

# Chapter 6

## The Use of Stable Isotopic Tracers in Metallomics Studies



Maria Montes-Bayón and Jörg Bettmer

**Abstract** Mass spectrometry represents an essential technique in Metallomics studies. It permits the identification of metal-containing molecules as part of the metallome as well as their quantification at low concentration levels. The technique becomes even more powerful in combination with the use of isotopically enriched species. Provided that they are stable, these isotopically labelled species can be easily distinguished from their natural counterparts by mass spectrometric techniques. This capability permits that these species are used for accurate and precise quantitative experiments and/or metabolic studies applying inductively coupled plasma as ionization source. In this chapter, we present the different concepts of using stable isotope tracers and isotope dilution analysis as quantification strategy. Besides some fundamental aspects, various examples from Metallomics studies, for instance, on the preparation of isotopically enriched metalloproteins and determination by isotope dilution analysis or the exploration of the biological pathways of Se species, are shown in order to demonstrate the usefulness of isotopes.

**Keywords** Isotopes · ICP-MS · Isotope dilution analysis · Tracer studies · Metalloproteins · Metallometabolites

### Abbreviations

AQUA	Absolute quantification
CRM	Certified reference material
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
ESI	Electrospray ionization
FAZ	Fractional absorption of zinc

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GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma-mass spectrometry
IDA	Isotope dilution analysis
IPD	Isotope pattern deconvolution
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MALDI	Matrix-assisted laser desorption and ionization
MC-ICP-MS	Multicollector-inductively coupled plasma-mass spectrometry
MELD	Model for end-stage liver disease
MeSeCys	Se-methyl selenocysteine
MS	Mass spectrometry
NTI-MS	Negative thermal ionization-mass spectrometry
PCR	Polymerase chain reaction
RSD	Relative standard deviation
SeCys	Selenocysteine
SeMet	Selenomethionine
SOD	Superoxide dismutase
TIMS	Thermal ionization mass spectrometry

## 6.1 Introduction

### 6.1.1 *The Possibilities of Using Stable Isotope Tracers in Metallomics Studies*

#### 6.1.1.1 From Nonmetals to Metal/Metalloid Tracers

The use of isotopic labels has been successfully applied for a number of years for the identification and quantification of important biomarkers in the study of biological systems. Mainly, the concept is based on the observation that chemically identical but isotopically unique chemicals will exhibit identical behavior in chromatographic and mass spectrometric analyses. Thus, molecules containing elements with different (stable) isotopic signatures that can be obtained through a number of chemical labelling and chemical label-free methods are used for this aim. Commonly, molecules containing labels of  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$ , etc. have been widely obtained and used for a number of years in metabolic experiments performed in bacteria and mammals already in the 1950s. For instance, stable isotope-labelled fatty acids and amino acids were applied to animals and in rare cases also to man. Absorption and metabolism were followed, for example, by appearance of the label in individual compartments and in metabolic end products such as water or small gaseous compounds, such as  $\text{CO}_2$ ,  $\text{N}_2$ , and  $\text{H}_2$ .

The availability of mass spectrometry (MS) after the introduction of ESI and MALDI around the year 2000 inspired the design of *in vivo* labelling experiments with cells or whole organisms, with the aim to analyze molecular alterations in different chemical compartments. As stated in a recent review article (Lehmann 2017), for the first time, with LC-MS/MS an analytical system was available with a molecular specificity and data collecting capacity that had a chance to cope with the complexity of a cell or an organism. Within this frame, stable isotope labelling was found to be essential for quantitative LC-MS/MS analyses. This situation in combination with analytical progress in other fields (PCR, DNA sequencing, microarray technology, immunofluorescence techniques) finally laid the ground for the field of comprehensive protein analysis, named *proteomics*.

Since biomarkers take many forms as cell responses to stimuli and can be manifested during transcription, translation, and/or metabolic processing, researchers have relied upon mixed-isotope labelling coupled with MS to perform relative quantification of biomarkers between two or more biological samples. This has been applied in the field of “quantitative proteomics” either at the peptide (using the AQUA peptides) or the protein level using stable isotope-labelled intact proteins. With the advent of mass spectrometry-based proteomics, new concepts for *in vivo* labelling were developed tailored to detect proteome expression differences following a stimulus or a genetic manipulation.

But not only tracing or metabolic experiments have made use of stable isotopes. The use of C, H, O, or N isotopically labelled compounds as internal standards to improve the quantification of small organic molecules has been increasing since the year 2000. This has been specifically the case in clinical chemistry where quantifications with both high precision and accuracy (trueness) are required. Therefore isotope dilution in combination with GC-MS or LC-MS has been extensively applied in this field, covering quantification of biogenic amines, steroid hormones, amino acids, drugs, and drug metabolites, among others. In particular, newborn screening for inborn metabolic errors is firmly established in numerous developed countries, and it is usually conducted by isotope dilution analysis with isotopically enriched amino acids. Forensic analyses and doping control are also growing areas of isotope dilution applications.

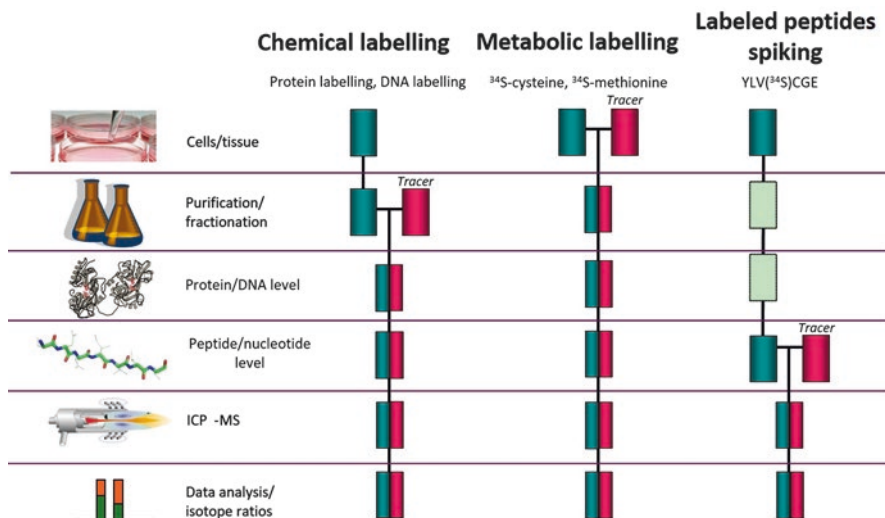
From the experience acquired over years in the field of “organic” mass spectrometry, the extension of the work to isotopically labelled hetero-elements (defining as such, those elements besides C, H, O, and N) occurred naturally. Such development has been associated to the use of inductively coupled plasma (ICP) as ionization source for mass spectrometry. ICP-MS does not provide structural information since the molecules are disrupted into their atomic components once they reach the ionization source. Nevertheless, this, a priori, disadvantage represents the important strength of ICP-MS since it reduces the sample complexity and it has led to an innovative research approach named Metallomics. Similar to other “omics” discipline, e.g., proteomics or genomics, Metallomics is defined as the global investigation and characterization of the entirety of metal and metalloid species such as metalloproteins or other metal-containing biomolecules (*the metallome*) within a defined biological entity. Metallomics is a transdisciplinary research area with an impact on geochemistry, clinical biology and pharmacology, plant and animal physiology, and nutrition.

Thus, the isotopically labelled elemental species have been used in the field of Metallomics with two different purposes (as previously in the fields of proteomics and metabolomics): (i) as quantification tool (by applying isotope dilution analysis, IDA, as the main quantification strategy) and (ii) in the form of metabolic tracers to address the fate of labelled elements or elemental species in biological systems. As in the case of organic mass spectrometry, the use of isotopically enriched elemental species permits to have the ideal “internal standard” for quantification. The major asset of isotope dilution is its high metrological quality over the traditional methods of standard additions, internal and external calibration. Isotope dilution can be considered as an evolution of internal standard methods that are very advantageous to correct for matrix effects and (partial) analyte losses (Meija and Mester 2008). In addition, the feature of ICP-MS to provide “virtually” species-independent ionization will permit the application of other quantification strategies such as the species-unspecific isotope dilution which is an outstanding feature of this technique. Such quantification possibilities open new and extraordinary venues in the field of Metallomics studies (Heumann 2004; Bettmer 2010; García Alonso and Rodríguez González 2013). These different quantification aspects will be covered in the next section. Regarding the use of isotope labelling for stable isotope tracing experiments, several studies can be found in combination with ICP-MS as detector for metabolic tracing experiments in cell, fish, or plant models. For instance, the suitability of a food fortification procedure has been conducted by comparing zinc absorption in adults from rice porridges prepared from a hydroponically biofortified rice labelled intrinsically with  $^{70}\text{Zn}$  with the same variety but fortified at point of use with  $^{70}\text{ZnSO}_4$  to the same total zinc content. Or the differential incorporation of several isotopically labelled inorganic Se species commonly used as foodstuff enrichment sources in root and leaf extracts of ryegrass (*Lolium perenne L.*) which had been co-exposed to two labelled Se species. Most recent publications have revealed the use of isotopically enriched hetero-elements as tracers has permitted to extend the traditional speciation experiments even beyond, for instance, in the case of nanoparticles’ fate in environmental and biological systems. Intrinsic labelling of metal-containing engineered nanoparticles with stable isotopes seems to be a useful tool for the highly sensitive and selective detection of these species in the environment and organisms, thus enabling tracing of their transformation, uptake, distribution, and clearance. Figure 6.1 shows, schematically, the different levels of labelling experiments that can be used for metabolic experiments.

All these aspects of the applications of isotopically labelled species in Metallomics studies will be illustrated in the next sections in order to point out the principles of the methodologies as well as a summary of the most remarkable existing literature and the unexplored possibilities for this type of applications in the growing field of Metallomics.

#### **6.1.1.2 The Use for Quantification: Isotope Dilution Analysis (IDA)**

The quantification of any compound bound to metals represents a highly challenging task in Metallomics studies. The great variety of binding forms, in which the metal can be found in biological systems, requires sophisticated strategies to



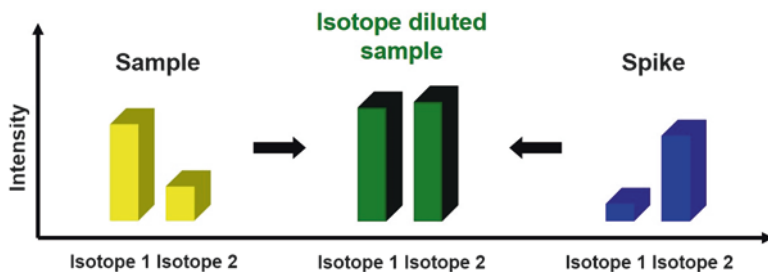
**Fig. 6.1** Different possibilities of labelling to conduct Metallomics experiments: at the purification level, at the metabolic level, or at the quantification level. (Modified from Bettmer et al. 2009)

characterize them completely and finally to quantify them. Usually, not all compounds forming the metallome are quantified, but a number of relevant or selected ones. Among traditional quantification strategies, isotope dilution analysis (IDA) is considered as a method providing highly accurate results. Potential sources of error are usually well understood and can be controlled. Therefore, IDA is often named a “definitive method” (Heumann 1992).

The application of IDA of metals requires certain basic prerequisites. First of all, the element of interest needs to have at minimum two stable isotopes.<sup>1</sup> This excludes per se important metals from being acquirable with IDA – Na, Al, Sc, Mn, Co, As, Y, Nb, Rh, I, Cs, and Au – and the four lanthanides Pr, Tb, Ho, and Tm. All other metals can be principally accessed. Secondly, the metal of interest needs to be available in an isotopic composition different from the natural one. The most capable composition consists of the depletion of the naturally most abundant isotope and the enrichment of one isotope with naturally low abundance, so-called spike or spike solution. Virtually all metals highly enriched in one isotope are commercially available. The third requirement is that the mass spectrometric method needs to be free of interferences. With respect to ICP-MS – as probably most often used quantification technique for metals in Metallomics studies – spectral interferences on two isotopes of the element of interest have to be eliminated completely as they can influence the measurement of the isotope ratio (R) of the sought metal. This requires either collision/reaction cell instruments or those that provide an enhanced mass resolving power.

The basic principle of IDA is represented in Fig. 6.2. Briefly, a sample containing the sought metal in its natural isotopic composition with unknown concentration

<sup>1</sup>In a few cases, a long-lived radioactive isotope can be also used for IDA, e.g.,  $^{129}\text{I}$  or  $^{60}\text{Co}$ .



**Fig. 6.2** Basic concept of isotope dilution analysis

( $c_{\text{sample}}$ ) is mixed with a spike solution of known isotopic composition (different from the natural, e.g., enriched in the naturally lowest abundant one) and concentration ( $c_{\text{spike}}$ ).

After a complete mixing, the resulting isotope ratio of the mixture ( $R_m$ ) is established between the one of the metal being present in the sample and the one of the added spike, reflecting the unknown concentration of the metal in the sample by the following equation (Eq. 6.1). Detailed description of the mathematical derivation of the isotope dilution equation can be found in the literature (Heumann 1992; Rodríguez-González et al. 2005).

$$c_{\text{sample}} = c_{\text{spike}} \frac{m_{\text{spike}}}{m_{\text{sample}}} \frac{M_{\text{sample}}}{M_{\text{spike}}} \left( \frac{A_{\text{spike}}^2 - R_m A_{\text{spike}}^1}{R_m A_{\text{sample}}^1 - A_{\text{sample}}^2} \right) \quad (6.1)$$

With

$m_{\text{sample}}$ ,  $m_{\text{spike}}$ : the mass of the sample and the mass of the added spike.

$M_{\text{sample}}$ ,  $M_{\text{spike}}$ : the atomic masses of the element in the sample and the spike, respectively.

$A_{\text{sample, spike}}^{1,2}$ : the isotope abundances of isotope 1 and 2 in the sample and spike, respectively.

Equation 6.1 reveals that the measurement of  $R_m$  is sufficient for the determination of the unknown concentration of the analyte. It is recommended to use exactly the same mass spectrometer for the determination of the isotope ratios and the isotopic abundances in order to consider potential mass bias and detector dead time effects. Once these parameters are controlled, the isotope ratio  $R_m$  is equal to the ratio of signal intensities ( $I$ ) of the isotopes 2 and 1 that are measured during the experiment. The isotope dilution analysis bears the advantages of highly accurate and precise results provided that a complete mixture between the analyte in the sample and the added spike has occurred. This can be explained by the fact that important sources of errors influencing the quality of analytical results are virtually eliminated due to the measurement of isotope ratios. After a complete isotope mixing, any analyte losses during sample preparation and measurement do not affect the quality of the results as the analyte present in the sample and the added spike are

affected in the same manner. Instrumental changes in sensitivity do not influence the determination of isotope ratios. Therefore, the use of an isotopically enriched spike is considered as the perfect internal standard for the determination of a sought element. The main application field of IDA is the determination of total element concentrations, but it has also turned out to be very useful for specific compounds, e.g., elemental species. Therefore, it is not surprising that IDA has entered the field of Metallomics studies due to its ease of use and expected analytical figures of merit that permit high-quality investigations to follow a sought metal in biological systems. Two main analytical strategies have been applied to support Metallomics studies that will be explained in the following two sections: “Species-Specific IDA” and “Species-Unspecific IDA.”

### 6.1.1.3 Species-Specific IDA

The basic idea of species-specific IDA is the conceptual application of IDA for the accurate and precise determination of a specific compound or compounds. The first examples making use of elemental mass spectrometry dated back to the late 1980s. Inorganic selenium species were separated by ion chromatography and detected off-line by thermal ionization mass spectrometry (TIMS) (Heumann and Großer 1989). A few years later, the first application with online coupling of HPLC and ICP-MS appeared on the example of iodine species (Heumann et al. 1994).

In order to apply species-specific IDA, several prerequisites need to be fulfilled. Like for IDA of the elements, in general, the metal requires minimum two stable isotopes. As the sought analyte is a metal-containing compound, this compound needs to be available in an isotopic composition different to the natural one. Some low-molecular metal compounds enriched in one isotope are commercially available (e.g., methyl-Hg, butyl-Sn compounds, etc.), but usually it requires the lab-made synthesis of the sought compound. In some cases, this might be challenging because the synthesis is usually carried out at the low mg range (Demuth and Heumann 2001). In the case of metalloproteins and other metal-containing complexes, the exact identity has to be guaranteed (Deitrich et al. 2007).

Such a prepared spike, in this case species-specific, needs to be characterized in terms of isotopic composition, purity, and concentration. This spike can be then added in a known amount to a sample with the sought species with unknown concentration. After complete mixing between the spike and the species in the sample, any analyte losses, species degradation, or transformation does not influence the accuracy of the analytical result. Those processes might affect the species in both isotopic compositions to the same extent. The next step is to separate the sought species from other compounds containing the same metal as they would disturb the measurement of  $R_m$ . Most common techniques are gas and liquid chromatography and electrophoretic and field-flow fractionation techniques and can be easily coupled online to ICP-MS. In the case of slab gel electrophoresis, sample introduction into the ionization source can be realized by laser ablation. In general, transient signals are produced, e.g., as an eluting peak from a chromatographic column.

By continuous monitoring of two isotopes following peak integration, the isotope  $R_m$  can be determined and the corresponding concentration results from Eq. 6.1. Therefore, species-specific IDA denotes a specific case of total elemental determination using IDA. The main limitation is availability of isotopically enriched species. Low-molecular metal-containing species, provided that they are stable, are relatively easy to be synthesized. In the case of high-molecular species, e.g., metalloproteins, the synthesis and purification requires more sophisticated strategies. Although a huge number of metal-containing compounds are principally accessible by species-specific IDA, an alternative strategy has been provided for those applications where isotopically enriched compounds are not available.

#### 6.1.1.4 Species-Unspecific IDA

Species-unspecific IDA, also known as post-column IDA, was developed on the basis of HPLC coupled to ICP-MS (Rottmann and Heumann 1994a, b). First examples were dedicated to the analysis of metals associated to dissolved organic material (DOM). Due to its attractiveness and universal applicability, the concept of species-unspecific IDA has entered various applications (Heumann 2004; Rodríguez-González et al. 2005; García Alonso and Rodríguez González 2013).

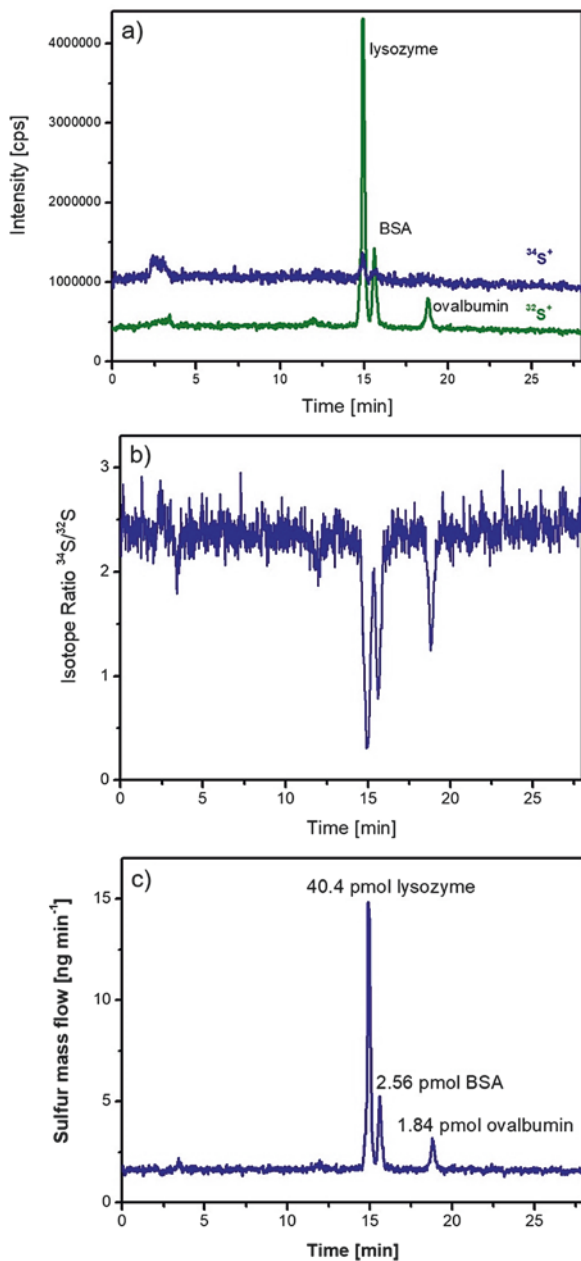
The basic idea consists of a separation of the species of interest, e.g., by means of liquid or gas chromatography, or electrophoresis. A solution (in the case of gas chromatography a volatile species in inert gas) is continuously added containing the element in an isotopically altered composition to the eluent of the separation unit (post-column). In contrast to species-specific IDA, this spike can have any chemical form available of the sought element and does not have to be identical to the species of interest. For instance, the determination of iron-containing proteins can be achieved by the use of an isotopically enriched Fe(III) solution by monitoring two relevant Fe isotopes (Del Castillo Busto et al. 2006). However, it has to be guaranteed that the chemical form of the spike and the sought compound, e.g., a metalloprotein, show identical or very similar transport and ionization efficiencies. Figure 6.3a represents a typical chromatogram on sulfur-containing proteins using  $^{34}\text{SO}_4^{2-}$  for post-column isotope dilution (Zinn et al. 2008).<sup>2</sup> As the spike (in a known concentration and isotopic composition) is continuously added to the eluent of the chromatographic system, the trace for  $^{34}\text{S}$  shows higher signal intensity than the naturally higher abundant  $^{32}\text{S}$  (natural abundances of  $^{32}\text{S}$  and  $^{34}\text{S}$  are 94.93% and 4.29%, respectively). The sulfur-containing compounds eluting from the column, of course, consist of a natural isotopic composition; a fact that is reflected in the observed signal intensities (Fig. 6.3a). Out of the recorded intensity chromatogram, one can easily transfer it to an isotope ratio chromatogram<sup>3</sup> (Fig. 6.3b). During no specific sulfur-containing compounds elutes from the column, the

<sup>2</sup>Although sulfur is shown in this example, the principle is the same for any other metal suitable for species-unspecific IDA.

<sup>3</sup>In this specific case, the isotope ratio of  $^{34}\text{S}$  and  $^{32}\text{S}$  was used.



**Fig. 6.3** (a) Intensity chromatogram of three proteins monitoring  $^{32}\text{S}$  and  $^{34}\text{S}$ , (b)  $^{34}\text{S}/^{32}\text{S}$  isotope ratio chromatogram, and (c) resulting mass flow chromatogram of S. (Adapted from Zinn et al. 2008)



observed time-dependent isotope ratio ( $R_t$ ) results from the one of the spike ( $R_{sp}$ ) and the blank contribution of the mobile phase. Once a sulfur-containing compound elutes from the column, its natural isotopic composition gets mixed with that one of the spike solution (isotope mixing). This results in a change of the observed isotope ratio into the direction of the natural one (in this case  $R_{nat}(^{34}\text{S}/^{32}\text{S}) = 0.0452$ ) depending on the present mass of the sought element in the eluting compounds (Fig. 6.3b).

In order to extract quantitative information for each compound detected in a chromatogram, the isotope ratio chromatogram needs to be converted into a so-called mass flow chromatogram (Fig. 6.3c). For this, Eq. 6.2 is applied in each point of the chromatogram.

$$\text{Mf}_{\text{sample}} = \text{Mf}_{\text{spike}} \frac{M_{\text{sample}}}{M_{\text{spike}}} \left( \frac{A_{\text{spike}}^2 - R_t A_{\text{spike}}^1}{R_t A_{\text{sample}}^1 - A_{\text{sample}}^2} \right) - \text{Mf}_{\text{blank}} \quad (6.2)$$

where

$\text{Mf}_{\text{sample}}$ ,  $\text{Mf}_{\text{spike}}$ , and  $\text{Mf}_{\text{blank}}$  are mass flow of the sample, the spike, and the blank, respectively, and  $R_t$ , time-dependent isotope ratio (isotope 1/isotope 2).

With a known mass flow of the spike (it can be usually calculated from the concentration of the spike and its flow rate) and of the blank, the remaining unknown is the time-dependent isotope ratio ( $R_t$ ). It is therefore continuously determined during the chromatographic run as shown in Fig. 6.3b. The mass of the sought element present in a specific compound detected is now calculated by integration of the peak, e.g., lysozyme in the chromatogram. The product of mass flow and time (equivalent to the area of the integrated peak) will result in the mass of the element in this compound that eluted from the chromatographic or electrophoretic column. In order to correlate the detected mass to the initially present concentration in the sample, the recovery rate of each compound has to be known or to be determined. The reason is that the isotope mixing, an indispensable requirement for IDA, occurs post-column. Therefore, the determination of the column recovery is absolutely needed in order to guarantee accuracy and precision of the obtained results using species-unspecific IDA (Meija and Mester 2008; Bettmer 2010).

As a spike of any chemical composition can be used in its isotopically enriched form, species-unspecific IDA can be denoted as a generic approach for the determination of metallo-compounds. Therefore, its widely spread applicability in Metallomics studies can be found on metal-containing metabolites, metalloproteins, and metalloenzymes.

#### 6.1.1.5 Examples on Metallomics Studies Making Use of IDA

Traditionally, species-related IDA methods were proposed for low-molecular compounds of elements like Se, Hg, Pb, and Sn (Demuth and Heumann 2001; Ruiz Encinar et al. 2004; Monperrus et al. 2005; Poperechna and Heumann 2005).

These compounds can be clearly considered as forming part of the metallome in a certain biological system.

Se-containing amino acids and other Se-containing metabolites have been in the focus of extensive research. Selenium is an essential element and considered to be cancer-protective agent. In order to determine its various chemical forms (Gammelgaard et al. 2008; Fairweather-Tait et al. 2010), IDA methods, mainly with detection by ICP-MS, have gained great interest (Pedrero and Madrid 2009). Species-specific IDA was applied to the determination of selenomethionine in selenium-enriched yeast (Hinojosa Reyes et al. 2004).  $^{77}\text{Se}$ -enriched methionine was synthesized in *S. cerevisiae* in a multiple-step procedure and used for further quantification studies of selenomethionine in yeast samples. A similar approach was used for the determination of selenomethionine in human serum (Ruiz Encinar et al. 2004). The application of a modified capillary HPLC-ICP-MS interface allowed lowering the absolute detection limits to the 75 fg level. By nano HPLC-ICP-MS, accurate quantification of selenium-containing peptides was achieved by species-unspecific IDA (Giusti et al. 2005).

An important task in Metallomics studies is the quantification of metal-containing biopolymers like metalloproteins and metalloenzymes. During the analysis, it is especially critical and important to maintain the analyte intact, e.g., any metal loss will affect the accuracy of ICP-MS detection. Sample preparation and separation methods need to be adjusted to the integrity of the sought metalloproteins. Besides non-denaturing electrophoretic techniques, the most commonly used separation techniques are size-exclusion, ion-exchange, and ion-pairing chromatography (Montes-Bayon et al. 2006). As mentioned in item 6.1.1.4, species-unspecific IDA does only require a spike solution with the isotopically enriched element but in any chemical form available and maintainable. Therefore, its application to structurally challenging biomolecules like proteins is relatively straightforward. Many studies have been shown on the determination of selenoproteins (Hinojosa Reyes et al. 2003; Jitaru et al. 2008; Kirby et al. 2008) and on the determination of metallothioneins (Schaumlöffel et al. 2002). In the latter work, the combination of capillary electrophoresis with species-unspecific ICP-MS turned out to be an important quantitative tool for the studies of various metallothionein isoforms. The simultaneous introduction of spike solutions of Cu, Zn, Cd, and S allowed the authors to quantify the different protein forms and, moreover, to determine the stoichiometric composition through the sulfur-to-metal ratio (Schaumlöffel et al. 2002).

Generally more accurate is the application of species-specific IDA. In the case of metalloproteins, this requires their synthesis in an isotopically altered form. In a few examples, the removal of the naturally present metal ions followed by the incubation with a spike solution can be sufficient. Examples are superoxide dismutase and transferrin as summarized in Table 6.1. Other metalloproteins require the use of biochemical procedures, usually involving the cloning of a certain gene for the (over)expression of the sought protein in cell cultures or *Escherichia coli*. The metalloprotein is then expressed in the presence of a spike solution of required metal (Table 6.1).

**Table 6.1** Examples on metalloproteins that have been synthesized in an isotopically enriched form

Metalloprotein	Enriched isotopes	Method of synthesis	Reference
Rusticyanin	$^{65}\text{Cu}$	Protein was expressed cell cultures, isolated, purified, and incubated with an excess of $\text{Cu}^{2+}$ solutions	Harrington et al. (2005)
Ferritin	$^{57}\text{Fe}$	Cloning and overexpressing the <i>Phaseolus vulgaris</i> ferritin gene pfe in <i>Escherichia coli</i> in the presence of $^{57}\text{FeCl}_2$	Hoppler et al. (2008)
Superoxide dismutase	$^{65}\text{Cu}$ , $^{68}\text{Zn}$	Cu and Zn as metal cofactors of the enzyme were removed under different conditions and replaced by $^{65}\text{Cu}^{2+}$ and $^{68}\text{Zn}^{2+}$	Deitrich et al. (2007)
Human transferrin	$^{57}\text{Fe}$	Apo-transferrin was saturated with an excess of $^{57}\text{Fe}^{3+}$	Del Castillo Busto et al. (2006)
Plastocyanin	$^{63}\text{Cu}$	Cloning, heterologous overexpression, purification, and reconstitution of a variant of the type 1 copper protein plastocyanin from <i>Synechocystis</i> in <i>Escherichia coli</i> in the presence of $^{63}\text{Cu}^{2+}$	Hann et al. (2006)
Hemoglobin	$^{57}\text{Fe}$	Preparation of $^{57}\text{Fe}$ -enriched protoheme and apo-hemoglobin followed by reconstitution of hemoglobin	Brauckmann et al. (2016)
SEPP1 and GPX3	$^{76}\text{Se}$	Incubation of <i>Escherichia coli</i> after vector cloning in the presence of $^{76}\text{Se}$ -methionine in <i>E. coli</i>	Konopka et al. (2016)
Carbonic anhydrase	$^{67}\text{Zn}$ , $^{68}\text{Zn}$	Incorporation into carbonic anhydrase: effects on isotope enrichment	Pessôa and Arruda (2017)

Analytically well documented is the analysis of human transferrin glycoforms in a certified reference material (CRM 470, human serum) and various serum samples (Del Castillo Busto et al. 2006). Transferrin is the main iron-transporting protein in serum, and their glycoforms deficient in carbohydrate (CDT, carbohydrate-deficient transferrin) are used as clinical biomarkers. Both IDA strategies, species-specific and -unspecific, were compared for the achievable accuracy and precision using anion-exchange chromatography. In this case, an excellent agreement was found that also revealed a quantitative recovery of the analytes from the column.

Another well-studied metalloprotein (metalloenzyme) in this context is superoxide dismutase 1 (SOD1) containing one Cu and one Zn ion in each subunit. In 2007, the first report appeared on the preparation and characterization of bovine SOD enriched in  $^{65}\text{Cu}$  and  $^{68}\text{Zn}$  (Deitrich et al. 2007). This spike was later used for the species-specific IDA using non-denaturing gel electrophoresis coupled to laser ablation-ICP-MS (Deitrich et al. 2010). The enriched SOD remained stable during the entire method and tested it successfully in complex matrices like bovine liver. However, detection limits were still improvable. Better detection limits could be obtained by anion-exchange chromatography coupled to ICP-MS as shown on the analysis of SOD in red blood cells (Nuevo Ordóñez et al. 2011).

Based on these and other related studies, the application of IDA methods for the analysis of metalloproteins has captured great interest by European metrological institutes. The aim is to provide measurement procedures to achieve comparable and traceable results (Swart 2013; Swart and Jakubowski 2016). An example is the extension of SOD determinations by the use of a species-specific double and triple IDA (Gleitzmann et al. 2016). The calculated mass fractions of SOD1 in human red blood cells showed excellent agreement and showed again the potential of IDA methods in the accurate and precise determination of metalloproteins and metalloenzymes.

### 6.1.1.6 Stable Tracers for Metallomics Experiments

Enriched stable isotopes are nowadays indispensable tools for experiments regarding the fate of minerals and trace elements in biological systems. Their use has increased rapidly since the first experiments conducted in 1960s. Today stable isotope tracers are used in various scientific areas, including Metallomics, to investigate pathways of minerals, trace elements, and heteroatom-containing biomolecules in diverse biological systems, ranging from estimating human absorption of essential elements such as iron to studies regarding the bioaccumulation of mercury in aquatic food webs or tracking the fate of nanoparticles in the environment.

Most of the isotope tracer experiments conducted nowadays are based on the previous radioisotope tracer experiments and, somehow, similar as recently revised in an interesting review article (Stürup et al. 2008). However, the use of enriched stable isotopes tracers has the important advantage of dealing with non-toxic species that can be applied in experiments involving living organisms without any toxicological concerns. On the other hand, the main drawbacks of enriched stable isotopes is that, unlike radioisotopes, they are often affected by the presence of a significant amount of the selected isotope as part of the natural abundance of the element present in the biological system under investigation. Hence, a relatively large amount of the enriched stable isotope tracer has to be administered to induce a detectable shift in the tracer isotope signature in the biological sample of interest. In addition, the sensitivity that can be achieved with regular radioactivity detectors to perceive low levels of emitting material is usually very high in comparison to what can be achieved with the regular ICP-MS systems. Considering that the tracers undergo a significant dilution on their way to the compartment or organism of interest, the experiments have to be carefully designed using an adequate concentration of isotopic tracers that permits to detect changes on the isotopic signatures of the elements of interest with conventional ICP-MS instruments.

In general terms, the modalities of label isotope experiments that can be applied for Metallomics studies can be divided into two different categories based on the experimental type of information that is intended to be extracted out of the performed experiment:

- *Qualitative isotope distribution information*: in this case, the objective of this experiment is to follow the distribution of an isotope tracer in a given system and determine the compartments or organisms into which the tracer is incorporated by measuring the variation on the measured isotope ratio with respect to the control sample.
- *Quantitative isotope information*: these experiments involve, additionally, the calculation of a mass balance of the tracer isotopes added to the given system (this is the determination of the absolute concentration of the incorporated tracer in every specific compartment).

The choice of one or the other depends on the biological process under investigation and on the information required. Indeed, qualitative isotope experiments are by their nature simpler than quantitative experiments, but they permit to monitor the fate of an element or more often a specific biomolecule containing the hetero-element of interest in a biological system. In addition, qualitative isotope distribution experiments are ideal for investigating species transformations during metabolism or the transfer of isotopically labelled compounds through a biological system. Regarding the quantitative experiments, the main consideration is that since they are mass-balanced experiments, the isotope tracer flux in and out of the system under evaluation should be estimated, and such scenario requires the use of “closed” systems for which the fluxes in and out can be controlled. This is relatively complex to achieve and, in this case, animals and humans are ideal models to conduct quantitative isotope balance experiments, as excretion can be estimated by collecting urine and feces. Potted plants are also well-established models since the uptake of nutrients and atmospheric deposition can be controlled in growth chambers and even volatile species production can be obtained with the adequate setup. The extension on the use of these well-established models out of the laboratory requires the preparation and maintenance of artificial biological systems. Such scenario turns into a very complex process involving not only addition and measurement of the isotope tracer but also the control and determination of all of the other parameters defining the biological system under investigation.

In general terms, some other considerations that have to be taken into account when designing a Metallomics experiment using isotopic tracers (qualitative and quantitative) are detailed below:

- (a) The cost: the use of enriched isotopes with a low natural abundance is typically more expensive than those with a high natural abundance due to the difficulty of preparation, which inevitably increases the cost of the experiments.
- (b) Uncertainty considerations in qualitative studies are less critical than in quantitative isotope experiments, and factors like mass bias, detector dead-time, etc. can often be ignored, as long as the mass spectrometric method applied is interference-free and the isotope ratios can be measured with a precision of better than 1% RSD. However, in the case of quantitative experiments, careful uncertainty measurements have to be taken into account which implies complicated mathematics to be applied in the calculation of the concentrations.

- (c) The methodologies for quantitative experiments make use of advanced matrix algebra to resolve the contributions of several different enriched stable isotope tracers to the isotope pattern including isotope pattern deconvolution (IPD) using multiple linear regression analysis. A recent contribution of a transgenerational marking study where the transfer of a Sr isotope double spike ( $^{84}\text{Sr}$  and  $^{86}\text{Sr}$ ) from female spawners of the common carp (*Cyprinus carpio L.*) to the center of the otoliths of their offspring was studied by LA-MC-ICPMS refers to the requirement of a full metrological protocol for data processing during isotope pattern deconvolution of the enriched Sr isotope double spike (Zitek et al. 2014). In this example the authors include, as specific aspect, the control of the blanks (considering the presence of Kr, Sr, and Rb), the variation of the natural Sr isotopic composition in the sample, the mass bias effect, the interferences (Rb), and the total combined uncertainty.

Regarding the nature of the tracer to be used in the experiments, two different scenarios can be considered: (i) the use of pure isotopically labelled elements and (ii) the use of pure isotopically labelled elemental species. Both possibilities, with the corresponding advantages and limitations, will be briefly discussed in the following section.

#### 6.1.1.7 Isotopically Labelled Elements

Isotopically labelled elemental species (normally minerals and trace elements) have been used in biological systems for the evaluation of their biosynthetic incorporation into the studied model organism. One of the inherent problems with enriched stable isotope labels is the lack of “isotopically pure” tracers; there will always be traces of other isotopes. In fact, most commercially available enriched stable isotopes are 95–99% pure (in the best case), and although the content is certified, the uncertainty over the certification of minor isotopes is often very large (up to 100% in some cases). Thus, a previous step for characterization of the isotopic abundances and concentration of the tracer to be used is mandatory before starting any experiment. Such process is often expensive and time-consuming. This is a problem, in particular, for quantitative isotope experiments, as it cannot then be assumed that only one (pure) isotope is added to the biological system. Thus, this factor must be taken into account, so more isotopes have to be measured and a mass balance for each isotope of the element is needed. In the same way, the uncertainty over the certification of the enriched isotopes needs to be included in the overall expanded uncertainty of the experimental results.

Biosynthetic incorporation of isotopic elemental tracers in biological systems has been conducted in a number of applications. Perhaps the studies of elemental incorporation in plants and, in lesser extent, nutritional studies have been the most successful ones. Elements like Se, Zn, and Fe have been used in the labelling of plants with enriched isotopes taken up through the roots using one or more than one of the isotopically labelled elements. Some examples of these particular applications will be given in the next sections.

### 6.1.1.8 Isotopically Labelled Elemental Species

In this case, all the considerations that have been documented for the isotopically labelled elements still apply although additional aspects have to be taken into account. The most troublesome fact of using isotopically labelled species is the need for having these species commercially available, with high purity (not only regarding isotopic composition but also regarding the possible presence of other species). In addition, species stability referring to the absence of modification of the structure due to other processes than the process under study needs to be taken into account. Very few isotopically labelled species are nowadays commercially available in the market to conduct this type of experiments; among them some organo-metallic species (no so much in use for Metallomics experiments) like tributyltin or methyl mercury can be found. In addition, organometalloid species like Se-labelled Se-methionine can be also purchased.

Apart from those, labelled elements in their different oxidation states like Cr(III)/Cr(IV), Fe(II)/(III), or Se(IV)/Se(VI) can be also obtained in a relatively easy way. If the labelled species is not commercially available, the experiment has to be initiated by its synthesis with the corresponding increment of cost and feasibility of the study. Recent experiments involve also the use of isotopically labelled metallic NPs for addressing the fate of this emerging species in biological or environmental media. In this case, many other important considerations on the characterization of starting isotopically enriched material have to be taken into account like size and shape, etc. This will be further explained in the following sections.

## 6.2 Applications on the Use of Stable Tracers for Metallomics

In the next sections, some relevant examples that have been selected from the literature related to the application of the use of isotopically labelled elements and elemental species will be revised.

### 6.2.1 *Iron and Iron Species in Plant and Human Tissues*

In agricultural practice, the deficiency of Fe in crops (also called Fe chlorosis) is characterized by a marked decrease in leaf chlorophyll that, in turn, results in losses in crop quality and yield; the most common cause of Fe deficiency is low soil Fe bioavailability, due to the occurrence of this metal in oxy-hydroxide insoluble forms. The efficiency of Fe(III) chelates such as Fe(III)-ethylenediamine di(o-hydroxyphenylacetic) acid (o,oEDDHA) as an Fe fertilizer is due to the remarkable stability over a wide range of pH values and the low reactivity in soils. However, this compound presents two different stereoisomers that might affect



the way for its uptake by plants. Thus, a new approach to study plant Fe uptake and translocation using this compound is the use of multiple-stable isotope tracer methodologies, which also provide the possibility to carry out long-term experiments and provide relatively “closed” systems for quantitative calculations. The aim of the work was to address the uptake, movement, and distribution of Fe and ligands from racemic and meso Fe(III)-o,oEDDHA chelates, when applied simultaneously to the roots of Fe-deficient plants (Orera et al. 2010). For this aim, sugar beet plants grown in nutrient solution were treated with a mixture of racemic and meso-o,oEDDHA isomers, each one labelled with a different Fe stable isotope ( $^{54}\text{Fe}$  or  $^{57}\text{Fe}$ ). Then, the contents of the Fe stable isotopes in different plant parts were determined by ICP-MS, whereas those of the ligands (racemic and meso-o,oEDDHA) were determined by HPLC-ESI/TOFMS. The authors could establish that the meso isomer appeared to be the major contributor to the exceptional efficiency of Fe(III)-o,oEDDHA to deliver Fe to plants in nutrient solution, with rates of xylem transport and total uptake about twofold higher than those found for the racemic isomer. Both isomers of the chelating agent were incorporated and distributed by plants at similar rates, in amounts one order of magnitude lower than those of Fe.

Similarly, iron uptake and accumulation in the brain of rodents was evaluated using a similar working principle. Iron deposits in the brain are a common hallmark of Alzheimer’s disease and Parkinson’s disease. This spurred the hypothesis that iron may play a functional role in the pathogenesis of neurodegenerative disorders through free radical damage. Short-term studies using radiotracers suggested that brain iron uptake is small as compared to other tissues in adult rodents. This led to the assumption that brain iron uptake must also be marginal in humans after brain development is complete. Thus, in these experiments, the authors applied a novel approach to determine directly the fraction of iron that was transferred over time from diet to the brain and other organs in adult rats by adding a known amount of a stable iron isotope ( $^{57}\text{Fe}$ ) with drinking water to adult rats over 4 months (Chen et al. 2014). In this case the isotope ratio measurements were taken using negative thermal ionization mass spectrometry (NTI-MS) instead of ICP-MS. In addition, an enriched  $^{58}\text{Fe}$  label was used for quantification in order to determine the amount of natural iron in the sample following isotope dilution principles. Isotopic enrichment of a tissue sample was calculated as the percentage change of the  $^{57}\text{Fe}/^{56}\text{Fe}$  isotope ratio in the sample compared to natural iron. Transfer rates from diet to tissues were calculated as total moles of tracer recovered in the sample/organ relative to total moles of tracer consumed with the drinking water. From the amount of dietary iron consumed and the transfer rate, the amount of non-tracer iron taken up from the diet and transferred into the different body tissues was also calculated.

Using this approach, a lot of physiological data could be extracted in a single experiment. For instance, isotopic enrichments could be calculated and were found to range from 27.0% for blood down to 5.32% for muscle. This can be explained by considering that the blood has the highest turnover rate of all body iron pools due to constant renewal of red blood cells and hemoglobin synthesis.

In contrast to the blood, iron turnover of muscle tissue is low. The authors explain this effect due to the fact that iron in muscle is mainly present as myoglobin for oxygen storage and is only released/replaced when muscle cells are catabolized. In addition, an efficiency of iron transfer from the feed to the liver ( $0.132 \pm 0.019\%$ ) was about ten times lower than for blood ( $1.48 \pm 0.11\%$ ). Iron uptake of the heart and kidney was lower by three orders of magnitude as compared to the blood and even lower for the brain. However, differences between tissues were much smaller when the fraction of iron taken up from the diet was expressed relative to total tissue iron content.

The most significant finding of the conducted experiments was that iron uptake by the adult brain in a mammalian model was small as compared to other tissues but considerable when measured relative to brain iron content in normal adult rats. About 9% of brain iron could be traced back to the diet over a period of 4 months, a time span that can be compared to 7–10 human years. This became possible by using a stable iron isotope for tracing instead of short-living radiotracers and by measuring directly the fraction of iron in the brain that originates from diet.

Finally, iron labelling shows also a great potential in pharmacokinetic experiment as observed in a recent publication aimed to determine Fe-species absorption by administration of hemin to rat models. The iron in heme is in its oxidized state which is a highly unstable form. In its reduced state, named hemin, Fe is in the form of iron(III) protoporphyrin. Thus, the use of hemin to evaluate iron species absorption instead of heme is very meaningful considering the instability of the heme molecule. This molecule was labelled on  $^{58}\text{Fe}$  and extrinsically administrated to rats intravenously to obtain the concentration-time profiles of this compound in rat plasma. This study represented a proof of concept for the methodology to be applied in further pharmaceutical applications (Zhao et al. 2014).

### 6.2.2 Zinc in Biological Studies

Over approximately the past 20 years, there has been a steadily growing body of experience and expertise in the application of zinc stable isotope techniques to investigate whole body human zinc homeostasis and physiology (Krebs and Hambidge 2001). Of greater fundamental importance, zinc has three stable isotopes for which the natural abundance is sufficiently low to allow their utilization as “tracers.” These are  $^{67}\text{Zn}$  (natural abundance 4.1%),  $^{68}\text{Zn}$  (18.8%), and  $^{70}\text{Zn}$  (0.6%). The availability of these three stable isotopes of zinc in low natural abundance concentration makes it possible to administer all three tracers essentially simultaneously via different routes. For instance, dual stable isotope tracer techniques to measure fractional absorption of zinc (FAZ) require only a single measurement in plasma or urine of both orally and intravenously administered zinc tracers. In this experiment, only a fraction of the oral zinc dose is enriched with the tracer, and a different zinc stable isotope is injected intravenously. Urine monitoring possibly with multiple venipunctures and/or long fecal collection periods are needed depending on the

endpoints measured (Tran et al. 2015). Analysis of two stable isotopes in urine permits calculation of FAZ; the following equation was used:

$$\text{FAZ}(\text{urine; \%}) = \text{enrichment}(\text{oral / intravenous}) \times \text{dose}(\text{intravenous / oral}).$$

The following assumptions need to be taken to use the previous equation in order to use this strategy for estimation of FAZ:

- (i) That both isotopes (oral and intravenous) enter the plasma at the same time.
- (ii) That the isotope enrichment in plasma and urine are the same after 40 h (enough time has elapsed since isotope administration so that the intravenous and oral tracer enrichments are decaying proportionally).
- (iii) That urine enrichments of both isotopes are similar for plasma.
- (iv) That the extrinsic label isotope reflects that of the intrinsic zinc in the diet.

Using the multiple spiking techniques, it has been possible to establish that while the fractional absorption of zinc from a meal depends on the quantity of exogenous zinc and on specific dietary factors such as phytic acid, the fractional absorption does not appear to be dependent on the size of the rapidly exchanging pool of the host. In contrast, the quantity of endogenous zinc excreted via the intestine is positively correlated with both the amount of absorbed zinc and the zinc “status” of the host, and thus this process has an equally critical role in maintaining zinc homeostasis. Thus the challenge remains for research at the whole body level to carefully characterize zinc distribution and exchange under diverse circumstances, while research at the cellular level must elucidate the regulatory processes and the factors to which they respond.

Closely related to these studies is the evaluation and use of Zn-fortified crops as means of increasing Zn uptake in humans. Measuring zinc absorption from biofortified crops is an important first step before demonstrating their efficacy in improving zinc status. Some studies have reported zinc absorption from staple foods, including wheat, rice, beans, millet, and maize, which were genetically biofortified with zinc by plant breeding. Some of these studies used extrinsic labelling of the biofortified and control meals, assuming that the food’s endogenous zinc and the extrinsic zinc label mix in a common pool in the gastrointestinal tract. However, there are several reasons why isotope exchange with native zinc, either with or without biofortification, may not be complete. The exchange may be influenced by physiologic factors, such as residence time of the meal in the stomach or small intestine, the physical state of the meal (liquid or solid), the physical state of the isotopic label, and the tightness with which the native zinc is bound to other food constituents. Thus, some recent experiments compare zinc absorption in adults from rice porridges prepared from a hydroponically biofortified rice labelled intrinsically with  $^{70}\text{Zn}$  with zinc absorption from a control rice of the same variety and phytic acid content, fortified at point of use with  $^{70}\text{ZnSO}_4$  to the same total zinc content (Brnić et al. 2016). The observed results showed that zinc absorption from rice biofortified with zinc hydroponically turned out to be similar to zinc absorption from rice fortified with zinc immediately before consumption. Such finding

indicated that zinc entering plants as a result of soil fertilization could be well absorbed by humans and that biofortification by this route is likely to be an efficacious intervention strategy.

Very recent experiments have also used Zn isotopic tracers to address the fate of ZnO nanoparticles in environmental samples. Some authors suggested few years ago that stable isotopes tracing may prove valuable for monitoring nanoparticles in the field and addressing emerging research questions but very few studies have been conducted so far. As previously explained, besides the costs, there are several technical challenges in creating stable isotope-labelled nanoparticles and tracing their fate in biota including: (1) availability of suitable isotopically enriched precursor for the synthesis of the material; (2) quantitative measurement of the newly accumulated metal, distinguishing that from background concentrations, particularly while working at low exposure concentrations with essential metals like Zn; (3) detection of the tracer in the animal tissue following short-time exposures at environmentally realistic concentrations. Despite these limitations, a recent publication reports on the synthesis of isotopically unique  $^{67}\text{ZnO}$  nanoparticles and the advantage of using these to determine whether  $^{67}\text{Zn}$ -enriched nanoparticles could be detected in animal tissues after short dietary exposures (Dybowska et al. 2011). For this aim, freshwater snails (*L. stagnalis*) were fed with diatoms mixed with different amounts of the synthesized  $^{67}\text{ZnO}$  nanoparticles (made by forced hydrolysis) and then fed them unlabelled food to clear their gut. The  $^{67}\text{Zn}$  retained after gut clearance was used to define assimilation. They also fed snails diatoms labelled with  $^{67}\text{Zn}$  to show how a tracer can improve experimental sensitivity, thereby overcoming the limitations imposed by the high natural concentrations of total Zn in animals and the environment. The obtained results revealed that a Zn concentration as low as  $1 \text{ mg g}^{-1}$  can be detected in freshwater snails exposed to about  $15 \text{ mg g}^{-1}$  of Zn when an enriched stable isotope of Zn is used as a tracer ( $^{67}\text{Zn}$ ). In contrast, exposures 333 times higher were required to detect Zn uptake if no tracer was used. Thus, the enriched stable isotope technique was found to be sufficiently sensitive to determine the uptake rate of Zn in *L. stagnalis* at an exposure equivalent to the lowest concentrations of Zn that might be expected in environmental media. The authors concluded that without the tracer, uptake rates could only be determined at concentrations equivalent to some of the most contaminated Zn conditions (or much longer exposure times), reducing the feasibility of studying a range of environmentally realistic exposure conditions.

### 6.2.3 Selenium Experiments

Selenium has been an element widely studied also on the frame of isotopically enriched labelled studies. Selenium is both essential and toxic for mammals; the range between the two roles is narrow and not only dose dependent but also related to the chemical species present in foodstuff. First experiments were conducted already in the year 2006 by the group of Suzuki in which the metabolic pathway for

the formation of Se-methyl selenocysteine (MeSeCys) was precisely traced by referring to those for selenomethionine (SeMet) and selenite by applying a tracer method involving multiple Se stable isotopes (Suzuki et al. 2006). Monomeric selenoamino acids are major forms in some selenium-accumulators, such as SeMet accumulators (grains such as wheat and mushroom) and MeSeCys-accumulators (garlic and onion). These selenium species get converted into the common intermediate selenide for the synthesis of either selenoproteins for utilization or selenosugar for excretion. Therefore, this work aimed to determine the precise mechanism underlying the transformation of MeSeCys into selenide in comparison with that of SeMet and selenite. For this aim, male Wistar rats were depleted of endogenous natural abundance selenium with a single  $^{80}\text{Se}$ -enriched isotope, and then  $^{76}\text{Se}$ -MeSeCys,  $^{77}\text{Se}$ -SeMet, and  $^{82}\text{Se}$ -selenite were orally administered simultaneously. Organs and body fluids were obtained after a number of days and subjected to speciation analysis.

MeSeCys and SeMet were detected in organs in their intact forms, suggesting that selenoamino acids are absorbed and delivered to organs in their intact forms, as in the case of general amino acids, and then transformed for utilization and excretion. However, MeSeCys and SeMet were not detected in the urine, suggesting that selenoamino acids are not excreted into urine in their intact forms. The SeMet-specific delivery and presence in the pancreas were also observed for MeSeCys at a comparable level to in the case of SeMet. As for the transformation of MeSeCys and SeMet into selenide, MeSeCys is considered to be transformed into methylselenol through the  $\beta$ -lyase reaction and then demethylated into selenide for the syntheses of selenoproteins for utilization and selenosugar for excretion. On the other hand, SeMet is suggested not to be transformed into methylselenol, but into selenide through the trans-selenation reaction followed by the  $\beta$ -lyase reaction. The authors suggest that SeMet and MeSeCys transformed into selenide through the  $\beta$ -lyase reaction but differently; SeMet is transformed into SeCys through the trans-selenation reaction and then into selenide through the  $\beta$ -lyase reaction for SeCys, while MeSeCys is transformed into methylselenol through the  $\beta$ -lyase reaction for MeSeCys and then through the demethylation reaction into selenide.

Similarly, a recent work on a non-hyperaccumulator plant, ryegrass (*Lolium perenne* L.), was chosen as model system for being an easy and fast to grow plant. Moreover, ryegrass is one of the most important forage varieties in terms of contribution to pasture composition, and an increase of Se accumulation was previously evidenced in greenhouse experiments, as well as nonnegative effect on plant yield, when Se was supplied as selenite or selenate forms at low concentration. In this work (Di Tullo et al. 2015), the authors used two stable isotopes,  $^{77}\text{Se}$  and  $^{82}\text{Se}$ , to label, respectively, selenite and selenate sources added to hydroponic growth media (double spike) in order to monitor respective bio-incorporation of these two species in roots and leaves of ryegrass. HPLC-ICP-MS was used for Se species determination after water extraction and enzymatic hydrolysis of freeze-dried samples. Through the use of isotopically enriched tracers, it was shown that inorganic selenium could be taken up and bio-transformed, i.e., converted into organic compounds and/or incorporated into Se-containing proteins. In general, total selenium

measurement showed that supplementation with Se(VI) resulted in similar total Se level in whole plant compared to that enriched with Se(IV). However, allocation in plant compartments and metabolism of Se depends on the nature of initial inorganic species.

### 6.3 Isotopic Signatures as Intrinsic Tracers in Metallomics Studies

Isotope fractionation is the general term referring to altered isotope abundances when no isotopically labelled species has been added into the sample. It has been reported that isotope fractionation can occur in the physical and chemical transformation of many elements, including Fe, Zn, Cu, and Cd within biological processes. Therefore, variations in isotope ratios of the heavy elements could provide new insights into biochemical processes, and they have been recently investigated as potential tools for diagnosis. For instance, iron stable isotopes now begin to serve as tracers of nutrient uptake paths, processes, and efficiencies in humans. In particular, Fe isotope fractionation effects may serve as a tool to identify long-term differences in dietary Fe absorption between individuals. Iron isotope ratios have been commonly measured to study the absorption and utilization of dietary Fe in humans by applying isotopically enriched Fe as tracers. Sophisticated mass spectrometric techniques, mostly based on the use of multicollector (MC-ICP-MS), allow the measurement of small changes in Fe isotope ratios produced by natural processes. Variations in the  $^{56}\text{Fe}/^{54}\text{Fe}$  isotope ratio of 1 to 2‰ can be induced in natural samples both by microbial activity and abiotic processes. Therefore, the accuracy and precision of such measurements have to be carefully addressed in this kind of experiments.

Also, all humans contain a specific iron stable isotope signature in their blood. Human blood and muscle tissue are enriched in the light iron isotope,  $^{54}\text{Fe}$ , by 1 or 2‰ over the heavy iron isotope,  $^{56}\text{Fe}$ , when compared to initial surveys of the human diet. Similarly, the accurate measurement of Fe isotopic fractionation has permitted to establish that men and women differed in the Fe isotope composition of their blood. This can be ascribed to different reasons, including the fact that iron losses from the body are biased in favor of the discharge of heavier Fe isotopes and that light Fe isotopes are preferentially absorbed in the intestine (Walczyk and von Blanckenburg 2002).

Similar studies have been also conducted in plants. The isotope fractionation associated with the growth of higher plants is substantial and also generates systematic plant-specific patterns. Some of the work published in this regard hypothesizes that these patterns mirror the two different strategies that plants have developed to incorporate iron from the soil: reduction of Fe(III) in soils by plants results in the uptake of iron, which is depleted in  $^{56}\text{Fe}$  relative to  $^{54}\text{Fe}$  when compared to the available Fe in soils; complexation with siderophores by plants results in the uptake of iron that is heavier than that in soils. Furthermore, younger parts of strategy I plants

get increasingly depleted in heavy isotopes as the plant grows, while strategy II plants incorporate nearly the same isotope composition throughout. These results point to an entirely different translocation mechanisms between strategy I and II plants. Thus, the conclusion is that plant metabolism represents an important cause of isotopic variation in the biogeochemical cycling of Fe (Guelke and von Blanckenburg 2007).

Besides iron, isotopic fractionation of copper (Cu) and zinc (Zn) during plant uptake has been also reported. The results show that the fractionation patterns of these two micronutrients are decoupled during the transport from nutrient solution to the root. In roots, an enrichment of the heavier isotopes for Zn has been found, but an enrichment of isotopically light Cu was simultaneously found, suggesting a reduction of Cu(II) possibly at the surfaces of the root cell plasma membranes. This observation applies for both graminaceous and non-graminaceous species and confirms that reduction is a predominant and ubiquitous mechanism for the acquisition of Cu into plants similar to the mechanism for the acquisition of iron (Fe) by the strategy I plant species (Jouvin et al. 2012).

Recent studies in mammals reveal also variations in Cu, Fe, and Zn natural isotope compositions in organs, body fluids, diets, and feces of mice and sheep. Large and systematic isotope variability was observed, notably in the  $\delta^{66}\text{Zn}$  in the liver and  $\delta^{65}\text{Cu}$  in kidneys, but significant differences exist between mice, sheep, and humans, especially in the  $\delta^{66}\text{Zn}$  value of blood. The results are interpreted with reference to current knowledge about the fact that the light isotopes preferentially fractionate into “softer” bonds involving sulfur such as cysteine and glutathione, whereas heavy isotopes fractionate into “harder” bonds involving nitrogen (histidine) and even more oxygen, notably hydroxides, phosphates, and carbonates. Bonds involving the reduced forms  $\text{Cu}^+$  and  $\text{Fe}^{2+}$  are enriched in the light isotopes relative to bonds involving the oxidized  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  forms. The isotopically heavy Cu in the kidneys may reflect isotope fractionation during redox processes and may be relevant to ascorbate degradation into oxalate (Balter et al. 2013).

Very recent publications try to correlate isotopic fractionation results with certain disease conditions, in particular for the diagnosis and prognosis of liver cirrhosis. Significant differences in the isotopic composition of Cu were observed between the reference population and the patients. A wide spread in  $\delta^{65}\text{Cu}$  was observed within the cirrhosis population and could be linked to the severity of the disease. Patients with end-stage liver disease showed a significantly lighter serum Cu isotopic composition. Many clinical parameters used for the diagnosis and monitoring of liver diseases, i.e., the levels of aspartate aminotransferase, De Ritis ratio, prothrombin and international normalized ratio, albumin, bilirubin, Na, and C-reactive protein, correlated well with the  $\delta^{65}\text{Cu}$  values, as did the ceruloplasmin level and the ceruloplasmin/Cu concentration ratio. The isotopic composition of serum Cu appeared to reveal the synthetic and hepatocellular function of the liver synergistically with inflammation and fluid retention in the cohort studied. A relevant relationship was also observed between  $\delta^{65}\text{Cu}$  and scores of mortality risk, such as the model for end-stage liver disease (MELD). Thus, the isotopic composition of serum Cu is suggested as potential new approach for the prognosis of liver

disease, and although further investigation is required, for evaluation of the mortality risk in end-stage liver disease and prioritization of liver transplants (Costas-Rodríguez et al. 2015). In this vein, a decrease of  $\delta^{65}\text{Cu}$  in the serum of cancer patients has been also assigned to the extensive oxidative chelation of copper by cytosolic lactate. The potential of Cu isotope variability as a new diagnostic tool for breast and colorectal cancer seems strong (Télouk et al. 2015). Therefore, the measurement of isotope fractionation of essential elements in body fluids or tissues seems to hide an extraordinary potential for Metallomics experiments in human metabolism and diagnosis.

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