constants, and Vitamin B_{12} was used as a metal containing neutral marker. The LOD for this on-line CE–SRXRF system were ∼0.1 mol L^{-1} (0.5 ng), somewhat high when compared with ICP-MS detection. In the configuration presented, less than 0.5% of the fluorescence emission was collected, and CE–SRXRF was still in the initial stages of development. The authors believe that after optimization, the LOD could be enhanced by two–three orders of magnitude; however the high working voltage used in CE separation may result in breakage of the combination of metal ions with proteins. For this reason, the method is not applicable for the study of metalloprotein distribution in biological samples.

In 2002, Gao et al. [69] proposed a more suitable approach to separation and detection of metalloproteins in human liver cytosol. The procedure included gel filtration chromatography and isoelectric focusing separation of proteins, a step of gel drying and a SRXRF analysis of trace elements in the protein bands. The results showed that there were at least two Zn-, two

Cu- and about eleven Fe-containing protein bands present in human liver cytosol. Despite these results, further improvements are necessary for the quantitative determination of trace elements in protein bands. Gao et al. [14] also detected metalloproteins in human liver cytosol by SRXRF after SDS-PAGE separation. The gel was dried immediately after protein electrophoretic separation, and the metal contents at certain sites on the gel were then determined by SRXRF. After analysis, the gel was stained and distained. More than 35 bands could be visually distinguished in the sample. A series of SRXRF spectra were collected along the electrophoretic direction. With this procedure six Zn-, at least four Fe- and one Cu-containing protein bands were present in the samples.

Recently, Weseloh et al. [70] combined SDS-PAGE with SRXRF to analyze metal-loaded apoazurin (non-covalently bound metals) and selenoproteins in rat testis homogenate (as an example for a covalently-bound trace element). The apoazurin sample was incubated together with 2 mmol L^{-1} ZnCl in order to replace the Cu(II) ion in the molecule by Zn(II)-ion, and the selenoprotein sample was appropriately prepared. All samples were separated by SDS-PAGE, blotted onto a PVDF-membrane, and stained with Coomassie-blue. The selenoprotein distribution determined by SRXRF was compared to that of ^{75}Se-labeled selenoproteins from rat testis, which had been prepared as described by Behne et al. [71]. The distribution of several elements along the separation lane was determined in a zinc-loaded apoazurin sample. Zinc and chlorine were found to be present in the protein band, while iron, tin and calcium were detected along the lane. For the selenoprotein sample, only one distinct selenium peak was detected by SRXRF, and it was identified as the seleno-enzyme phospholipid hydroperoxide glutathione peroxidase by means of an autoradiogram. Additionally, bromine, copper, lead and zinc were also detected along the whole separation lane, probably caused by contamination. The authors conclude that the combination procedure proposed could be applied in the identification of trace element-containing proteins in which the metal or metalloid is either covalently bound or present in the form of a stable complex. In cases of weaker ionic metal–protein complexes, there is the problem of metal loss, and native PAGE has to be employed. They also pointed out the considerable risk of contamination from different steps during the preparation and separation processes.

Verbi et al. [37] investigated protein bands from in vitro embryogenic *callus* using micro-SRXRF after SDS-PAGE separation. The protein bands ranged from ~14 to 86 kDa, and each band was scanned using synchrotron radiation. Two protein bands (81 and 14 kDa) presented a heterogeneous iron distribution through the protein clusters, suggesting that different proteins could be linked to this metal. The 53 kDa protein measured presented iron, calcium, copper, potassium and zinc. Once again, heterogeneity of metals distribution along the protein band was observed. Metal-binding protein quantification was done, after microwave oven decomposition of each gel protein band, by synchrotron radiation total reflection X-ray fluorescence (SR-TXRF), FAAS and FAES. Ca, Cu, K, Fe and Zn were determined by SR-TXRF, Ca and Mg by FAAS, and Na by FAES (Flame Atomic Emission Spectrometry). The quantitative and qualitative results were in agreement, besides good precision between SR-TXRF and FAAS results. Another interesting result was obtained for Fe and Mg. They were only quantified in two protein bands (81 and 14 kDa for Fe and 86 and 27 kDa bands for Mg), indicating that both metals can play an important role in the biochemical events where these proteins participate.

3.5. Flame atomic absorption spectrometry (FAAS) and electrothermal atomic absorption spectrometry (ETAAS)

Atomic absorption spectrometry techniques are widely used to quantify metallic ions in biological materials. Generally, the determination of metallic species can only be performed in a gaseous medium. For this reason, the principal and most critical step in all atomic spectrometry procedures is atomization. Thus, there are available several different atomizers in atomic spectrometry to accomplish this task.

In FAAS, the analyte (usually present in an aqueous medium) is converted to a mist that is carried into the flame by a gaseous oxidant or reductant flow. In the flame, the solvent present in the sample is evaporated and the resulting solid particles are transformed into gaseous atoms and elementary ions. This technique shows desirable characteristics, such as high analytical frequency, good selectivity and low cost. However, the quantification limits obtained from FAAS are in the mg L^{-1} range. ETAAS presents lower detectivity (in the μg L^{-1} range) and it can be employed for trace element quantification. This detectivity is reached because the sample is atomized in a short period of time, the residence time of the atoms in the optical path is longer than in the FAAS technique, thus promoting better atomization efficiency. In the ETAAS technique, the samples are first evaporated at a low temperature. In a following step, the sample is ashed at a higher temperature, generally in an electrically heated graphite tube. After ashing, the current is rapidly increased and sample atomization occurs in a period of a few milliseconds to seconds. The ETAAS technique shows some limitations, such as slowness (requires several minutes per element), some chemical interferences and relatively high cost [72,73].

FAAS and ETAAS are important analytical techniques and may be employed for quantification of metals bound to proteins, generally after chromatographic or gel electrophoretic separations [9]. However, there are only a few examples in the literature that report the use of spectrometric techniques when proteins are separated by electrophoresis.

Scancar et al. [74], employed size exclusion chromatography with UV and FAAS and ETAAS detection to investigate the proportion of metals bound to protein in serum and continuous ambulatory peritoneal (CAPD) fluids of dialysis patients. Identification of metal binding proteins in spent CAPD fluids was performed in protein containing fractions after the size exclusion chromatographic separation by SDS-PAGE. Research about chronic diseases, such as renal insufficiency, is very important because these diseases provoke imbalances of elements in the human body. Due to renal malfunction some elements such as Al, Cr, Fe, Si and Sr, may be accumulated in the body, while others such as Se, Br, Zn, Li and Rb, may be depleted. In patients undergoing continuous ambulatory peritoneal dialysis, depletion

occurrence of some elements may also be induced by the losses of metal binding protein during the treatment. Specifically, it was possible to identify Cu, Al, Fe and Zn bound to different proteins presenting high MW. Aluminum and iron were presumably bound to transferrin, copper was most probably bound to albumin and zinc to albumin and globulins.

Wróbel et al. [75] also used ETAAS to verify to which human serum proteins the aluminium was actually bound. For that, gel electrophoresis was employed for further separation of fractions, firstly obtained by HPLC. In this work, a polymeric anion-exchange column (Protein-Pak DEAE-5PW) in the HPLC separation was used to minimize the risk of Al losses. Speciation studies of this element in bio-fluids are important to establish the mechanism of its toxicity. In this work, synthetic serum solutions and diluted normal serum samples were used. The results indicate that transferrin was the only serum protein to which aluminium was bound (it contains about 90% of total serum aluminium). However, in presence of desferrioxamine (a drug used in renal patients to sequester Al), the Al was partially displaced to form proteins of low MW.

Gomez et al. [76] monitored metals in *Hypericum perforatum*, and its pharmaceutical derivatives, using absorption and emission spectrometry. The extracts or herbal teas of this plant (known as St. John's wort) are used for treatment of chronic diseases like depression. However, some kind of adulterants and metal species can be present in these samples, and investigations about quality and the identification of adulterants in phytopharmaceutical products are very important. Related to adulterants, CE was used as a separation technique to analyze caffeine, theobromide, theophylline and ephedrine present in antidepressive herbal products. The metals selected for determinations, and the techniques employed to measure their concentrations were, respectively: Li, Na and K by FAES, Ca, Cu, Mg, Mn and Zn by FAAS and Ni by ETAAS. The results obtained indicated that their concentration levels were similar to those recommended by the World Health Organization. In addition, the samples under investigation did not show any adulterants in the electropherograms.

3.6. Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS is a versatile analytical technique that is often coupled to different separation techniques. This hyphenation allows carrying out metal speciation on a routine basis. Hyphenated techniques based on coupling chromatographic separation techniques with ICP-MS detection are established as an important analytical tool for speciation analysis. Analytes include coordination complexes of metals with larger proteins and metallothioneins as well as selenoproteins and metal/semimetal binding to carbohydrates [77].

The inductively coupled argon plasma (ICP) is an excitation source used to generate ions that are introduced to the mass spectrometer. The ions produced by the plasma are accelerated, magnetically separated and counted, according to their mass-to-charge ratios, using an electron multiplier. It is widely used due to its great selectivity, very low detection limits for trace-metal and metalloid-containing compounds (ng L^{-1} range) and multi-element/multi-isotope capabilities [78]. In addition, the wide linear dynamic range of ICP-MS routinely exceeds six orders of magnitude, allowing detection of both major constituents and trace components at the same sample dilution. Moreover, the multi-elemental capability of ICP-MS enables the observation of individual isotopes, which permits the use of isotopic-dilution techniques for internal standardization and also to monitor species transformations that may occur during sample pre-treatment or separation. Due to isotopic resolution, it is possible distinguish between metal (heteroelement)-containing species and metal-free species in a HPLC or CE effluent or, via laser ablation, in a 2D gel spot [1].

In spite of all of these advantages, there are some problems that are commonly found in the use of ICP-MS for speciation analysis. An important point is the solvent composition when ICP-MS is coupled with HPLC or CE. Excessive amounts of organic solvent can result in plasma instability, as well as a build-up of carbon residues on the sampling cone. Another common problem is the sample matrices. Biological and environmental samples contain very complex matrices and the use of a buffer or a solution of high ionic strength is often required to obtain good separation. However, high salt concentrations in the effluent entering the ICP-MS can result in signal suppression due to increased space-charge effects that defocus the ion beam [79,80].

There are some examples in the literature that use ICP-MS for metal-binding protein determinations, mainly after their separation using CE.

Olesik et al. [24] pointed out the importance of speciation of the metals. Therefore, the authors proposed an efficient and reliable method for elemental speciation. In this research, they coupled CE to ICP, thus discussing some potential capabilities and problems of this hyphenated technique. One of the advantages for combining CE and a detector such as ICP-MS is that all species could be measured from one injection. In addition, there is no necessity to separate ions with the same charge from different elements when using ICP-MS. Consequently, there is no need to add complexing ligands to the sample. The authors pointed out that the CE–ICP-MS LOD, based on concentration, was poorer (from 10 to 600 times) than traditional ICP-MS. This was due to the fact that the sample amount entering the plasma per second was less than for typical ICP-MS (30 times less analyte enters in the plasma per second for CE–ICP-MS). Other potential difficulties in this technique include sample losses and chemical matrix effects. In the first case, the ions can be retained in the CE capillary by chemical absorption or be trapped in the electrical double layer that forms near to the capillary wall. In the second effect, components in the sample can affect the detectivity or the electrophoretic separation. To minimize the magnitude of matrix effects it might be useful to reduce its presence in the sample. The authors reported that CE–ICP-MS could be optimal for some applications, but that some aspects, as indicated above, must be taken into account.

Recent publications have been related applications of CE–ICP-MS to biomolecule, especially those about characterization of metallothioneins (MT). As examples, Cd, Cu, S and Zn were simultaneously detected in metallothioneins isoforms

of rabbit liver and a further purified MT-1 preparation (metallothionein 1) by Schaumlöffel et al. [81]. The approach for quantification of these metals was done via sulfur measurements. For this purpose, the MTs were separated by CE and the metals quantified by ICP-MS. The CE–ICP-MS system was used to perform on-line isotope dilution. The eluent of the CE capillary was continuously merged with the make-up liquid before the mixture was nebulized into ICP-MS for on-line isotope ratio measurements. With this procedure, it was possible to characterize the stoichiometric composition of the metalloprotein complexes and to quantify the metals. The MT-1 quantification precision was 11.3%. For MT isoforms, the quantification was more difficult due to sample complexity. There was a larger amount of matrix in the metallothionein preparations than for further purified MT-1. Despite this fact, CE–ICP-MS could be used to investigate MT isoforms.

Metalloproteins present in cytoplasmic fractions from *Escherichia coli* were detected and quantified by Binet et al. [82] using LA–ICP-MS. The species used in this study were grown under stress conditions, related to Cd and Zn concentrations. The results were compared to organisms that did not have stress conditions. The metalloprotein cells were firstly separated by native PAGE, it thus becoming possible to identify to which protein the cadmium or zinc were binding. Different protein loadings of cytoplasmatic fractions were investigated. The results suggested that the metalloprotein species synthesized under stress conditions presented a greater metal concentration than those synthesized without stress conditions.

Lustig et al. [46] used samples from human and rabbit serum in their work. Isoelectric electrophoresis and 1D native-PAGE separated the metal-binding proteins. In both samples, Ga and In were detected by ICP-MS. The authors also demonstrated the efficiency of this technique for metal speciation at low concentrations. They identified that In and Ga bind exclusively to transferrin in rabbit and human serum, although the latter was partly stripped during electrophoresis.

In the same year Lustig et al. [83], speciated platinum in serum samples (rabbit and human). They used 2D native electrophoresis for separation and ICP-MS as the metal detection technique. The 2D native electrophoresis is not commonly used for this propose due to several factors (mainly lack of resolution and poor repetivity). Accordingly, it was necessary to develop procedures to reach the improved goals of resolution and recovery. This was especially important for those high MW proteins present in serum.

The studies related to hyphenated techniques of MT bioinduction in rat were limited to Zn-induced proteins. In contrast, Polléc et al. [84] investigated Cd–MTs complexes. They proposed some studies of CZE–ICP-MS and HPLC–ICP-MS interface designs for the characterization of metal complexes in metallothioneins in rat liver and kidney. For that, two sets of samples from different rats (exposed and unexposed to Cd) were used. The electropherograms of liver samples showed four peaks for Cd. With this technique it was possible to define the mixture of Cd– and Zn–MT complexes from liver samples. In kidney sample, two peaks were clearly detected. These peaks have also been referred to the mixture of Cd– and Zn–MT complexes.

4. Ligand identification

Mass spectrometry is an important tool in biochemical research capable of analyzing small and large molecules. It is used for proteins, peptides, oligonucleotides, etc. with detection limits in the picomole or femtomole range. This tool allows determining the exact molecular weight of compounds by separating molecular ions according their mass-to-charge ratio (m/z) and, additionally, significant structural information.

The molecular ions are formed by inducing a gain or loss of charge (e.g. electron ejection, deprotonation or protonation). The common ionization techniques in biomolecule analysis are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI).

In some cases, ligand characterization is done using the synergetic effect between ionization techniques (MALDI and ESI) and mass spectrometry (MS) techniques, thus resulting in MALDI–TOF-MS and ESI-MS hyphenated techniques. In this way, it is possible to make the information of complex characterization both comparable and complementary.

4.1. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry

Advances in mass spectrometric methodologies over in recent years have had a great impact on the identification and characterization of gel-separated proteins. Specially, the development of MALDI ionization techniques has allowed mass spectrometers to be used for routine protein characterization.

MALDI–TOF-MS is a technique where the analytes, embedded in an excess of a specific absorbing matrix, are protonated and desorbed into the gas phase following a laser pulse. The mass-to-charge ratio of the analyte ions is determined by their time-of-flight. Thus, amino acid sequence information can be obtained by interpretation of the fragmentation pattern of peptide ions (proteolysis). The mass spectrometric-derived information probes peptide or peptide fragment sequences that are compared with databases make possible the identification those proteins of interest.

One feature of this technique is its ability to detect biomolecules in complex mixtures in the presence of larger molar excesses of salts and buffers. Another feature is that MALDI–TOF-MS exhibits good detectivity providing the identification of low femtomole quantities of proteins.

Dihazi et al. [28] optimized the in-gel digestion protocols for proteins in dried gels. They improved the protocol optimizing the enzymatic degradation within acrylamide, thus reducing the amount of analyte necessary for identification. The sample used was aldolase from rabbit muscle, in 10% SDS-PAGE. The work described the procedures for in-gel digestion of aldolase from dried and moist polyacrylamide gels, submitting them to MALDI–TOF-MS analysis. The mass spectrum of aldolase tryptic digestion obtained from dried gels did not show significant differences when compared to those from the moist gel. Therefore, the protocol for in-gel digestion development proved efficient for both sample types. This result makes it possible to identify, by MALDI–TOF-MS analysis, proteins obtained dur-

ing earlier work or conserved in dried polyacrylamide gels at room temperature for years.

Recently, in 2003, Marvin et al. [85] presented a review of MALDI–TOF-MS and its applications in clinical chemistry and biology. Thus, disease markers could be identified in combination with 1D and 2D gel electrophoresis separations, followed by data base researching. The authors reported that MALDI–TOF-MS has been developed as a primary investigative tool to characterize a number of cancer, arthritis, allergy and Alzheimer diseases. Therefore, much proteomic data have been obtained by coupling these techniques. Finally, this review described the applications in detection and speciation of bacteria, and for genotyping.

Chen et al. [86] did a proteomic comparison between tissues at different conditions using MALDI–TOF-MS analysis. Proteomic analysis is a good alternative strategy to discover proteins that undergo changes in expression level, and may underlie differences in phenotype. In this work, the authors used young and old human brain tissues, separating by 2D gel electrophoresis. This study was important to investigate the molecular mechanism of human brain aging and the associated brain diseases. The 2D gels showed changes in the relative intensity of protein spots, and the expression-changes were identified by MALDI–TOF-MS. Five protein spots, found down-regulated in older brains (whose expressions did not differ significantly), were identified.

4.2. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS uses an electrohydrodynamic spraying technique of liquids to produce gas-phase ions from sample molecules present in a diluted solution. These ions are subsequently transferred into a mass analyzer.

A strong electric field creates a fine spray of highly charged droplets that generates the gaseous ionized molecules. For effective ion formation, special conditions such as voltage, flow rates and solution parameters (conductivity, concentration, solvent composition) are required. This type of ionization is highly favorable to the formation of multiply charged molecules that allow the analysis of high-mass ions with a relatively low instrumental m/z range, in addition to giving a better mass accuracy (detectivity is in the picomole range).

Rosenberg [87] has presented a review of the use of ESI-MS for structural elucidation of metal complexes and characterization and quantification of small organometallic species. In that work, a lot of examples for determination of low-molecular-mass metal species, metal complexes and organometallic compounds by ESI-MS is provided. Biomolecule and metalloprotein characterizations and investigation of metal interaction are also emphasized. The author indicates that ESI-MS is normally used for detection of metalloproteins after multidimensional separation. This technique is ideal to detect high MW metallocompounds, due to the softness of the ionization process that allows labile complexes to be transferred intact into the gas-phase.

Lobinski and co-workers give a number of examples for the structural elucidation of various metallothioneins and their isoforms using ESI-MS [84,88–90]. Poléc et al. [84], have analyzed a mixture of MT complexes from rat tissues by ESI-MS. The investigation of the peaks obtained from HPLC–ICP-MS chromatograms and from CZE–ICP-MS electropherograms of MT fractions allowed the detection of one major class of metallothionein isoform in kidneys, and two MT isoforms in liver (Cd–MT-1 and Cd–MT-2). The authors concluded that in spite of rat sample complexity, Cd complexed with MT isoforms could be characterized using a combination of these techniques. Thus, a screening for metal–MT complexes became possible, as well as a procedure to define the molecular mass of the complexes present.

Montes-Bayón et al. [91], pointed out selenium speciation in wild-type and genetically modified *Brassica juncea* (Indian mustard). For that, Se species proceeding from plant tissues were separated by HPLC in conjunction with ICP-MS for sensitive and selective Se monitoring. This procedure allowed comparing Se species found in these two types of tissues and observing that genetic modification did not confer to the plant the ability to accumulate more Se. Results showed only that genetically modified *B. juncea* enlarges the tolerance to Se by increasing the production of non-proteinogenic seleno amino acids. The use of ESI-MS permitted characterization of some unknown Se species produced during Se metabolism, and the confirmation of others initially proposed in the genetically modified plant. The presence of the Se-methylselenomethionine in plant extracts was reported for the first time in this study [91].

5. Conclusions and challenges

Besides the large number of studies focusing on proteomics, it is clearly noted that studies focusing on metal-binding proteins or metalloproteins are still in their infancy in terms of science, mainly if one considers the function, mapping and quantification of these kinds of proteins. In fact, it is easier to find studies related to proteins than to metalloproteins. In this way, this review demonstrates some possibilities for metal mapping and quantification in metal-binding or metalloproteins using hyphenated or non-hyphenated analytical techniques. Electrophoresis was pointed out as a separation technique due to its inherent detectivity, resolution and easy implementation. In fact, it is commonly used for proteomics studies, mainly if 2D PAGE is considered.

By analyzing the potentialities, nuclear techniques (such as autoradiography or INAA) present specificity (by marking proteins with an isotope) and extremely good detection limits. Similar characteristics describe mass spectrometry. However, when the objective is to map metals along the protein clusters, X-ray techniques tend to be better (mainly micro-SRXRF) due to the size of the beam, which permits metals to be detected/quantified in different positions among the proteins expressed in the gel. Sample treatment is minimized as well.

By using hyphenated techniques (such as CE–SRXRF, LA–ICP-MS, etc.) some figures of merit can be improved due to the synergism achieved, thus making the characterization of those imperceptible metals ions involved in biological studies possible.

Since only nowadays is metallomic studies receiving significant attention, some challenges may be considered, thus opening other fields of study, such as sample preparation, quality control,

instrumentation, etc. Sample preparation can be pointed out in this sense, since soft processes for those extraction procedures used in non-denaturing system are imperative, mainly if protein conformation studies are indicated, as well as to avoid metal loss during extraction for metalloprotein analysis. Another challenge concerns contamination due to buffers and reagents. Due to these low concentrations of metals to be determined, important efforts in quality control must be made to assure the quality of information obtained. Many metal or semi-metal complexes can adhere onto the walls of an electrophoretic capillary as well as onto a HPLC stationary phase. Once again, other strategies in terms of extraction procedures are needed.

Finalizing, metal-binding or metalloprotein evaluations can contribute in an effective way to proteomic studies, because they give a new view for understanding the mechanisms of those proteins that depend on the presence of different metals in their structures. Like genomics and proteomics, a new and vast field of study (metallomics) is now becoming a reality.

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