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SPECIES-SELECTIVE ANALYSIS FOR METAL–BIOMACROMOLECULAR COMPLEXES USING HYPHENATED TECHNIQUES

(Technical Report)

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Species-selective analysis for metal–biomacromolecular complexes using hyphenated techniques (Technical Report)

Abstract: Analytical chemistry of metal complexes with biomacromolecules based on the coupling of a high resolution separation technique with an element or species selective detection technique is critically discussed. The role of size-exclusion chromatography (SEC) with on-line atomic spectrometric detection is evaluated for the characterization of the metal distribution among the fractions of different molecular weight. Attention is given to the conditions for the separation of metallated biomacromolecular isoforms and subisoforms by anion-exchange and reversed-phase HPLC. Techniques for interfacing chromatography with atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP AES) and ICP mass spectrometry (ICP MS) are assessed. The potential of electrospray (tandem) mass spectrometry for the on-line determination of the molecular mass of the eluting protein is highlighted. Perspectives for capillary zone electrophoresis (CZE), microbore and capillary HPLC with ICP MS and electrospray MS detection for probing metalloproteins are discussed. Applications of hyphenated techniques to the analysis of real-world samples are reviewed.

INTRODUCTION

Metals are commonly found in nature as natural complexes with biomacromolecules (e.g. oligo-and polypeptides, proteins, DNA restriction fragments, polysaccharides). Peptide-complexed metal ions are known to perform a wide variety of specific functions (regulatory, storage, catalytic, transport) associated with biochemical processes [1,2]. The largest interest is attracted by essential elements which include some transition metals such as, e.g. Fe, Cu and Zn associated with ferritin (Fe, Cu, Zn), β-amylase (Cu), alcohol dehydrogenase (Zn), carbonic anhydrase (Cu, Zn) and other proteins [1,2]. Homeostatic control, metabolism and detoxification of toxic elements (e.g. Cd, Hg) by their interaction with metallothioneins (MTs) have been in the focus of ecotoxicology and clinical chemistry [3,4]. Detoxification mechanisms of plants exposed to heavy metals involve synthesis of small thiol peptides (phytochelatines) able to chelate heavy metals due to the high cysteine content in the molecule [5]. The complexation of divalent cations with the carboxylic acid groups of uronic acids from plant cell walls polysaccharides (pectins) is well established [6].

Metals (Pt, Au, Ru) are components of many therapeutic drugs; the mechanisms of their interactions with proteins and DNA are a key to understanding their activity [7]. The interest in elemental speciation in breast milk is stimulated by the species-dependent difference in bioavailability of trace elements by formula-fed and by breast-fed children [8]. Speciation of biomacromolecular metal complexes in foodstuffs is stimulated by similar health concerns.

The key challenges to analytical chemistry of metalloproteins include: (i) selectivity with regard to the different iso-and subisoforms, (ii) selectivity with regard to metal, and (iii) sensitivity able to cope with the noninduced (background) levels in real-world samples. The impossibility of meeting these three criteria by analytical instrumentation available as well as the often low thermodynamic stability of the analytes are at the origin of the fact that speciation of metals of vital interest in biological fluids still remains unknown to a wide extent.

In the last decade a universally accepted approach to speciation analysis has been offered by hyphenated (coupled, hybrid) techniques that are undergoing a rapid and continuous development [9–13]. They are based on a combination of a separation technique (gas chromatography (GC), HPLC, or (CZE)

with a sensitive element detector (atomic absorption, plasma emission or mass spectrometer). The rapidly growing popularity of ICP MS [14–16] and electrospray ionization MS [17,18] opens new exciting possibilities for a more precise characterization of metalloproteins in terms of metal content and molecular mass, respectively.

The critical evaluation of the state-of-the-art and the perspectives of quantification and characterization of metalloproteins by coupled techniques is the topic of the present report.

SPECIES OF INTEREST

Species of interest include biomacromolecular metal complexes found in plants (e.g. polysaccharides, phytochelatins), biological fluids (e.g. proteins, porphyrins) and animal tissues (e.g. metallothioneins). Analytes studied in biochemical speciation research are presented in Tables 1a and 1b.

Table 1a Analytes in biochemical speciation analysis

Metallopeptides	Metallodrugs	Miscellaneous
phytochelatins (Cd, Cu, Zn)	cisplatin (Pt)	amino acid-complexed metals
metallothioneins (Cd, Cu, Zn)	carboplatin (Pt)	metalloporphyrines
metalloenzymes	auranofin, aurothiomalate, aurothioglucose	ferrocene derivatives
transport proteins [albumin (Cu, Al), transferrin (Fe, Al]	Ru-imidazole(indazole) complexes	DNA restriction fragments (Fe, Mn, Co, Pb, Cd)
	Tc-imaging agents	cobalamines, cobanamids (Co)
		seleno animoacids (Se)
		organoarsenicals, arsenosugars (As)

Table 1b Metals and the principal metal-binding polypeptides

Metal	Proteins of interest
Fe	haemoglobin, myoglobine, cytochromes, peroxidases and catalases, ferritin, transferrin
Cu	β-amylase, carbonic anhydrase, ceruloplasmin, ferroxidase, albumin and transcuprein, metallothioneins, superoxide dismutase
Zn	alcohol dehydrogenase, carbonic anhydrase (carbonate dehydratase) and thermolysin, metallothioneins, superoxide dismutase
Ca	calmodulin, parvalbumin, thermolysin and proteolytic peptides of calmodulin
Cd	alcohol dehydrogenase, metallothioneins
Al	transferrin, albumin

SAMPLE PREPARATION FOR SPECIATION ANALYSIS OF METAL–PROTEIN COMPLEXES

Biological fluids (full blood, plasma, synovial fluid, breast milk)

Sample preparation of serum prior to HPLC included filtration of sample on a 0.45-μm or 0.2 μm filter [19]. Erythrocytes were subject to three freeze-thaw cycles to lyse the cells [20,21], followed by a 10-fold dilution with a buffer and centrifugation to remove fragments of membranes. The supernatant was further diluted. Breast milk should be centrifuged to remove fat; precipitation of casein with 1 M acetate is optional [22]. Dialysis and purification by size-exclusion chromatography is required if further separation by RP HPLC should be untertaken [22].

Plant and animal tissues

Washing cells in a Tris-HCl buffer (pH 8) containing 1 M EDTA to remove metal ions reversibly bound to the cell wall was recommended [23]. In the majority of works SEC [23–30] has been prefered to heat treatment [31–35] for the isolation of the metallothionein fraction from the tissue cytosol. Guidelines for the preparation of biological samples prior to quantification of MTs were discussed with particular attention given to the care necessary to avoid oxidation [36].

Soluble extracts of tissues and cultured cells are prepared by homogenizing tissue samples in an appropriate buffer. Neutral buffers are usually used for extraction since Zn starts to dissociate from protein complexes at pH 5. Cd and Cu are removed at lower pH values. A 10–50 mM Tris-HCl buffer at pH 7.4–9 is the most common choice [23,27,33–35]. For cytosols containing Cd-induced MTs dilution factors up to 10 have been used whereas for those with natural MT levels equal amounts of tissue and buffer have been found suitable.

Metallothioneins and phytochelatins are prone to oxidation during isolation due to their high cystein content. During oxidation disulfide bridges are formed and the MTs either copolymerize or combine with other proteins to move into the high molecular weight fraction. Since species of interest may be oxidized by, e.g. oxygen, Cu(I) or heme components, the homogenization of tissues and subsequent isolation of MTs should be normally performed in deoxygenated buffers and/or in the presence of a thiolic reducing agent [40]. β-Mercaptoethanol is added as anti-oxidant [38,42]. Other components added during homogenation include 0.02% NaN_3 (an anti-bacterial agent) [43,44] and phenylmethanesulfonylfluoride (proteases inhibitor) [29,39,40,43,45]. The homogenization step is followed by centrifugation. The use of a refrigerated ultracentrifuge (100 000 ***g***) is strongly recommended.

As a result two fractions: a soluble one (cell supernatant, cytosol) and a particulate one (cell membranes and organelles) are obtained. Only the supernatant is usually analysed for biomacromolecules thus limiting the number of species of concern to those being cytosoluble. It is recommended to store supernatant at −20 °C under nitrogen prior to analysis [42]. For example the extraction efficiency by sucrose–Tris at pH 8 was 25–30% of the total metal [34]. Other workers using experimental animals (rats and mice) found that 50–80% Cd could be solubilized using similar extraction procedures [34]. The percentage of metals in cytosols is typically 50–85% and 30–57% for Zn and Cd, respectively [46,47].

For studies of metallothioneins, a heat-treatment (at 60 °C for 15 min) of the cytosol extracts is recommended to separate high-molecular fraction that coagulated from the supernatant (containing MTs which are heat stable). Such a treatment reduces the protein load on the HPLC column not only improving the separation of MT isoforms but also prolonging the column lifetime. Enzymolysis in simulated gastric and gastrointestinal juice was proposed for meat samples [34,35]. Enzymatic solubilization of edible plant, fruits and vegetables was reported [48].

Filtration of the cytosol (0.22 μm filter) before introducing it onto the chromatographic column is strongly advised [34,42,49]. A guard column should be inserted to protect the analytical column particularly from effects of lipids, that otherwise degrade the separation [36]. Any organic species that adhere to the column can also bind inorganic species giving rise to anomalous peaks in subsequent runs [34]. A new guard column should be used for each injection to prevent adsorption by ligands with a high affinity for cadmium that would otherwise interfere with subsequent injections; an extensive column cleanup was necessary [34]. To avoid contamination of the analytical column by trace elements, buffers should be cleaned by cation-exchange on Chelex-100 [34,49,50].

ANALYTICAL TECHNIQUES

The various possibilities of on-line coupling between a separation technique and an element (moiety, species) specific detector for speciation analysis of metal biomacromolecular species include different modes of HPLC or capillary zone electrophoresis (CZE), in terms of separation, and flame AAS, ICP AES, ICP MS or electrospray MS, in terms of detection. The choice of a hyphenated system depends primarily on the research objective. The separation component of the coupling system will be favoured when the characterization of a maximum number of iso- or subisoforms is of interest, whereas the detector component becomes crucial when a high sensitivity is required.

The presence of a metal bound to the biomacromolecule is the prerequisite of using an element-specific atomic spectrometric detector such as an atomic absorption, ICP atomic emission or ICP MS spectrometer. The ADLs of several femtograms of the latter often make it possible to detect noninduced metal biomacromolecular complexes in on-line mode (in contrast to immunoassays and off-line fraction-collection techniques). The linear range and multi-element capability of these detectors make them a rapid alternative to metal-saturation assays. Electrospray MS is unmatched to characterize metalloprotein isoforms in terms of molecular mass.

An important problem is often the interface between chromatography and spectrometry as the separation conditions may be not compatible in terms of flow rate and mobile phase composition with those required by the detector. High concentrations of methanol in reversed-phase HPLC negatively affect the plasma but enhance the electrospray ionization process. On the other hand, the relatively concentrated buffers used in ion-exchange chromatography suppress the signal in electrospray. The nanoliter sample volume and low microliter per minute flow rates make the dead volume and sensitivity critical in AAS and plasma source techniques. They can be handled, however, by ESI MS, especially with a nano- or microsprayer. The above considerations make the choice and optimization of the best hyphenated system for a given task difficult and require some experience.

Separation of metal–protein complexes

Successive ultrafiltration through membranes with molecular weight cut-offs of 500, 5000 and 30 000 has widely been used to study the distribution of metal-species as a function of molecular weight [44,51–53]. The metal and the protein was determined in the filtrate and retentate by an off-line technique, usually graphite furnace AAS owing to its high sensitivity. Due to the poor resolution, the methods allow only a rough speciation and are time consuming. A more efficient and less cumbersome is size-exclusion chromatography which can be used as a stand-alone technique but also can precede a finer analysis by reversed-phase chromatography.

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is based on the molecular sieve effect and enables species to be separated according to their size, and to a lesser extent, shape. The average time a substance spends in the pores (determined by its size which for a given shape) can usually be related directly with its molecular weight. The resolution of SEC being insufficient for the discrimination of the small amino acid heterogeneities, the technique serves for the separation of the high molecular weight fraction from the sample.

Packing. Separation by SEC should be independent of the analyte's charge but in practice the stationary phase surface displays charged properties so that a mixed-mode separation is observed. This makes the choice of packing critical. The two categories of packing used included silica and organic polymers. At the nanogram metal levels significant silanophilic effects including metal losses in the presence of low ionic strength eluents on silica-based SEC supports were reported [37,39,40,54]. A co-polymeric styrene-divinylbenzene SEC support that provided a symmetrical peak with negligible losses of Cd during chromatography was proposed [39,40]. The average pore size of the packings used varies from 100 to 1000 Å. For a detailed speciation of Se in human milk, columns with a separation range up to 250 kDa should be used [22]. The combination of two SEC columns provides a wider molecular range [22].

Mobile phase. The optimum eluent should assure the minimum competition between buffer and cytosolic ligands, and between these ligands and the gel. The separation by SEC with H_2O as mobile phase prevented structural changes, denaturation of proteins, and destruction of protein–metal complexes [55]. In practice, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material. Dilute buffers, in general, may cause adsorption of low molecular weight proteins on the column packing. When silica-based packings cannot be avoided, the addition of a noncomplexing salt (e.g. 100 mM NaCl) to the mobile phase is necessary in order to suppress the residual silanol activity of the column packing [54]. At such conditions no significant exchange of Cd occurred but occasional (for Hg) or appreciable losses (for Zn) were observed for other metals [54].

An addition of EDTA proposed by some authors to minimize metal ion-gel interactions [41] was found unsuitable by others [34], because of the occasional presence of anomalous Cd peaks in subsequent runs. Polymeric supports suffer from the deposition of excess free Cd^{2+} which interacts with the analytes, often causing severe degradation in peak resolution [40]. Because the weak complexing character of Tris is not sufficient to compete with the polymeric support for Cd^{2+}, complexation with β-mercaptoethanol is advised [40].

The wide variety of buffers reported in the literature makes it relatively easy to chose a suitable one for the detection technique to be used. Up to 30 mM Tris-HCl was found to be well tolerated for ICP MS whereas 20 mM formate or acetate buffer in 10% methanol are acceptable for the ESI. Addition of 0.03% NaN_3 as a retardant of bacterial activity is advised to protect the column from damage resulting from the bacterial growth when real-world samples are analysed.

Analysis time. This is a function of the column size and the flow rate. Although the columns up to 120 cm were used the standard 30 × 7.6 mm column is a good choice. At a flow rate of 1 mL/min, the separation requires ≈ 20 min to complete. The separation of MT-bound and unbound cadmium within 3 min on a 4.0-cm SEC column was reported [45]. The choice of small-bore columns with size-exclusion packings on the market is still limited but such columns enable a rapid characterization of various metal-containing molecular weight fractions in unknown cytosols by direct injection nebulization ICP MS or ESI-MS.

In many applications SEC HPLC-ICP MS has been used as a semiquantitative technique used to monitor relative changes of analytical signals in a well defined series of samples. Quantification of signals has usually been done using peak area calibration either by converting (by removing the column) the measurement system into the flow-injection mode after completing the chromatographic run [48,56], or using a calibration graph if standards were available [40,45].

One of major problems in the analysis of real world samples is the unavailability of standards. Only a very restricted number of standards (some serum proteins, metallothioneins) can be used for peak identification. In most applications further signal characterization by orthogonal (complementary) chromatographic techniques is necessary.

Reversed-phase chromatography (RPC)

Fractions separated by SEC can be further characterized by reversed-phase chromatography between a nonpolar stationary phase (usually a covalently bound C_8 or C_{18} linear hydrocarbon), and a relatively polar mobile phase. Reversed-phase HPLC seems to be superior to SEC and ion-exchange for the separation of metal-biomolecule complexes because the packing material for RPC is principally free of ligands for metals [42]. Since hydrophobicity of a polypeptide primarily dictates its retention in RPC, gradual elution of individual species of a mixture is achieved by decreasing the polarity of the mobile phase by the addition of methanol or acetonitrile. Isolation of MT-isoforms by RP-HPLC was reviewed [57].

Wide-bore (4–5 mm) 15–25 cm long columns are the most frequently used. The narrow-bore and microbore columns are expected to gain in significance soon because of their higher sensitivity and resolution. The presence of 10–50 mM of buffer is necessary. Tris-HCl buffer is the most widely used for the separation of metallated iso-forms. The resolution of the isoforms is affected by pH [58].

The high content of organic modifier makes RP HPLC poorly compatible with ICP MS. The use of Pt-cones, a desolvation unit and the addition of oxygen to the nebulizer gas are highly recommended. Loss in sensitivity cannot apparently be avoided. On the other hand, the RP separation conditions are close to ideal for ESI MS detection which is expected to gain popularity in the near future [59].

Anion-exchange chromatography

Fractions, that have a negative charge in aqueous solutions can be separated using anion exchange chromatography. High performance liquid chromatography is usually used but the use of a fast-flow anion-exchange column (40 cm × 3 cm) was reported [24]. Separation efficiency and recovery were the same as for the conventional method, but analysis time was reduced to one-third at the expense of sensitivity [24]. Usually, metallothioneins, prior to anion-exchange chromatography are pre-separated by

SEC. Anion-exchange methods have been developed for the separation of metallothioneins [59a,60] and serum proteins [61].

A weak anion exchanger with diethylaminoethyl functional groups has been used in the majority of works concerning the separation of metal complexes with MTs. Aqueous buffers with a linear concentration gradient are used as eluents. The high potential of separation of the MT-1 and MT-2 isoforms by anion-exchange is not fully exploited in the coupled systems because the common end-concentration of 0.25 M of buffer is not so easily tolerated by ICP MS and suppresses the electrospray ionization. Strong quaternary ammonium anion exchanger was used to separate aluminium carrying serum proteins [61].

Other separation techniques

Affinity chromatography using heparine-agarose [61a] or heparin–Sepharose CL-6B column in series with a column of reactive blue 2-Sepharose CL-6B [62,63] with off-line detection was proposed for the separation of selenoproteins in serum.

Polyacrylamide gel electrophoresis (PAGE) has been applied with good results to proteins, sodium dodecylsulfonate is used as a denaturating agent to break the protein aggregates [64–67]. As a result all proteins regardless of their identity are imparted the same free-solution mobility so their separation is controlled by size with smaller proteins migrating more rapidly than the larger ones. PAGE was used for the separation of proteins which had been labelled by ^{75}Se prior to radiography [68]. The problem is the lack of an element-selective detector to be used in on-line mode. Laser ablation ICP MS may offer an attractive off-line alternative [69].

Capillary electrophoresis has been applied for a long time to the separation of metalloprotein. An excellent review of this work is available [70]. Interfaces of CZE with ICP MS are beginning to be used for metalloproteins [71,72].

Detection techniques

The requirements and limitations concerning an interface between HPLC and atomic spectrometry vary in function of the separation mode and the detector used. Size-exclusion chromatography shows generally good compatibility in terms of flow rates used (0.7–1.0 mL/min) and mobile phase compositions (unless concentrated buffers are used) with AAS and ICP techniques. Organic solvents are not a problem in anion-exchange chromatography but the buffers used often exceed 0.1 M which are likely to clog the nebulizers. In RPC the organic solvent has to be managed, whereas the buffer concentration is seldom a problem. The flow rate depends strongly on the column geometry. Varying the column inner diameter from 8 mm to 0.18 mm allows the change of the flow rate from 10 mL/min to 2 μL/min so it is compatible with the flow rate required by the nebulizer used.

Atomic absorption spectrometry (AAS)

The metals which preferentially bind to MTs (Cd, Zn, Cu) are among the elements that give the most intense response in AAS. Off-line analysis of MT-bound metals after HPLC by fraction collection and graphite furnace (GF) AAS analysis has been a common approach [35]. Being less sensitive than GF AAS, flame AAS offers the possibility of an on-line approach. This technique is compatible both with the flow-rates and with the mobile phase composition (including organic solvent) commonly used in HPLC. Taking into account the widespread availability of this technique, it is no wonder that HPLC-AAS was the first hyphenated technique to be used for the determination of MT-isoforms [73].

The basic interface is very simple. The outlet of a UV detector is coupled to the nebulizer of an atomic absorption spectrometer and metals of interest are determined at their characteristic lines. Calibration graphs were reported to be rectilinear up to 200 μg/mL of metallothionein and up to 20 μg/mL of metal [30,42].

Several advanced interfaces based on thermospray have been proposed [43,74,75]. A simple and inexpensive thermospray interface was described that could be connected to a flame AAS system without the need for modification of the nebulizer and burner assembly [75]. Detection limits at the low ng level

were reported [75]. Another design included a silica transfer line attached to the HPLC column end and positioned with a guide tube in a thermospray tube, which was fitted with a heating jacket. The superheated mobile phase then passed to a pyrolysis chamber fitted with gas inlets for H_2 and O_2. Calibration graphs were linear over two orders of magnitude from near the detection limits (subng) with aqueous or methanolic mobile phases [74]. A micro-atomization interface (made of quartz tubes) which was fuelled by hydrogen and could operate with 100% aqueous mobile phases used in HPLC was developed [43]. The detection limit obtained for HPLC-AAS of Cd-MTs was over 200-fold lower than that obtained with a conventional flame AAS detector, and ≈ 30-fold lower than that obtained by a thermospray-enhanced flame AAS system [43].

The primary interest in HPLC-AAS lies in its simplicity, wide availability and compatibility with mobile phases used in HPLC. AAS is not truly a multi-element technique; nevertheless, with the latest instruments, up to 4 elements can be measured simultaneously, which is fully sufficient for practical applications. Despite the fact that detection limits obtained with advanced interfaces almost match those of ICP MS, the primary field of application of HPLC-AAS is the characterization of MT-isoforms after metal saturation protocols.

Inductively coupled plasma atomic emission spectrometry (ICP AES)

In comparison with flame AAS, ICP AES offers lower (at the 1 ng/mL level) detection limits [31,32], but lower solvent compatibility. High methanol or acetonitrile (> 10%) contents may not be easily tolerated. The unmatched advantage of ICP AES is the possibility of the monitoring of sulfur together with metals. A detection limit for S below 10 ng/mL is common. ICP AES instruments with axial plasmas, recently commercialized, seem to offer lower detection limits for transition metals. Multi-element capability is an advantage of instruments equipped with a polychromator. The sensitivity of coupling of ICP AES is generally inadequate to cope with the levels of most elements in noninduced samples.

Inductively coupled plasma mass spectrometry

ICP MS has been enjoying the largest popularity because of its multi-element character, low detection limits and the possibility of the use of stable isotopes instead of radioactive ones. HPLC-ICP MS with enriched stable isotopes is a unique analytical method by which speciation of both endogenous elements and external tracers can be achieved in a single experiment. A wider expansion of high resolution ICP mass spectrometers (with potentially lower detection limit and larger freedom of interferences) is hampered by the prohibitive cost of instrumentation and the high maintenance costs.

For multi-element SEC HPLC-ICP MS, the elements of interest were divided into groups for the sake of optimal sensitivity. Within each group the replicate time, dwell time and number of sweeps per reading were optimized. A separate injection was made for each group [76]. Eluents should not contain elements that give polyatomic interfering ions in an ICP.

Conventional pneumatic nebulization interface. The interface is straightforward—a piece of narrow-bore tubing that connects the outlet of the LC column with the liquid flow inlet of the nebulizer. Typical LC flow rates of 0.5–2 mL/min are within the range usually required for pneumatic nebulization. The limitation is the low (1–5%) transfer efficiency, losses in spray chamber, and thus degraded sensitivity. Aqueous eluents with the buffer content up to 50 mM are tolerated. Organic solvents influence negatively the plasma performance because of the increasing instability of the plasma (until extinction in extreme cases) and deposition of carbon on the sampling cone and torch. The use of a water-cooled spray chamber and an increase in RF power (up to 1.7 kW) can help to reduce the solvent load to the plasma and to increase its stability. The addition of oxygen (1–3%) to the nebulizer gas flow can help to minimize carbon deposition and clogging of the sample cone at the expense of the cone's lifetime [23,76]. Salts can cause short-term signal depression or enhancement and cause blockage of the nebulizer and the sampling cone [78]. Potential of LC ICP MS for trace metal speciation was discussed [14,79]. In general, ICP MS requires more dilute buffers to be used and tolerates lower concentration of organic solvents than ICP AES. Post-column split and makeup with water to lower the concentration of the organic modifier is a possible remedy, however, at the expense of sensitivity [45].

Direct injection nebulizer (DIN) interface. The DIN is a microconcentric pneumatic nebulizer with no spray chamber; it nebulizes the liquid sample directly into the central channel of the ICP torch. The low dead volume (<2 μL) and the absence of a spray chamber of the DIN minimize post-column peak broadening and facilitate the use of microbore LC columns and liquid flow rates (30–100 μL/min) [80–82]. Another advantage is the fast sample washout with minimal memory effects.

The carbon deposits on the sampler and skimmer orifices are less important than in the case of pneumatic nebulizers. Adding O_2 to the aerosol gas to prevent clogging by the salts dissolved in the mobile phase was not necessary for SEC [80]. Organic solvents in reversed phase chromatography require, however, the addition of oxygen to keep the plasma stable [81,82].

Hydraulic high pressure nebulizer (HHPN) interface. The HHPN [83] not only enhances the sensitivity by one order of magnitude but also allows an optimum adaptation to the low flow-rate by choosing an optimal nebulization nozzle (10 μm instead of 20 or 15 μm) [84]. The HHPN is also less affected than the pneumatic nebulizer by high salt content of the analysed solution. A dedicated desolvation unit can cope with methanol concentrations usually used in reversed-phase chromatography.

Electrospray MS

Electrospray ionization allows multiply charged molecular ions to be obtained without any fragmentation. The envelope of peaks in a mass spectrum is a manifestation of protons attaching to the species to give the multiple-charging phenomenon which is the basis for the accurate (0.1%) molecular weight determination of polypeptides.

Despite the enormous potential applications of this technique, the characterization of metalloprotein isoforms and its complexes with metals are still rare [40,59,85–87]. The formation of stable ions in the gas phase, even with metallated metallothioneins allows one to draw information about the number and identity of metals bound to the protein from the characteristic spectra for the apoproteins and metal-saturated proteins at acidic and alkaline pH [85,86]. The method can be used to determine accurately and rapidly how many and what cations are incorporated in each molecule of protein. A single measurement provides information about molar distributions and estimates the relative abundances of various complexes in the sample.

Pneumatically assisted electrospray (ion-spray) produces stable ion currents for a wide range of sample flow rates from 5 to 50 μL/min and is compatible with buffers (<10 mM) used in microbore HPLC. The coupled RP HPLC-ESI MS allows the on-line determination of sequentially eluting MT isoforms [59,87]. In studies with sheep MT, the four isoforms predicted from gene sequencing information were resolved by acidic RP HPLC-ESI MS and their masses determined to within one mass unit of the predicted apoprotein values [88]. The combination of the resolving capability able to discriminate between MTs that vary by one amino acid only with the possibility of the instantaneous, very precise on-line determination of molecular mass opens the way to identification and cataloguing of the naturally occuring MT isoforms [59]. Major isoforms of commercial rabbit liver, MT-1 and MT-2, showed nine and seven different subisoforms, respectively, the molecular masses of which could be precisely determined [59].

QUALITY CONTROL

During speciation analysis the major obstacles are contamination, break-up of the original metal-protein bond during the separation process, and insufficient detection limits of the element ion in the eluate.

Contamination risk

The risk of contamination at the level of sampling is as acute as in trace analysis for total metals and the same precautions should be applied. The ubiquitous presence in the laboratory environment of iron and zinc, and to a lesser extent copper makes the contamination problem particularly acute. Protocols for sampling of milk and standard specifications for cleaning the material used were discussed in detail [22]. The necessity to extend the procedure is likely for other biofluids. Chromatographic buffers were cleaned by elution over Chelex 100 and afterwards checked for contamination [22]. A silica-based scavenger

placed in the proximity of the injection valve was proposed to retain any Al and Fe originating from buffer solutions and recipients [19]. A metal-free HPLC–ETA AAS for the separation of the proteins and of the inorganic/organic metal species of interest was described [19].

Stability of species and acid–base equilibria

Biomolecules are easily subject to deterioration. As part of an investigation into the stability of organometal complexes in human milk, different buffers (varying in molarity and pH) were compared with H_2O as the mobile phase in size-exclusion chromatography (SEC) in order to investigate differences between the Zn distribution pattern and the total amounts of Zn per ligand. H_2O was found to be a more suitable mobile phase than the buffers with regard to contamination. A low pressure LC (LPLC) method was developed to investigate the possibility of a transfer of Zn among the proteins during SEC; casein and metallothionein were chosen as competitive Zn ligands [89]. A possible Zn-transfer from one protein to another was examined by combining a Zn-containing protein with a Zn-free one. No change of the Zn-status of the protein was detected, indicating a stable protein–metal complex under the experimental conditions.

Since the complexation equilibria between complexes of metals with biomacromolecules and 'free' metals are strongly pH-dependent, the control of pH of the mobile phase is crucial irrespective of which separation technique is going to be used. Acidic pH are responsible for the depletion of metals leading gradually to apo forms. For instance, Zn is lost from MT at pH 5, at pH 3.0 Cd_4 adducts of isoform are present and at pH 2 copper still remains attached. Various intermediate partially metallated forms occur at various pH ranges. The buffer chosen should therefore ensure that speciation of the analyte is not altered during its passage through the column.

Recovery

For biological macromolecules SEC columns are generally used to separate the compounds as a function of their size. The recovery with SEC was reported problematic because the result tended to suffer from poor protein recovery caused by adsorption of proteins on the stainless steel tubing [89]. Buffer solution (pH 7.4) should correspond to the physiological conditions of the body in order to avoid denaturation of the milk proteins during the chromatographic separation. Spiking cannot be regarded as an aid to the verification of recovery since a selective enhancement of metal concentrations is possible due to the free metal-binding capacities of certain proteins [90]. Also, a depletion of metal ions added to the mobile phase was observed in the separation of proteins with free metal-binding capacities [90]. The addition of metal salts can result in severe modifications of the intrinsic metal speciation [90].

Standardization and accuracy

To establish the identities of the elution peaks measured with an UV-detector, purified human milk proteins were used. The molecular weight association of the proteins is performed after calibration of the column with protein standards. There is a need of independent methods for separation and for detection in the same fluid to obtain unambiguous results. More important is that the lack of sufficiently pure calibration standards does not allow for unambiguous identification of signals in HPLC and CZE chromatograms whereas the accuracy of the assessment of the molecular mass in SEC leaves a lot to desire.

ICP MS instrumental instability due to the clogging of the plasma torch, sampling and skimmer orifices is common. The correction is possible by postcolumn addition of an internal standard.

AREAS OF APPLICATION

Biomedical

Trace metal complexation in blood and blood plasma

Metals are distributed in serum among different proteins; the distribution has important pathological implications [1,2]. The largest interest is attracted by essential elements which include some transition

metals such as iron, copper and zinc. Especially iron is important, which can be bound to proteins, such as, e.g. haemoglobin, myoglobine, cytochromes, peroxidases and catalases. It can be associated with sulfur in ferredoxins, while in invertebrates it is present as the oxygen-carrying pigment, hemerythrine. Metalloproteins binding essential and heavy metals in serum [21,80] and in erythrocytes [20,21] have been studied. Availability of many serum proteins standards makes peak identification possible for some elements (e.g. Fe, Cu). Several metals are associated with proteins: ferritin (Fe, Cu, Zn), β-amylase (Cu), alcohol dehydrogenase (Cd, Zn) and carbonic anhydrase (Cu, Zn). Al and Fe were found to be bound to transferrin and to citrate [19]. Six-metal binding protein fractions were identified in a human serum CRM [80].

Interest in speciation of toxic elements focuses mainly on lead and cadmium. In human serum lead was found in at least three molecular weight fractions. The major part of lead was coincident with copper and was found to be bound to ceruloplasmin. Of the protein-bound Pb recovered, 80% was reported to be contained in a protein of apparent molecular mass 240 kDa and 20% in protein of apparent molecular mass 45 kDa [20]. The major lead binding site in erythrocyte was identified as hemoglobin [21].

Cobalt protoporphyrin, iron protoporphyrin (hemin) and zinc protoporphyrin were separated by HPLC and detected by ICP MS, the method was used to quantify zinc protoporphyrin from the blood of a lead-poisoned patient [91]. The binding of metal cations both for essential elements (e.g. Mn, Fe) and toxic ones (e.g. Pb, Cd, Co) to DNA restriction fragments was studied by SEC HPLC-HR ICP MS [92].

Experimental conditions for species-selective analyses of metal-containing proteins in clinical samples are presented in Table 2.

Selenoproteins in human and animal tissues

Both of the common inorganic selenium species (selanate and selenite) are toxic whereas Se-containing amino acids are biologically useful and can be used for biosynthesis of selenoproteins. In contrast to the metal-protein complexes selenium is not associated by coordination but forms covalent C-Se bonds. The major protein is selenoprotein P in serum which is used as a biochemical marker of selenium status [93,94]. Se-containing proteins were widely studied in in breast milk [8] and rat tissues [68]; more than 25 Se-containing proteins or protein subunits were detected.

HPLC-ICP MS with enriched stable isotopes has been used to study speciation of both endogenous elements and external tracers with a ^{82}Se tracer. It has allowed the simultaneous differentiation of the fate of injected and endogenous Se [95–97]. However, full identification of Se peaks was not possible, several of them were assigned on the basis of their molecular sizes in the Se SEC profiles [95–98].

Metallodrugs and interactions with proteins

A wide range of Tc compounds (e.g. Tc-labeled anti-bodies, Tc-mercaptoacetyl glycine complex) are used for diagnostic imaging of renal, cardiac and cerebral functions and of various forms of cancer. Platinum (cisplatin, carboplatin), Ru^{3+} (*fac*-[$RuCl_3(NH_3)_3$]) and gold (auranofin) compounds are well-known in cancer therapy whereas some other gold compounds (aurithiomalate, aurothioglucose) are important anti-arthritic drugs.

SEC HPLC-ICP MS is a fast method allowing the evaluation of the kinetics of metallodrug binding to individual proteins and serum [99]. This is required for: (i) estimation of desactivation of the drug (e.g. cisplatin in serum), (ii) estimation of the transport efficiency[e.g. complex of Ru(III) drugs with apotransferrin], (iii) finding suitable 'release agents' for metallodrugs bound to serum proteins (e.g. citrate, EDTA, tartrate). The biologically active (e.g. anti-cancer) species is usually an unchanged metallodrug (e.g. cisplatin or carboplatin) that shows no significant anti-tumour activity after being bound to a plasma protein. However, some ruthenium-based anti-tumour drugs (e.g. *trans*-HInd($RuInd_2Cl_4$)) do not lose their anti-tumour activity and the transferrin-bound species exerts a considerable higher inhibitory effect compared to a free complex. To show an anti-tumor activity, the complexes must be released inside the cells. The addition of chelating agents such as citrate, EDTA, ATP to the metallodrug-plasma protein adduct leads to a new 'free' metallodrug [99].

Ion-pairing reversed-phase [100–102] and anion-exchange [103 104] chromatography have widely

Table 2 Species -selective analysis of metal-containing proteins in clinical samples

Sample	Sample preparation	Separation mode: column	Mobile phase	Detection	Ref
Serum	0.1 M Tris-HCl	SEC: Synchropak GPC 300 250 mm × 2 mm × 5 μm	0.1 M Tris-HCl (pH 6.9)	ICP MS	[80]
Serum	Filtration (0.45 μm)	anion exchange: Bio-gel TSK-DEAE-5PW (100 mm × 7.5)	Linear gradient from 0.05 M Tris-HCl (pH 9.2) to 0.05 M Tris-NaCl (pH 9.2).	HPLC/ETA AS	[19]
Pb, Fe, Cu, Zn and Mg in serum and red blood cells	Hemolysis of red blood cells by freezing and thawing. Centrifugation.	SEC: TSK G 3000 SW (300 mm × 7 mm)	0.1 M Tris-HCl pH 7.2	ICP MS	[21]
milk whey for Se, Fe and Zn	Centrifugation of the whole milk to separate off fat	SEC: Asahi-Pak GS 620 (7.6 × 500 mm × 9 μm) + Asahi-Pak GS 520 (7.6 × 500 mm × 9 μm)	buffer pH 7.4; 20 mM NaH_2PO_4 50 mM NaCl	ICP AES	[8]
Cu, Se, Zn, Fe and S in mice liver		SEC: Asahipak GS 520 (50 cm × 7.6 mm × 9 μm)	0.05 M Tris-HCl buffer; pH 7.4 (1.0 mL/min)	ICP MS	[95]
plasma, red blood cells, liver and kidney of rats, Fe, Cu, Zn, Se		SEC: Asahipak GS 520 (50 cm × 7.6 mm × 9 μm)	0.05 M Tris-HCl buffer, pH 7.4 (1 mL/min)	ICP MS	[96–98]
Al in neuroblastoma cells		SEC: Superdex-75 HR (30 cm × 10 mm)	0.03 M Tris-HCl, pH 7.2 (0.9 mL/min)	ICP MS	[105]
rat urine		SEC: Asahipak GS-320 (50 cm × 7.6 mm i.d.)	0.05 M Tris-HCl buffer, pH 7.4 (1 mL/min)	ICP MS	[97,98]
Cobalt protoporphyrin, iron protoporphyrin (hemin) and zinc protoporphyrin in blood	Mixing of blood with a 20:1:1 mixture of acetone, pyridine and sterol, centrifugation, drying of the supernatant at 40 °C, dissolution of the residue in 5 mL of MeOH	RP: Hypersil SAS C, column (25 cm × 4.6 mm i.d.x 5 μm)	68% MeOH of pH 4.5	ICP MS	[91]
Cu, Zn and Al in the femur, brain and kidney of guinea pigs		SEC: Progel TSK-HW55S (25–40 μm)	0.12M Tris-HCl; pH 8.2 (0.75 mL/min)	ICP MS	[49]
erythrocytes	Lysisis, dilution 1 + 9 with 0.05 M NH_4HCO_3, centrifugation at 18 000 ***g***, dilution 1:2 with a solution containing 5% NH_3, 0.5% w/v EDTA and 0.5% w/v Triton X-100	SEC: Superdex 200 HR 10/30 column;		ICP MS	[20]

been used to study metabolites and hydrolysis products of platinum [100] and gold [101–104] drugs in urine [101–103] and blood [102 104] of patients undergoing the therapy.

Experimental conditions for species-selective analysis in metallodrug research are presented in Table 3.

Miscellaneous

The interest in speciation in breast milk is stimulated by the bioavailability of trace elements for formula-fed children is different than that of breast milk fed children because of different speciation [8]. Speciation of Se, Fe and Zn in human milk was discussed [6]. Lacroferrin exists in four molecular forms in nature as does transferrin, and is dominant in human milk [22].

Understanding the Al neurotoxicity makes the identification of Al species in neuroblastoma model cells important, a low molecular compound containing Al was found after long-term incubation with inorganic aluminium [105].

Foodstuffs

Research in this area has been triggered by health concerns, applications published so far have concerned the bioavailability of toxic elements such as lead, aluminium and cadmium. Some of these studies involve the use of simulated gastro-intestinal digestion procedures [34,106], cooking [34] or enzymolysis [107] to monitor the changes in the chemical speciation of cadmium [34,50], zinc [49], aluminium [106] and lead and REE [107]. Speciation study of 24 elements in tea leaves and tea infusion [76] was presented.

The relatively high concentration of Al in tea has been the subject of concern, since for many people tea is the major source of Al in the diet. The excretion of Al is fairly effective, however, toxic effects can occur for patients with chronic renal failure [76,106]. It was assumed that the metal binding ligands in tea infusion are large polyphenolic compounds occuring widely in tea and other plants [76].

Speciation of lead in plants, vegetables and wine has attracted attention because of the well-proved toxicity of this element. Lead was found to be associated with one major macromolecular (5–15 kDa) species and with one to three minor compounds depending on the wine sample. The dominant species which accounted for 40–85% of lead was identified as the lead complex with the dimer of a pectic polysaccharide (Rhamnogalactorunan II). Other lead species found in wine that showed the molecular weight in the range 1000–3000 Da were not identified [48].

Experimental conditions for species-selective analysis of foodstuffs are given in Table 4.

Ecotoxicological

Metallothioneins

The best known area is research on metallothioneins (MTs) which are a group of nonenzymatic low molecular mass (6–7 kDa), cysteine-rich metal-binding proteins, resistant to thermocoagulation and acid precipitation. The most studied are mammalian MTs that have been isolated from kidney, liver and brain samples; MTs isolated from mussels, plants and fungi have also been reported. Homeostatic control, metabolism and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg, As) trace elements by environmental biota has been in the focus of interest because of environmental and economic consequences. Also, being the product of genetic polymorphism characteristic of MT genes in animals and humans, MT-isoforms draw attention of studies of metal-mediated gene expression mechanisms [3,4]. Metallothioneins in mussels have widely been studied [40,45]. Individual and synergistic effects of Cu, Zn and Cd ions on the induction of metallothionein in cyanobacterium (the origin of trophic chains in an aquatic systems) were investigated [56]. The *in vivo* selenium association with cyanobacterial metallothioneins following the co-administration of Zn and selenite or selenate was examined [23]. Analysis for metallothioneins using coupled techniques has recently been reviewed [108].

Hyphenated methods of the determination of metallothioneins are presented in Table 5.

Phytochelatins

The synthesis of phytochelatins is a common response of plant cells upon exposure to heavy metals.

Table 3 Hyphenated techniques in metallodrug studies

Species determined	Sample	Separation mechanism	Column	Eluent	Detection	Ref.
cisplatin, its hydrolysis products and products of incubation with methionine, glutathione and cysteine		ion-pairing	(15 cm × 4.6 mm) of OD5 C18 (5 μm), a guard column (5 cm × 4.6 mm) of Spherisorb ODS-2 (5 μm)	5 mM-heptanesulfonate/10% methanol/0.1% formic acid or 0.01 M trichloroacetic acid (pH 2.6)	ICP MS	[100]
Ru-and Pt-based metallodrugs in serum	serum	size-exclusion	Progel TSK G 3000 PWxL (6 cm × 7.8 mm)	0.03 M Tris-HCl, pH 7.2 (0.9 mL/min)	ICP MS	[99]
gold-based anti-arthritis drugs: auranofin and myochrysine (sodium aurothiomalate) and their metabolites	urine, blood	ion-pairing	(25 cm × 4.6 mm) Spherisorb ODS-2 (5 μm),a 5-cm guard column of the same material	MeOH–25 mM ammonium formate of pH 6 (1:1) containing 10 mM tetra-*n*-butylammonium chloride	ICP MS	[101, [102]
auranofin metabolites	urine	anion-exchange	(15 cm × 4.6 mm; 7 μm) with WAX-diethylmethylamine packing material (Alltech)	gradient from 100% of 20 mM-Tris (pH 5.5) to 100% of 200 mM-Tris over 15 min	ICP MS	[103]
auranofin metabolites	blood	anion-exchange size-exclusion	(15 cm × 4.6 mm) of Alltech WAX 300 anion exchanger (30 cm × 7.5 mm) of Bio-Sil TSK 25	20–200 mM-Tris buffer (pH 6.5 25 mM-Tris buffer (pH 7.7)	ICP MS	[104]

Table 4 Species-selective analysis of metal-containing species in foodstuffs

Sample	Column	Eluent (flow rate)	Detection	Ref.
metals in tea	SEC: Superdex 75 HR 10/30	0.1 M CH_3COONH_4 pH 5.5 (1 mL/min)	ICP MS	[76]
B in radish roots	SEC: YMC-Pack Diol-120 (30 cm × 8 mm)	0.2 M $HCOONH_4$ pH 6.5 (1 mL/min)	ICP MS	[109]
Pb, Ba, Sr, REE in edible plants	SEC: Superdex-75 HR (30 cm × 10 mm)	0.03M formate buffer, pH 5.8	ICP MS	[107]
chicken meat	SEC: Pharmacia Superose-12 HR 10/30	0.12 M Tris-HCl pH 7.3	ICP MS	[49]
Cd in pig kidney	SEC: Pharmacia Superose-12 (2 cm × 5 mm)	0.12 M Tris-HCl, pH 7.5	ICP MS	[34,50]
Zn isotope chicken meat simulated gastro-intestinal digest	SEC: Pharmacia Superose-12 (30 cm × 10 mm)	0.1 M CH_3OONH_4 0.1% TFA, pH 6.0	ICP MS	[49]
vanadyl porphyrins in marine mussels	RP: LiChrospher RP-8 (250 × 4.6 mm × 5 μm)	MeOH-H_2O (9 + 1)	ICP AES	[110]
wine	SEC:Superdex-75 HR (30 cm × 10 mm)	0.03M formate buffer, pH 5.8	ICP MS	[48]

Table 5 Hyphenated methods of the determination of metallothionein

Sample	Separation mode: column	Eluent (flow rate)	Detection	Ref.
liver tissue	RP: LiChrospher 100 RP-8 (12.5 cm × 4 mm × 5 μm)	20-min linear gradient from 0.5 to 30% MeOH in 50 mM Tris-HCl buffer (pH 7.0) (1.5 mL/min) AAS [42]	AAS	[42]
Anacystis nidulans	RP: Capcellpak C_8 (15 cm × 4.5 mm)	CH_3CN/50 mM Tris-HCl pH 7.5 (9 : 91) and 0.1 mM-EDTA (1 mL/min) ICP MS [23]	ICPMS	[23]
fish tissue (bream)	RP: Merck Purosphere$^{\rightarrow}$ RP-18 (250 mm × 4 mm × 5 μm)	A: 30 mM Tris-HCl pH 7.0; B: acetonitrile linear gradient: 0–20 min: 0–12% B	ICP MS	[45]
liver or kidney tissue	RP: PLRP-S (15 cm × 4.6 mm × 8 μm)	gradient elution with 10 mM $(NH_4)_2HPO_4$ (pH 8.2)	AAS	[30]
Cd-induced rat liver	Anion-exchange: DEAE-Sepharose Fast Flow (40 cm × 3 cm) Tris-HCl, pH 8.6)	2-l linear salt gradient (limiting buffer, 250 mM	AAS,	[24]
rat liver	Anion-exchange: DEAE-5PW (7.5 cm × 7.5 mm)	gradient elution with 0–40% of 200 mM Tris-HCl (pH 7.4) in 10 mM Tris-HCl (1 mL/min)	AAS	[59]
	Anion-exchange:TSK DEAE-3SW	gradient elution with Tris-HCl (pH 7.2–8.6) (1 mL/min)	AAS	[60]
mussel tissue (digestive gland)	SEC: Progel TSK G3000 SW (30 cm × 7.8 mm)	50 mM K_2HPO_4–KH_2PO_4 buffer, pH 7.5 (0.7 mL/min)	ICP AES	[31,32]
mussel tissue (digestive gland)	SEC: GPC Waters ProteinPack 125 Sephadex (100 cm × 1.5 cm)	50 mM K_2HPO_4–KH_2PO_4 buffer, pH 7 (0.7 mL/min)	ICP AES	[33]
Cd-induced rat liver	SEC: Sephacryl S-100 (120 cm × 1.6 cm)	10 mM $(NH_4)_2CO_3$	AAS	
osprey blood, mussels	SEC: Progel-TSK, G 3000PWxL (30 cm × 7.8 mm)	30 mM Tris-HCl, pH 8.6	AAS, ICP MS	[39,40]
Anacystis nidulans	SEC: Asahipak GFA-30F (30 cm × 7.6 mm)	50 mM Tris-HCl pH 7.5 and 200 M $(NH_4)_2SO_4$/0.1 mM EDTA (0.8 mL/min).	ICP MS	[23]
M. galloprovincialis	SEC: Progel TSK G3000PWxL (30 cm × 7.8 mm)	30 mM Tris-HCl (0.8 mL/min)	ICP MS	[45]
cyanobacterium	DuPont GF-250 (250 mm × 9.4 mm)	50 mM Tris-HCl, pH 7.5, 0.2 M $(NH_4)_2SO_4$	ICP MS	[41]

Phytochelatins are small peptides that are able to chelate heavy metals due to the high cysteine content in the molecule. The synthesis of phytochelatins molecules with different chain length is induced by a wide variety of heavy metals (e.g. Ag, Bi, Cd, Cu, Hg, Pb, Sn, Zn) whereas the direct participation of phytochelatins in the chelation of heavy metals ions in plants has been verified for only few elements under *in vivo* and *in vitro* conditions [5]. On-line HPLC-ICP MS was used for the characterization of heavy metal phytochelatin complexes of different plant systems [38].

CONCLUSIONS

It is essential that the analytical capabilities in the area of biological speciation analysis be improved if we are to understand in more detail the role of trace metals in health and disease. Hyphenated techniques are an attractive tool for rapid, sensitive and comprehensive characterization and quantitative determination of metal macromolecular complexes in biological samples. The progress in ICP MS and electrospray (tandem) mass spectrometry, and the increasing speed and efficiency of modern separation techniques, are expected to bring valid alternatives to metal saturation assays, immunoassays and electrochemical techniques in the near future.

There is an urgent need for creating an interface between biochemists, whose studies on the role of biomacromolecular complexes are still hampered by the tediousness and labour-intensity of the analytical procedures applied, and analytical chemists ready to demonstrate the usefulness of their newly developed instrumental techniques. The availability of standards and certified reference materials would enable the validation of hyphenated techniques against the classical methods which would facilitate their entry to biochemical and clinical laboratories.

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