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Metallomics: Guidelines for terminology and critical evaluation of analytical chemistry approaches (IUPAC Technical Report)*

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Abstract: Definitions for the terms "metallome" and "metallomics" are proposed. The state of the art of analytical techniques and methods for systematic studies of metal content, speciation, localization, and use in biological systems is briefly summarized and critically evaluated.

Keywords: metals; metabolomics; metallomics; proteomics; speciation.

1. RATIONALE AND HISTORY OF THE USE OF THE TERMS REGARDING THE METAL-RELATED *-OMICS*

The knowledge of the complete genetic blueprint of an increasing number of organisms has resulted in efforts aimed at the global analysis and functional study of a particular class of components of a living organism and the emergence of different "-omics". The concepts of genomics (the study of genes and their function) and proteomics (the study of the set of proteins produced by an organism, their localization, structure, stability, and interaction) have become part of the everyday language of life sciences [1,2].

Metal ions are a vital component of the chemistry of life [3]. One-third of all proteins is believed to require a metal cofactor, such as copper, iron, zinc, or molybdenum [4], delivered as a simple or complex ion or a metal-containing compound (e.g., methylcobalamin). The intracellular concentration of several metals, their distribution among the various cell compartments, and their incorporation in metalloproteins are tightly controlled [5]. The understanding of mechanisms by which a metal is sensed, stored, or incorporated as a cofactor requires, in addition to the identification of metalloproteins, the characterization of the pool of non-protein molecules (products of enzymatic or biochemical reactions) interacting with metal ions or of metabolites of exogenous metallocompounds, such as metallodrugs. A systematic approach to the study of metal content, speciation, localization, and use in biological systems is becoming increasingly important [6].

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The term "metallome" was coined by Williams who referred to it as an element distribution, equilibrium concentrations of free metal ions, or as a free element content in a cellular compartment, cell, or organism [7]. The latter would therefore be characterized not only by its genome or proteome but also by the metallome, their inorganic complement. The meaning of the term "metallome" was then proposed to be extended to the entirety of metal and metalloid species present in a cell or tissue type [8,9]. The characterization of the pool of metal-containing species in living organisms and of their interactions with the genome, transcriptome, proteome, and metabolome requires dedicated analytical approaches to in vivo detection, localization, identification, and quantification, in vitro functional analysis and "in silico" prediction using bioinformatics [10]. The term "metallomics" was coined by Haraguchi to denote the ensemble of research activities related to metals of biological interest [11,12] (Fig. 1).

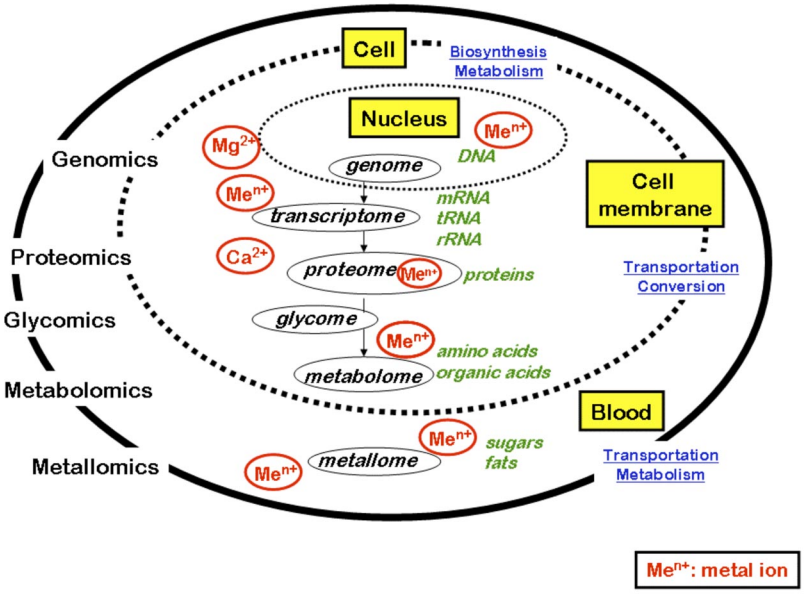

Fig. 1 Simplified model of biological system and related -omics sciences. The outer area surrounded with the continuous line is showing, e.g., an organ or whole body, and the inner area surrounded with the dotted line is showing a biological cell. Biological fluid, e.g., blood, is circulating in the intermediate area. The Mg^{2+} and Ca^{2+} ions are given as examples because of their large affinities with DNA and proteins, respectively, in the biological cell. Reproduced from ref. [12] with some modification.

Metallomics has been the topic of a number of reviews [13–15], special issues of edited journals [9,16,17] and of a Royal Society of Chemistry journal dedicated to the field, *Metallomics*. The terms "metallome" and "metallomics" have been used in different contexts. In addition, a number of related definitions proliferated, such as, for example, ionomics [18], heteroatom-tagged proteomics [19], or elementomics [20]. This report attempts to define the terms "metallome" and "metallomics", critically evaluates the available relevant analytical methodology, and highlights the concerned disciplines and research areas.

2. DEFINITIONS OF TERMS

metallome
Entirety of metal- and metalloid species* present in a biological system, defined as to their identity and/or quantity.

Note 1: The metallome can be determined in a bulk biological sample representative of the system [or its component(s)] or at specific location(s).

Note 2: The metallome can be characterized with different degrees of approximation, such as
- a set of total element concentrations,
- a set of metal complexes with a given class of ligands, e.g., proteins or metabolites, or
- a set of all the species of a given element (e.g., copper metallome).

Note 3: In contrast to the genome of which the analysis has a specific endpoint (the determination of a finite number of DNA sequences), the description of a metallome, like that of a proteome or metabolome [22], can never be complete. In particular, the numerous known and possible metal coordination complexes with biological ligands can be described only in terms of kinetic constants with defined thermodynamic equilibria.

metallomics
Study of the metallome, interactions, and functional connections of metal ions and other metal species with genes, proteins, metabolites, and other biomolecules in biological systems.

A metallomics study is expected to imply

- a focus on metals (e.g., copper, zinc, iron, manganese, molybdenum, nickel, calcium, …., cadmium, lead, mercury, uranium) or metalloids (e.g., arsenic, selenium, antimony) in a biological context;
- a link between the set of element concentrations or element speciation with the genome. This link may be statistical (an enrichment of an element coincides with the presence of a particular gene), structural (sequence of a metalloprotein is traceable to a gene) or functional (the presence of a bioligand is the result of a gene-encoded mechanism); or
- a systematic or comprehensive approach. The identification of a single metal species, however important, without specifying its significance and contribution to the system should not be referred to as metallomics.

3. METHODOLOGICAL APPROACHES TO THE METAL-RELATED -OMICS

3.1 Determination of the elemental composition of a biological system

The elemental composition of a biological system has been used in the literature as the basis of the following two types of "-omics" studies:

- ionomics: based on the building up of a large collection of mutants (differing by deletion of a particular gene) of a model organism, analyzing them for a considerable number of elements and linking the set of element concentrations with DNA sequences in order to detect metal-regulated genes [18,24];

*Specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure [21].

- metallogenomics: based on the purification of a large number of open reading frames from a given organism, heterologous expression of proteins and screening for those which contain metals in order to identify the metal-functional genes [25],

The data are acquired by the high-throughput analysis of bulk samples (often microsamples) but multielement analysis with spatial micro- or sub-micrometre resolution (e.g., tissue or cell elemental imaging) is becoming increasingly feasible and of growing interest.

3.1.1 Bulk analysis

The most suitable analytical technique is inductively coupled plasma-mass spectrometry (ICP-MS). It is based on the conversion of all the forms of an element present in a sample into element ions (usually singly charged) which are separated in a mass analyzer and counted by an ion detector [26]. The technique offers many advantages including femtogram-level detection limits, which are practically independent of the chemical form and the presence of matrix components, multielement and multiisotopic measurement capacity, and a large (10^9) dynamic range [26]. For these reasons, ICP-MS has practically replaced other techniques, such as ICP-atomic emission spectroscopy (AES), X-ray fluorescence (XRF), and instrumental neutron activation analysis (INAA), used in the past for the multielement determination of trace elements. State-of-the-art ICP-MS provides high-throughput analysis of microlitre or sub-microlitre samples in the continuous or flow-injection mode using dedicated micro- and nanoflow nebulizers [27].

ICP-MS suffers from interferences on a considerable number of isotopes. The understanding and awareness of these interferences by the analyst is a must. The use of a sector-field mass spectrometer at a required mass resolution or an ICP-MS equipped with a collision-cell is strongly recommended. The certitude that the analytically used signal corresponds to the expected isotope only is mandatory even in comparative analyses. The use of a certified reference material with the composition as close as possible to that of the analyzed sample is recommended for method validation and periodic quality assurance. Common sources of error are the selective or nonquantitative transfer of elements to the solution which requires a careful optimization of the sample digestion and minimizing contamination.

3.1.2 Spatially resolved analysis

A quantitative imaging (mapping) of the spatial distribution of elements in a solid sample (e.g., a thin section of a biological tissue) is possible by using a microanalytical technique, such as, e.g., X-ray absorption spectroscopy (XAS), particle-induced X-ray emission spectrometry, or laser ablation (LA) ICP-MS [28]. The analysis is usually not species-specific, although some specificity in terms of the oxidation state or the metal coordination environment can, in some cases (XAS), be obtained.

The high signal-to-noise ratio of ICP-MS makes it possible to acquire quantitative multielement concentration data (images) in thin (ca. 20 μm) section of biological tissues using direct LA with 10–150 μm resolution [29]. The image of the elemental distribution (ion current intensity as a function of tissue x and y coordinates and m/z ratios) for essential metals (e.g., iron, copper, zinc, manganese, molybdenum, cobalt, nickel), metalloids (selenium) and non-metals (phosphorous, sulfur, carbon) in the cross-section of analyzed tissue (e.g., brain, liver, kidney) can reveal some unique information for biological and medical research [30,31]. Inhomogeneous (often layered) site-specific metal distribution in tissue sections (as demonstrated in human brain different regions) can be obtained. The resolution can be decreased to the 100-nm level using nano-secondary-ion mass spectrometry (SIMS), nanoprobe synchrotron XRF, or near-field LA-ICP-MS [32], paving the way to metal distribution imaging in single cells and cell organelles.

Technically speaking, a commercial LA system using a Nd:YAG laser is usually coupled to a quadrupole ICP-MS without or with collision cell or double-focusing sector field ICP-MS. Modern commercial LA systems support the imaging of elements in tissues. LA-ICP-MS data are evaluated using dedicated imaging software developed in several laboratories. Detection limits of imaging LA-ICP-MS at the low μg g^{-1} and upper ng g^{-1} levels were reported. The measurement time for imag-

ing LA-ICP-MS of biological tissues (up to several hours) depends on the size of the analyzed area and the laser scan speed applied (varied from 20 μm/s up to 100 μm/s). For quantitative analysis, well-defined matrix-matched laboratory standards are required [33].

3.2 Analysis for metal-containing or metal-binding biomolecules

The analytical technique should produce a signal specific to a metal-containing biomolecule allowing its detection, identification, and quantification. The metallobiomolecules can be probed directly in a solid sample, by e.g., XAS or in a sample extract by electrospray MS. The ultimate resolution and sensitivity are offered by hyphenated (coupled) techniques which combine a separation by chromatography or electrophoresis with elemental or molecular MS detection.

3.2.1 X-ray absorption spectroscopy

XAS in the edge region, usually referred to as XANES (X-ray absorption near-edge spectroscopy), provides information about oxidation number, covalence (the number of pairs of electrons which a given atom shares with its neighbours), molecular symmetry of the site, and, hence, coordination number. The analysis beyond the edge region, referred to as EXAFS (extended X-ray absorption fine structure) [34], provides structural information about the atomic neighborhood of a metal(metalloids) being probed, such as the coordination number (number of ligands), the identity of the ligand atoms, and the distance between the metal and each ligand.

XAS is a quantitative technique but it is not particularly sensitive (a minimum analyte concentration at several tens of $\mu g\ g^{-1}$ level is required). In the case of a mixture of different species of the probed element present, the interpretation of the spectra becomes rapidly difficult so the technique is ideal when a single (or maximum two) dominant species are present. Identification (oxidation state or coordination center) is made by the comparison of the spectrum with that of a reference compound. XAS is invaluable for probing the kinetically labile complexes, which can readily dissociate or exchange ligands during sample handling and for the differentiation of the oxidation state, which in some cases [e.g., As^{III}/As^{V}] is readily modified during any extraction step. A representative example of applications includes the determination of quantitative distribution of zinc among the complexes with phosphate, cysteine, and histidine in plant (XANES) [35] or the identification of citrate as the major nickel-binding ligand in *Leptoplax* leaves (EXAFS) [36].

XAS, which is becoming popular owing to the increasing access to synchrotrons, gives the comfort of avoiding sample preparation, but its areas of application are limited. It can only confirm the presence of expected species (of a single element at a time) in a sample, but not find new ones.

3.2.2 Chromatography and capillary electrophoresis (CE) with element-specific detection

The most favored tool for a rapid semi-quantitative screening for the presence of metal-biomolecule complexes in biological samples has been the coupling of size-exclusion liquid chromatography (LC) and ICP-MS (for reviews of applications, see refs. [13,37]). The elution is monitored in the multielement (multiisotopic) mode, and as the column can be calibrated in terms of molecular mass, a chromatogram allows the fractionation of the metallobiomolecules as a function of size prior to detection. Fairly concentrated samples (e.g., 3–5 times diluted cytosol) can be analyzed, and conditions for non-denaturating separations can be readily optimized.

The resolution of size exclusion chromatography (SEC) is low, and the chromatographic purity of peaks is usually poor. The matching of the elution volume with that of a standard cannot be considered as a definitive proof of the species identity. Also, the control of the adsorbed metal ions on the stationary phase is important as they can exchange with the metal ions already complexed or can be scavenged by ligands from the sample leading to ghost peaks.

Among other types of non-denaturating chromatography (preserving the metal-biomolecule bond) anion-exchange high-performance liquid chromatography (HPLC) of metalloproteins [38] and hydrophilic interaction liquid chromatography (HILIC) [39] of metal-containing metabolites have at-

tracted particular attention. They offer much higher resolution than size-exclusion LC, and, especially, HILIC can be readily coupled with electrospray ionization (ESI)-MS for species identification.

CE offers a number of incontestable advantages for metal species analysis such as a small sample size (in the nanolitre range) required, high resolution, and the absence of stationary phase, and hence, the possibility of analysis of labile complexes. The development and commercialization of a dedicated CE-ICP-MS interface eliminated most of the problems of the earlier laboratory-made designs [40].

The rapidly developing application areas of CE-ICP-MS include metal-binding studies with pure compounds of biological origin, e.g., recombinant proteins [41,42], the determination of stoichiometry of metal-protein complexes [43–45], and the use of CE for fine (2^{nd} or 3^{rd} dimension) separations of metal-containing fractions isolated by chromatography [46,47].

The potential of some non-chromatographic techniques, such the coupling of field flow fractionation (FFF) with ICP-MS and high-field asymmetric waveform ion mobility spectrometry (FAIMS) for metallomic-type applications, merits a deeper insight.

3.2.3 Identification of metal-containing species by molecular mass spectrometry

ESI allows the production of ions of metal complexes in the gas phase, which can be then analyzed by mass spectrometry. The accurate mass determination should allow the determination of the empiric formula (and thus the metal-ligand stoichiometry) whereas the collision-induced dissociation (CID) mass spectra should deliver information on the structure of the ligand [48]. Paradoxically, the number of reports on the successful use of ESI-MS for the identification of metal-complexes has been remarkably scarce [13].

The problem is the vulnerability of ESI-MS to the presence of concomitant ions generated by matrix components which suppress the ionization of the species of interest. This concerns in particular the currently used quadrupole time-of-flight (QTOF) mass spectrometers for which the intrascan dynamic range is small. The ionization efficiency is critically dependent on the analyte chromatographic purity. Even if a single species of a given element is present (as detected by ICP-MS), hundreds of matrix compounds, transparent to the ICP-MS detection, may co-elute. Therefore, a purification step, using an orthogonal separation mechanism, is required prior to electrospray MS [49]. ICP-MS offers the possibility of monitoring the purification of the metalloprotein while accounting for its integrity (monitoring of the metal or, in some cases, the metal-to-sulfur ratio).

Column techniques (chromatography or CE) with the parallel ICP-MS and ESI-MS detection seem to offer the most adequate tool for the in vivo identification of metal–biomolecule complexes at the pico- and nanomolar levels, although the number of successful identifications to date has been limited and concerned relatively small proteins, such as metallothioneins. Further progress in the use of ESI-MS for identification of metal–bioligand complexes is expected with the wider availability of Fourier transform mass spectrometers, using either ion cyclotron resonance or electrostatic orbital traps, which offer a larger intrascan range and the possibility of the multistage fragmentation and mass spectrometry facilitating the species identification.

3.2.4 Gel electrophoresis with element-specific detection

Polyacrylamide gel electrophoresis (PAGE), employed either in the monodimensional or 2D mode, is considered the most adequate technique for the separation of proteins. The principal difficulty in its use for metalloproteomics is the need for the preservation of the metal–protein bond. Many metal-complexes with proteins are labile and can be destroyed by exchange with the metal impurities of the gel during separation and staining. Amongst the recommended precautions, the most important are: (i) the use of non-denaturating separation protocols, (ii) avoiding the presence of metal impurities in gels, and (iii) avoiding staining or the use of ultrapure staining reagents (as, e.g., in BlueNative electrophoresis [50]).

Metal-specific detection in the gels has enjoyed considerable interest for a long time, the principal techniques including autoradiography with its inherent use of radioactive isotopes (e.g., selenium-

75), and synchrotron radiation XRF and proton-induced X-ray emission (PIXE) with the need for hardly available facilities. LA-ICP-MS imaging, pioneered by Nielsen et al. [51], offers a competitive alternative for the in situ probing of the protein spots for the presence of metals and metalloids. The ablated analytes are swept into the ICP by a continuous stream of argon, and the ions are analyzed by MS. As a result, an electropherogram is obtained in which the quantity of a given element is a function of its position in the gel. Detection by LA-ICP-MS is a potentially fast and fairly robust technology, because no further reaction or derivatization step is involved, and the signal is, theoretically, directly proportional to the quantity of the analyte element in the gel [52].

LA-ICP-MS detection in 1D gel electrophoresis can be carried out in the lane scan mode [28]. In the spot-to-spot hopping mode [53] protein spots present in a 2D gel must be visualized prior to ablation. An alternative is the imaging mode [54]. The high stability and precision of LA-ICP-MS makes it possible to scan a gel in raster mode and thus to acquire element images. The analysis in the imaging mode is time-consuming. Scanning a gel of 2 cm^2 with a 100-μm resolution takes several hours, but the technique allows the identification of areas with increased metal concentrations regardless of the presence of intense protein spots. As the whole gel is scanned, the metalloproteins do not need to be visualized in either lane or imaging mode by staining, which minimizes the risk of metal loss.

Proteins containing covalently incorporated metals (e.g., selenoproteins) can be recovered from the gel and analyzed using the canonical proteomics protocols. The recovery of an intact metal-protein complex is much more difficult. Unless a mass spectrum of the metal-protein complex is provided, there is always a risk that the protein identified by the spot digestion and peptide mapping is different from that binding the metal in the same spot. Auxiliary data such as the presence of metallated peptides in mass spectra, in-gel EXAFS showing the metal–protein bond [55] or the desorption of the metalloprotein from the gel (or the blot) are required.

3.3 Analysis for biomolecules with metal-binding capacity

There is a class of analytical methods which does not target intact metal complexes but simply biomolecules showing an affinity to bind metals. Such molecules are typically isolated by immobilized metal affinity chromatography (IMAC). The analysis is qualitative and concerns the ligand only. The existence of the metal–ligand bond in vivo is assumed on the basis of the characteristics of the separation step.

IMAC resins use chelating agents (e.g., tridentate iminodiacetic acid) immobilized on a sorbent. Prior to use, metal ions are immobilized on the IMAC resin and are supposed to react with specific protein ligands in the sample. An IMAC protocol includes the saturation of an IMAC support (column/chip/chip array) with the metal of interest, washing off the excess of metal, introduction of a metal-depleted sample (metal depletion results in unoccupied metal-binding sites of proteins which facilitates binding to the IMAC metal) and the removal of the non-bound sample components followed by the elution of the retained proteins (with the metal-binding affinity) with a competing complexing agent. The proteins with the metal-affinity are recovered as apo(demetallated forms) and can be analyzed by any of the classic proteomics approach.

Alternatively, the retained proteins can be digested on-column. The metal-coordination center is expected to be preserved by the retention of the metal–peptide complex after the digestion of the protein [56,57], which seems to be a convenient way for the identification of the metal-binding motives

The IMAC approach enjoys a growing popularity with recent applications referring to the analysis of the zinc [56,57] and copper [56–58] proteomes in human hepatoma cell lines, uranyl proteome in human serum [59], bismuth proteome in *Helicobacter pylori* cell extracts [60], and Ni-proteome in human keratinocytes [61,62] and *Arabidopsis thaliana* roots [63].

IMAC does provide information on the presence of proteins with metal-binding sites but not on the metal–protein complexes present. Also, metalloproteins with a high metal affinity at functional sites, such as, e.g., superoxide dismutase (SOD), are likely to pass through the IMAC column due to

occupation of binding sites by physiological metals [57]. The IMAC approach is not quantitative, and minor proteins are likely to be masked by major ones.

3.4 Bioinformatics and prediction of the metal-binding patterns

This approach consists of searching metal-binding motives (which are expected to be preserved regardless of the organism studied) in putatively expressed proteins based on genome sequences present in data banks [64,65].

Substantial progress has been recently achieved for the identification of selenoproteins in a number of organisms, including humans. The original computer algorithms were based on the use of the canonical SECIS (selenocysteine insertion sequence) element (AUGA_AA_GA) as a signature for mammalian selenoproteins [66] and then refined to search for SECIS-containing genes with in-frame UGA codons [67]. The vast majority of genes encoding selenocysteine-containing proteins could be identified in this way.

Methods allowing the search for entire sets of metal-binding proteins are less advanced. Bertini et al. proposed an approach taking advantage of known consensus sequences, i.e., taking into account the nature and spacing of amino acids present in the metal-binding region; the distribution of iron-, copper-, and zinc-binding proteins in several archaea, bacteria, and eukaryotes was thus described [65]. The bioinformatic analysis of the protein sequence (by correlation with the relevant gene) allows the finding of the conserved metal-binding motifs and provides important clues to function and metal site structure [68]. This approach is critically dependent on the availability of consensus sequences for the binding of different metals.

Bioinformatics is also useful for the prediction of a metal-binding site based on a known 3D structure. It is possible owing to the conserved nature of the metal-binding site and its usual compact size [69]. The number of 3D protein structures is still limited but increases with the progress of structural genomics projects. Metal-binding sites can also be predicted by the combination of low-resolution structural data with sequence information [70].

The bioinformatic methods largely facilitate the identification of seleno- and metalloproteins but do not replace in vivo analysis, which is the sole way to say whether a given protein was actually biosynthesized or not.

4. INTEREST AREAS FOR METAL-RELATED -OMICS

Metal-related "-omics" are transdisciplinary research areas with an impact on biogeochemistry, clinical physiology and pharmacology, plant and animal physiology, and nutrition. An example of interdisciplinary interest is the detection, quantification, isolation, and characterization of metalloenzymes playing fundamental roles in a number of biological processes, including photosynthesis, respiration, and nitrogen fixation [71].

Biogeochemistry and environmental chemistry. Metals represent a link between the chemistry of the atmosphere and oceans from which life has evolved and the genomes and proteomes of living organisms [3]. The role of transition metals in microbial metabolism has been discussed with a particular focus on the deduction of geochemical signatures and phylogenetic relationships of prokaryotes from whole genome sequences and on the use of this link to infer geochemical aspects of the biosphere through time [72]. Model proteomes were reported to contain putative imprints of ancient shifts in trace metal geochemistry [73]. In environmental chemistry, a systematic view of the transition-metal metabolism requires an understanding of how organisms sense, adapt, and use these metals within the biodiversity characteristic of each specific ecosystem [6]. Environmental pollution with metals leads to the evolution of the protein expression signature and spurs the assignment of some proteins as environmental pollution biomarkers [14]. The correlation of sentinel organism proteomes with the metal contamination is one of the areas of environmental proteomics [14].

Plant biochemistry and physiology. The primary concern is the search for mechanisms responsible for the (i) mobilization of low solubility metals from soil, (ii) translocation within the plant, and (iii) sequestering metal ions in cytosol or in cellular compartments. Consequently, the identification and quantification of low-molecular, metal-containing metabolites is of interest in studies of (i) uptake and bioavailabity of essential elements, mainly iron and zinc with the aim to combat the micronutrient malnutrition in developing countries [74]; (ii) metal hyperaccumulation with a focus on phytoremediation and/or phytomining [75]; and (iii) plant defense mechanisms against heavy metal stress (including volatilization, appearance of inorganic insoluble forms, or induction of phytochelatins) [76].

Clinical chemistry and pharmacology. Despite their vital role as regulatory cofactors, metals can also be highly toxic and involved in pathophysiology of several diseases and human health risk assessment [77]. Most diseases associated with copper, iron, and zinc result from mutations in the genes encoding for metal-transporting proteins which can be identified by proteomics approaches [61,78]. A number of pharmaceuticals contain a metal in their structure and are referred to as metallodrugs [79]. They have anti-neoplastic function (e.g., cisplatin or ruthenium compounds), anti-inflammatory (gold), anti-bacterial (bismuth), and others. Metallomics studies will focus on the metabolism of these drugs, their transport, and interactions with biologically relevant molecules.

Nutrition and essential elements supplementation. The essential role of many trace elements, their implication in prevention of a number of diseases and the low levels of some of these elements in the diet in many countries are leading to increasing interest in the supplementation of food and feed with mineral elements, such as selenium [80], zinc, iron, manganese, and chromium. The relevant metallomics studies focus, on one hand, on the characterization of the chemical forms and their bioavailability in the supplements, and on the other hand, on the transport and fate in the target organisms, such as animals (meat, milk, and eggs from selenium-supplemented animals become functional food themselves) and humans [81,82].

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