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Speciation and isotope pattern deconvolution for inductively coupled plasma-mass spectrometry quantitative studies of mineral metabolism and supplementation*

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Abstract: Human breast milk can be considered as "ideal" food for the correct development of newborn babies and, for those that are not breast-fed, formula milk has to be used instead. Ideally, the composition of such formula milk preparations should closely resemble that of maternal human milk. Considerable differences between both in the total content of trace elements such as Fe, Cu, Se, Zn, and I and in their chemical form in both milk types have been demonstrated.

Speciation analysis in milk whey was carried out first by high-performance liquid chromatography (HPLC) with inductively coupled plasma-mass spectrometry (ICP-MS) elemental detection and showed that the observed element distribution patterns were very different in the investigated human and formula milks. Using complementary molecular mass techniques (i.e., MALDI-TOF), the identity and chemical characterization of some biomolecules (e.g., protein) with which metals are associated in each fraction was also established (by a typical heteroatom-tagged proteomics protocol). Attempts to assess the nutritional value of elemental supplements in formula milk with the aid of quantitative chemical speciation, using stable isotopes in combination with ICP-MS and isotope pattern deconvolution (IPD), proved to be successful to differentiate and quantify endogenous (natural) and exogenous (supplemented) Se or Fe trace levels. In particular, the application of such ICP-MS based techniques to study Se bioavailability from formula milk and metabolism in Se-supplemented lactating rats is discussed in detail. Quantification of selenospecies of endogenous (natural) and exogenous (supplement) Se in rat's urine is demonstrated and relevant information on possible Se biotransformations and its final catabolism from such results is discussed.

Keywords: endogenous and exogenous selenium; HPLC-ICP-MS; ICP-MS; isotope pattern deconvolution; selenium speciation; stable isotopes.

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INTRODUCTION

The determination of essential and toxic trace elements in nutrition has acquired great importance [1]. However, elements may occur in food in many chemical forms, i.e., as building blocks of essential macronutrients (e.g., proteins, carbohydrates, lipids, etc.) and micronutrients (e.g., vitamins, enzymes) and as minerals and trace elements. Today it is widely recognized that the nature and amount of a given element chemical species in a food matrix, rather than the corresponding element total analytical concentration, will determine its bioavailability (e.g., in human milk), metabolism, transport/storage in the body, and eventually its biochemical essential role and so its nutritional value. The precise definitions of the different terms including "speciation analysis" and "chemical species" are given elsewhere [2]. In other words, chemical element species information (apart from total elemental concentration) is needed for a sound assessment of how the considered element is absorbed, retained, metabolized, etc. and to decide whether its effects are beneficial (essential elements), toxic, or rather the element has no adverse impact at a specific concentration, but it can become a therapeutic drug at higher levels of concentration [3]. In brief, the consideration of the essentiality (or toxicity) of a given trace element is deeply related to its chemical form in the food. Therefore, aiming at assessing the role of trace elements in food and nutrition, problem-related chemical speciation is becoming a most important analytical strategy [4,5].

On the other hand, milk is a critical food for nutrition of the newborn because it represents the baby's single source of nutrients during the first months of his life. Such a rapidly developing organism is particularly sensitive to nutritional deficiencies (e.g., of essential trace elements) and, what is more, deficiency-derived disorders may be irreversible [6]. Thus, to secure this required level of nutrients, and so of trace elements, in baby's nutrition it is necessary to resort to appropriate "formula" milks. Ideally, the composition of such formula milk preparations would be as similar as possible to maternal human milk. Usually, the base of formulas is cow milk, and total trace element levels in cow milk are different from human milk elemental concentrations. This entails the common practice of essential trace elements supplementation and the need for their monitoring in formula milks.

The analytical determination of trace element levels in foods and supplements is usually carried out by atomic spectroscopy techniques based on radiation–matter interactions [7]. However, sensitivity, selectivity, multielemental capability, and isotope ratios measurement ability of inductively coupled plasma-mass spectrometry (ICP-MS) progressively favors the use of this latter technique, particularly for trace element speciation [8]. It should be borne in mind, however, that atomic techniques (and so the ICP-MS) provide elemental information only. That is, total elemental determinations can be secured by using ICP-MS but, as explained before, chemical speciation information is today strongly demanded to assess the nutritional value of natural foods, formula milks, and supplements. A typical strategy used to complement this ICP-MS elemental character is its coupling on-line with a powerful separation technique (e.g., HPLC, CE, or GC) of the species. Figure 1 shows a scheme of the so far most powerful "hybrid" technique for chemical speciation of trace and ultratrace elements in food: the HPLC-ICP-MS coupling, where the sought chemical species separation takes place in the chromatographic column while the ICP-MS (different mass analyzers are used today) will detect on-line, in real-time, and in an extremely sensitive and robust manner, the sought trace element. In this way, the observed retention time in the corresponding chromatographic peak is a first indication of the biomolecule where that element is present (in other words, retention times are indicative of the chemical nature of such biocompounds, e.g., of a given protein in a milk serum sample). As the m/z recorded in the ICP-MS provides simultaneous elemental information of trace elements, the described hybrid arrangement provides a powerful analytical tool to carry out a first approximation to multiple trace element speciation analysis. There are many examples in the present scientific literature illustrating the use of such hybrid techniques to tackle several metals speciation analysis in real samples of human milk and formula milks [9]. Moreover, absorbance in the UV–vis region can be also measured (see Fig. 1) concurrently to ICP-MS to show the presence of metal-biomolecule complexes, because of a charge-transfer absorption band.

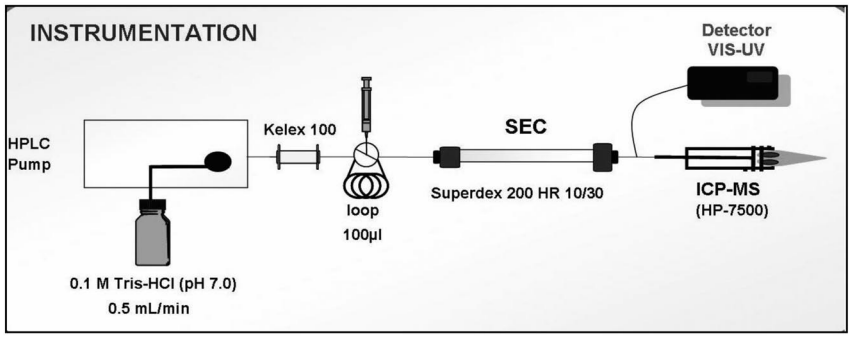

Fig. 1 Scheme of a typical coupling of HPLC-ICP-MS.

This paper is a brief overview of the work carried out in our laboratory in the last few years, illustrating two aspects of new analytical strategies to investigate mineral metabolism and supplementation issues: first, the present importance of qualitative chemical speciation in milk (and its implications to prepare appropriate and healthy formulas) with special reference to carry out molecular MS experiments, to confirm the chemical nature of the metal-biomolecules and, second, the use of stable isotopes and isotope pattern deconvolution (IPD) techniques to achieve the quantitative analysis of the unveiled metal-biomolecule species, a new aspect still in its infancy.

Studies on absorption from food and/or metabolism of essential or toxic trace elements, from a quantitative point of view (using stable isotopes and IPD), are now possible, paving the way for new nutritional and metabolism insights.

CHEMICAL SPECIATION IMPORTANCE AS ILLUSTRATED IN MATERNAL AND FORMULA MILK STUDIES

The distribution of essential and toxic elements among the different bulk fractions of milk (fat, caseins, and milk whey) is a previous step when aiming at investigating the particular species in which the trace element is present in human maternal or formula milks. Multielemental distribution patterns for human, cow, and formula milks have been compared in our research group [10]. Significantly different element distribution patterns were observed for each type of investigated milk. After removing fat and caseins (because about 70 % of total essential elements were found in human milk whey), the identification of the possible trace element species in such milk fraction (e.g., for Fe, Cu, Zn, I, and Se) has been reported, mainly using size exclusion (SE) high-performance liquid chromatography (HPLC) separation coupled to ICP-MS detection [11–13]. Figure 1 illustrates the employed instrumental set-up scheme with UV–vis (molecular) and ICP-MS (elemental) simultaneous detection.

At this point, it is important to stress the critical need for some previous sample preparation steps before the actual species separation and detection are accomplished. Carefully planned sampling, storage, fractionation, etc. of the investigated samples are also mandatory for a successful speciation analysis of trace elements in human and formula milks. Guidelines and more detailed information about such previous, but of prime importance, sample preparation steps can be found elsewhere [14]. After an appropriate sampling, storage, and preparation of the raw sample, a first analytical strategy based on the use of HPLC-ICP-MS for trace elements "screening" in the different chromatographic peaks can be extremely valuable. This has been reported for Ca, Mg, Fe, Cu, Zn, Se, I, etc. [11], but we will only illustrate here its use and information potential for the case of milk Cu speciation: first of all, the size exclusion chromatography (SEC) column in our speciation set-up (Fig. 1) was calibrated by measuring the corresponding retention times of selected proteins, covering the mass range expected in milk whey (e.g., α-lactalbumin, β-lactoglobulin, ovoalbumin, albumin, immunoglobulin G, etc.). In this way, the

HPLC-ICP-MS chromatograms for Cu in human milk whey, at different days of the assayed mothers lactating period, were obtained (see results in Fig. 2 for human and formula milk). As the SEC column is calibrated, we could assign preliminary molecular masses to each of the chromatographic peaks (milk whey molecules) separated. From those preliminary results, it was apparent that [11]: first, we could not find statistically different Cu chromatographic profiles (not only for Cu, but for the other trace elements as well) in human milk all along the whole lactating period studied (1 month); second, the speciation of Cu in maternal milk is complex, very rich in biocompounds and the element is mainly bound to several high-molecular-mass biomolecules; and third, in the formula milks, however, the Cu speciation pattern was much simpler than in maternal milks, with Cu^{2+} mainly bound to low molecular mass compounds (see comparatively the two chromatograms in Fig. 2). It can be said that those findings can be extrapolated, one by one, to the other trace elements investigated: Fe, Zn, Se, and I [11]. A main conclusion emerging from all our milk speciation results is the drastic difference in the trace elements chemical speciation between human maternal milk (Fig. 2a) and five different commercial formula milks investigated (Fig. 2b), virtually in all investigated essential trace elements. Therefore, formula manufacturing companies are well advised of this fact and of the subsequent convenience of starting to use trace element speciation information to manufacture more scientifically guided formula milks and substitute food for newborns.

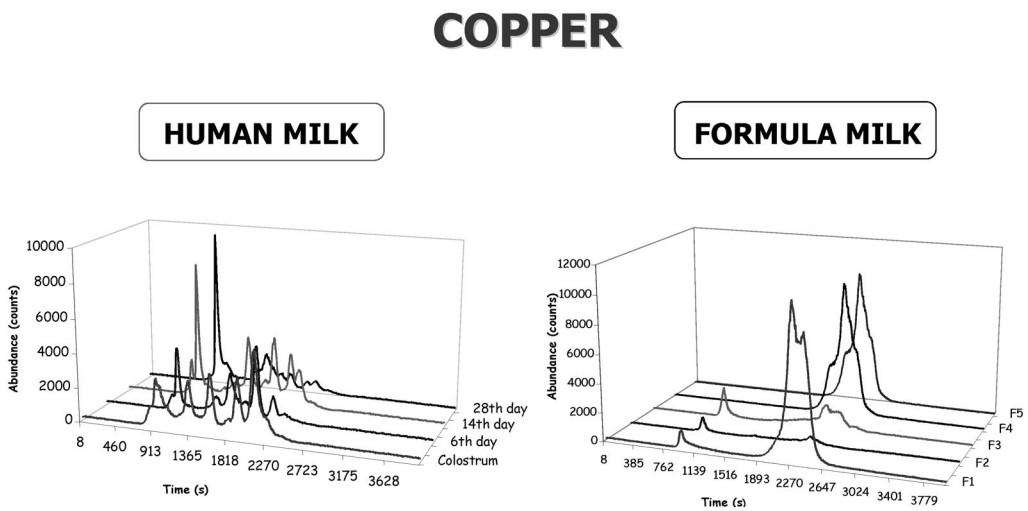

Fig. 2 Speciation results for Cu in human formula milk whey.

"Integrated" chemical speciation via mass spectrometry

The application of HPLC-ICP-MS strategies and techniques to separate and detect proteins, peptides, and amino acids has recently been proposed as an alternative tool to complement the well-established molecular MS-based techniques (e.g., MALDI and ESI sources). The outstanding capabilities of ICP-MS to track any desired "heteroelement" and its isotopes (meaning here any bioelement, except C, H, N, or O for which the ICP-MS is not appropriate) of a biomolecule in an almost interference-free, microgram per litre detection and in a robust manner for real biological samples, are today well documented. In fact, a new concept of element-driven proteomics has been introduced and termed "heteroatom(isotope)-tagged proteomics" [15,16]. In any case, the use of ICP-MS in such cases does not exclude or decrease the extensive use and great success of molecular MS-based techniques for analytical proteomic work. The point is that both, "elemental" and "molecular" ion sources, should be considered complementary.

This concept of using elemental ion sources (e.g., ICP-MS) along with molecular ones [e.g., MALDI-MS and ESI-$(MS)^n$] to get a speciation information as complete as possible for a given trace element can be termed "integrated" chemical speciation and, of course, is all based on using MS detectors. Figure 3 shows a workflow illustrating this concept as applied to trace element speciation in milk: as shown in this figure the first step should be to screen the heteroatom-containing proteins in multispecies, multielemental HPLC-ICP-MS experiments obtaining the chromatograms for individual elements (e.g., see in Fig. 2 the several peaks or fractions for Cu^{2+} in milk whey). Once the sought protein (peak) is identified, that fraction would be purified and/or preconcentrated and sent to matrix-assisted laser desorption/ionization (MALDI)-MS [or ESI-$(MS)^n$] for typical qualitative proteomic analysis. By using such powerful molecular techniques, the identity and chemical characterization of that particular biomolecule (e.g., protein) of the peak can be established (see step 2 in the flow chart of Fig. 3). In a third step, the particular ability of ICP-MS for quantitative analysis of the heteroatom selected would allow a final determination of the peptide/protein (if the stoichiometry heteroatom/protein is known or has been previously determined, e.g., via the second step).

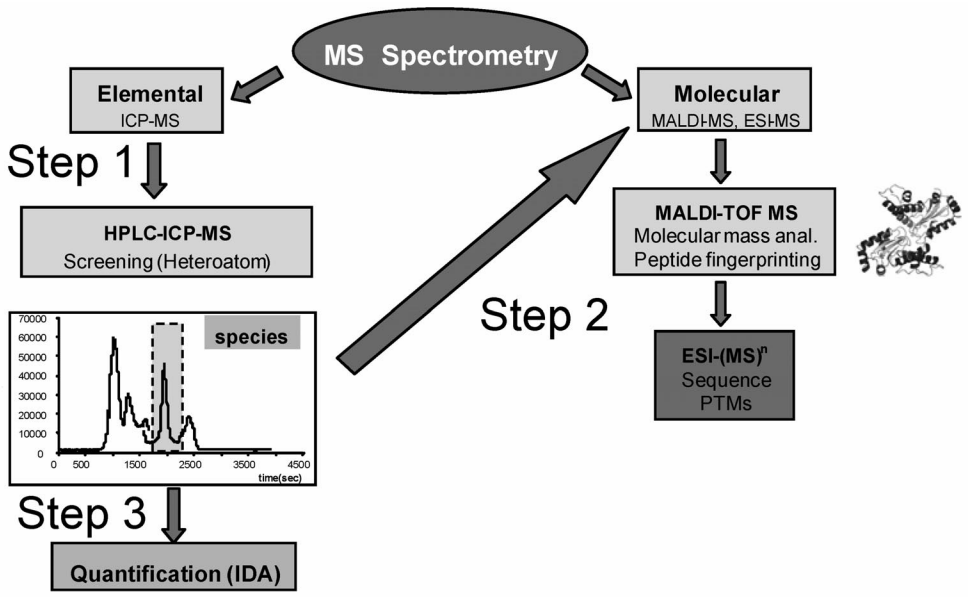

Fig. 3 Integrated speciation via MS, a general workflow.

In the example of Cu speciation in milk whey, the peak (fraction eluting at retention time 1800 s in Fig. 2) eluting at around 14 kDa was isolated, desalted, and preconcentrated (using a classical Centricon Ym-10) and its MALDI-MS analysis (in a matrix of sinapinic acid, acetonitrile, and trifluoroacetic acid) was carried out. The MS spectrum obtained of that fraction (see Fig. 4) showed a major MS peak at 13 995 mass (m/z), corresponding to the singly charged molecule of the milk protein α-lactoalbumin. Another peak, at half that mass (m/z = 7005), was also strong, corresponding to the doubly charged protein molecular mass (within our experimental error).

The nature of that fraction (protein) was confirmed by peptide mass fingerprinting of its tryptic digest as well. The measured m/z of the resulting peptides in the MALDI-TOF, after appropriate cleaning in a C_{18} Zip-tip, were matched with the proteomics MASCOT database, resulting in a good match for at least four peptides of the protein α-lactoalbumin.

Unfortunately, the metal (Cu^{2+}) bond with the biomolecule (protein) may be cleaved at the high acidity required in the MALDI matrix for efficient protonation. For this reason, usually only protein

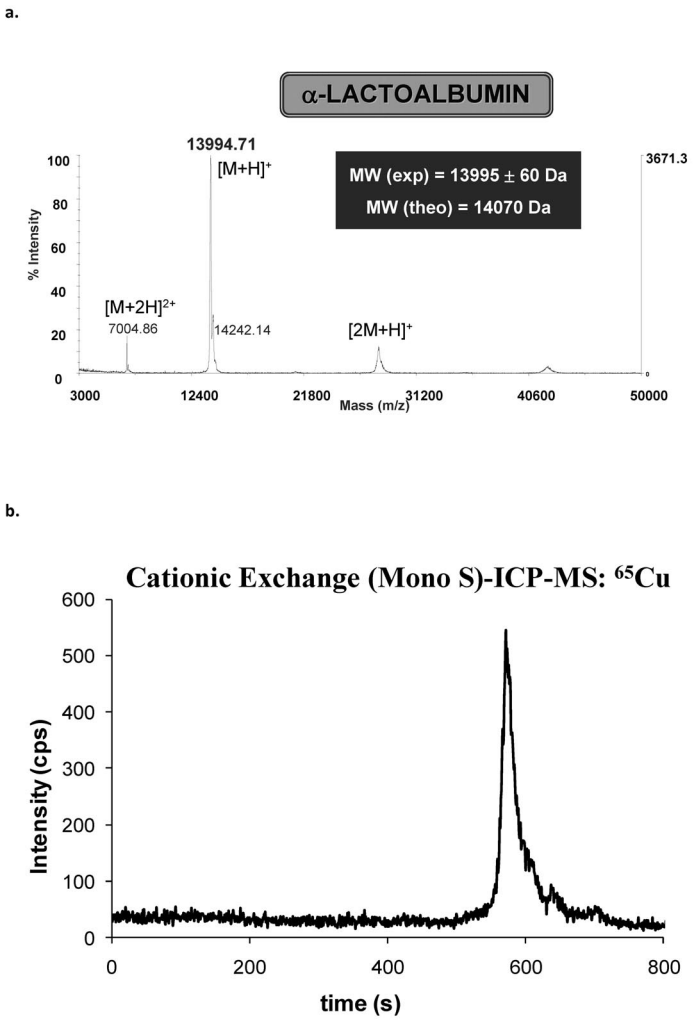

Fig. 4 (a) MALDI-MS spectrum of fraction at retention time 1800 s in Fig. 2. (b) Fraction corresponding to retention time 1800 s injected on cationic exchange column coupled to ICP-MS: isotope monitored ^{65}Cu.

present in the studied fraction can be identified by its molecular mass. However, the ICP-MS identifies the associated metal in the corresponding peak (fraction). The combination of both techniques allows a first metal-biomolecule identification.

Usually multidimensional (orthogonal) strategies are required for compound purification, previous to final identification by MALDI or electrospray ionization (ESI). For instance, the combination of a SEC initial separation and ion exchange (IE) chromatography, have been used here to purify the compound (proteins) in milk serum before sending them to MALDI analysis.

In the example of Cu^{2+}, the metal-binding proteins of the SEC fraction eluting at 14 KDa were then subjected to further separation in a Mono-S column (cationic exchange mechanism). The observed fraction, containing still the metal as certified by ICP-MS signals (Fig. 4b), was then collected, desalted, preconcentrated, and analyzed by MALDI. The obtained mass spectrum in such fraction confirms the presence of lactoalbumin.

Also, Fig. 4b shows the ^{65}Cu chromatogram obtained for the fraction corresponding to 14 KDa (from SEC) after injection in a cationic exchange column (Mono S) coupled to ICP-MS. In this way it

is possible to ensure the presence of metal-biomolecule complexes coordination. Concurrently to ICP-MS, absorbance in UV–vis can be measured to show the presence of metal-biomolecule coordination complexes and/or biomolecule (Fig. 1). On the other hand, it is well established that Cu^{2+} (and other metals too) is always strongly bound to α-lactoalbumin at pH around 7 [17].

The different metal contents (corresponding to all measured essential elements in that particular protein) were determined, by HPLC-ICP-MS (step 3 in Fig. 3), in the milks of all the lactating mothers who participated in the milk whey speciation exercise (carried out in collaboration with the Department of Neonatology from Hospital Central of Asturias, Oviedo). The observed figures were: Cu (7–35 %), Zn (2–6 %), and Fe (0–4 %) in the α-lactoalbumin peaks. They show that the metal contents in milk α-lactoalbumin may vary quite a lot, depending on the mother's diet, the day of the milk sampling, the individual mother sampled, etc., during the first month period assayed.

To conclude this section, it should be stressed that calculating absolute protein concentrations from quantitative element determinations in a given isolated biocompound (chromatographic peak) is not straightforward. First, the metal/protein stoichiometry should be known; second, the protein should be metal saturated, otherwise, we will obtain just the fraction of the existing protein bound to that metal (e.g., just around 30 % of total transferrin for Fe-transferrin in human blood serum); third, of course, any losses or contaminations of the measured metal in the whole analytical process would produce gross errors in the calculation of the absolute amount of metalloprotein originally present in the biological sample.

Se STABLE ISOTOPES FOR QUANTITATIVE INVESTIGATIONS ON Se METABOLISM AND BIOAVAILABILITY FROM SUPPLEMENTS: CONCEPT OF IPD

As said before, essential trace element total contents in formula milks turned out to be higher than those observed in human milk. Fortifications of the base product (cow milk) in formula milk could explain the comparatively high levels of essential trace elements observed in formulas. Moreover, speciation analyses have shown that trace element chemical speciation observed are drastically different as well. As bioavailability, absorption, and retention of the ingested supplements will depend on elemental speciation, to estimate adequately the nutritional value of elemental supplementation in formula milk not just qualitative but quantitative chemical speciation information is also needed.

The use of mineral stable isotopes is a very useful tool in metabolic and nutritional research. The most frequent application of stable isotopes in the field of minerals nutritional studies has been to determine dietary mineral absorption and availability and to investigate the flow of nutrients through the human body. Since stable isotopes are safe and nonradioactive, they can even be used in infants and pregnant women. Another advantage of stable isotopes includes the fact that multiple isotopes of a given element and/or multiple isotopes of different elements can be administered simultaneously or sequentially to investigate synergic toxic or detoxifying effects of a given element on the biological functions of the others [18]. Application of mineral stable isotopes requires that suitable analytical methods, providing precise and accurate isotope ratios measurement, are available. During the past three decades, analytical isotopic analysis methods were mainly based on mass spectrometry [fast atom bombardment MS (FAB-MS), thermal ionization MS (TIMS), gas chromatography MS (GC-MS)] and neutron activation analysis (NAA). Currently, ICP-MS is the most widely used technique for precise metal isotope ratio measurements due to its excellent analytical characteristics for the purpose [18,19].

In brief, the use of stable isotopes in connection with ICP-MS detection offers today a most useful approach in the field of nutritional studies. Moreover, measuring isotope ratios by ICP-MS and using appropriate mathematical calculations, based on IPD techniques, may also provide quantitative data about endogenous and exogenous essential or toxic elements and their metabolism. IPD is a mathematical technique allowing for isolating distinct isotope signatures from mixtures of natural abundance and enriched tracers [20,21]. Although still in its infancy, IPD has proved to be a very promising analytical tool for nutritional studies [22,23] and in speciation [21,24–27].

In particular, a methodology based on IPD in combination with ICP-MS or HPLC-ICP-MS was recently proposed in our laboratory to study Se metabolism in rats [22,27]. In these studies, two-week-old lactating rats were used and maintained in metabolic cages (three rats/cage). The first group (three rats) was fed with formula milk *ad libitum*. The second group was fed with the same formula milk but supplemented with ^{77}Se in the form of selenite at the recommended level (0.15 μg/g), for two weeks. Finally, a reference three rats group was fed with maternal milk during the same time. Urine and feces were collected daily. At the end of the two weeks supplementation period the rats were sacrificed and their blood and tissues were collected for further analysis. Se determinations in the samples were carried out by IPD analysis using ^{74}Se as the quantitation isotope spike. The samples, containing a mixture of natural abundance Se (endogenous) and the isotopically labelled tracer (^{77}Se enriched, exogenous), are spiked at the beginning of the analysis with isotopically enriched ^{74}Se. Three different Se isotope patterns are then present in the spiked sample (see Fig. 5): one for ^{nat}Se, another for ^{77}Se tracer, and the third for ^{74}Se.

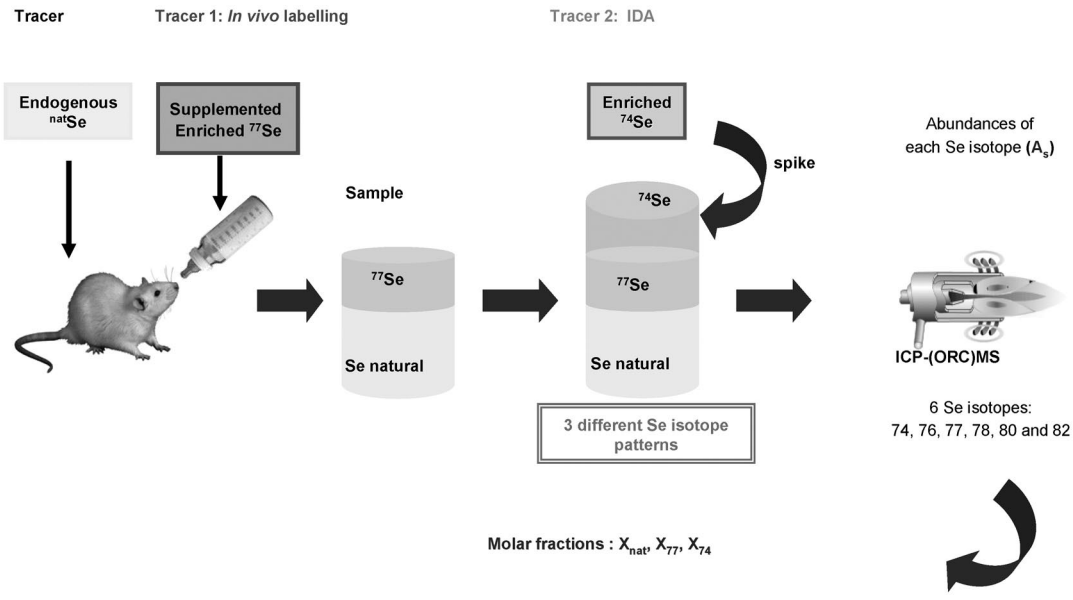

Fig. 5 Determination of total endogenous and exogenous Se in rat samples by IPD-ICP-MS.

The six Se isotope abundances in the sample (A_s) are related with individual isotope abundances (A^y_{nx}) of tracers used (^{77}Se, ^{74}Se) and ^{nat}Se (all of them are known) and with the molar fractions (x_{nx}) of ^{nat}Se, ^{77}Se, and ^{74}Se, according to this matrix form equation [22]:

$$\begin{bmatrix} A_s^{74} \\ A_s^{76} \\ A_s^{77} \\ A_s^{78} \\ A_s^{80} \\ A_s^{82} \end{bmatrix} = \begin{bmatrix} A_{nat}^{74} & A_{77}^{74} & A_{74}^{74} \\ A_{nat}^{76} & A_{77}^{76} & A_{74}^{76} \\ A_{nat}^{77} & A_{77}^{77} & A_{74}^{77} \\ A_{nat}^{78} & A_{77}^{78} & A_{74}^{78} \\ A_{nat}^{80} & A_{77}^{80} & A_{74}^{80} \\ A_{nat}^{82} & A_{77}^{82} & A_{74}^{82} \end{bmatrix} \cdot \begin{bmatrix} x_{nat} \\ x_{77} \\ x_{74} \end{bmatrix} + \begin{bmatrix} e^{74} \\ e^{76} \\ e^{77} \\ e^{78} \\ e^{80} \\ e^{82} \end{bmatrix}$$

Abundances of each Se isotope in the sample (A_s^y) were determined from the isotopic composition of Se in such sample, measured by collision cell ICP-MS, and corrected for the dead time of the detector, mass bias errors, and for BrH^+ and SeH^+ interferences [22,23]. In this way, the abundances of the six Se isotopes in the sample were evaluated and the unknown molar fractions can be also calculated from the above formula by minimization of the error (e), applying least-squares fitting [22].

Once the corresponding molar fractions of Se are known (x_{nat}, x_{77}, and x_{74}) the amount of natural Se (N_{nat}^{Se}) and of ^{77}Se-labelled Se (N_{77}^{Se}) in the sample can be easily calculated (because the moles of ^{74}Se added to the sample N_{74}^{Se} are known) by the following equations [22]:

$$x_{nat} = \frac{N_{nat}^{Se}}{N_{nat}^{Se} + N_{77}^{Se} + N_{74}^{Se}}$$

$$x_{77} = \frac{N_{77}^{Se}}{N_{nat}^{Se} + N_{77}^{Se} + N_{74}^{Se}}$$

$$x_{74} = \frac{N_{74}^{Se}}{N_{nat}^{Se} + N_{77}^{Se} + N_{74}^{Se}}$$

TOTAL ^{nat}Se AND ^{77}Se DETERMINATIONS BY IPD-ICP-MS

The described IPD methodology was first validated by determining total Se in human serum and urine reference materials (Seronorm™ Trace Elements Serum LOT MI0181, Seronorm™ Trace Elements Urine LOT Nº 2525): the sample with a certified amount of Se (natural isotopic abundance) was spiked with ^{77}Se (considered exogenous) and ^{74}Se (as quantitation tracer of both, natural and exogenous Se) before ICP-MS measurements. The results obtained after IPD calculations showed a good agreement with the Se present in the sample (endogenous) and with the ^{77}Se tracer added to it (exogenous) [22].

Then, the apparent Se absorption (difference between intake ^{77}Se dose and Se excreted in feces) and the apparent retention (difference between intake ^{77}Se dose and Se excreted in urine and feces) were calculated. Apparent absorption was not significantly different during the two-week study, obtaining an average of 98.8 ± 0.5 %: very little loss of exogenous Se, through biliary and intestinal pathways took place, and ^{nat}Se amounted to most of the Se observed in feces. This fact can indicate a long turnover of selenoproteins metabolism occurring in body tissues. Also, the cumulative ^{nat}Se excreted by the supplemented group (1470 ng) was much higher than that excreted by the nonsupplemented group (223 ng). That is, Se turnover in the body seems lower for low Se intakes. The apparent Se retention observed decreased gradually and an abrupt decrease was apparent in the 11th day, urine being the main excretory route for Se (endogenous and exogenous).

In the supplemented group, the molar fraction of natural Se in urine decreased slowly with the days of supplementation while ^{77}Se molar fraction increased (see Fig. 6). After 11–12 days there is a cross-over point in which the molar fraction of enriched ^{77}Se (supplement) becomes a bit higher than that of natural Se. Interestingly, in blood serum, about 60 % of ^{nat}Se was exchanged by ^{77}Se after the 14 days of supplementation and the ratio of ^{77}Se/^{nat}Se measured in urine after 14 days (r = 1.33) approaches ^{77}Se/^{nat}Se value measured in blood serum (r = 1.38).

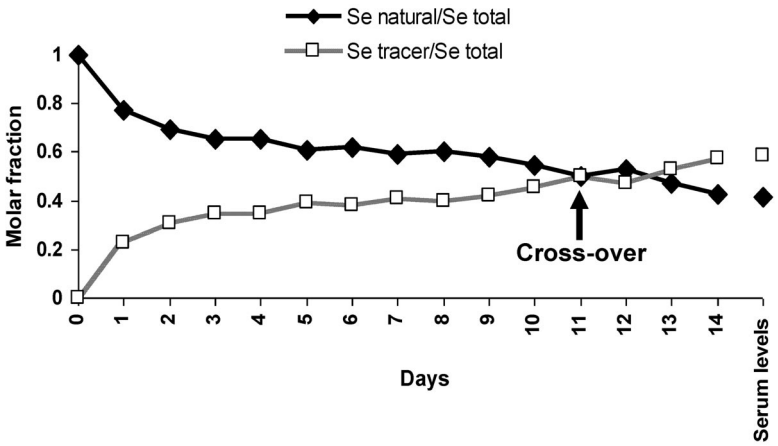

Fig. 6 Ratios of the observed molar fractions: natural Se/total Se and tracer Se/total Se in urine, as a function of the day of supplementation.

MULTIPLE SPIKE IPD FOR QUANTITATIVE SPECIATION OF ENDOGENOUS (NATURAL) AND EXOGENOUS (SUPPLEMENTS FATE) ELEMENTAL SPECIES

The determination of endogenous trace element species in biological material by isotope dilution analysis (IDA) has been widely reported (see ref. [28]). However, discrimination between endogenous and exogenous species had not been attempted before and can be attained using IPD, which is envisaged as an important tool for speciation analysis of Se metabolism in rats [22,27].

In such studies, the separation of selenospecies (containing natural and supplemented Se) in urine was performed by reverse-phase chromatography followed by ICP-MS on-line detection. Se determination in such selenospecies was carried out by post-column on-line isotope dilution analysis, by continuous mixing of the enriched ^{74}Se quantitation spike solution with the separated selenospecies, and IPD calculations at every obtained chromatogram point to obtain the corresponding mass flow chromatograms for ^{nat}Se and ^{77}Se [27]. In this way, the amounts of endogenous and exogenous selenocompounds in each peak, exiting the chromatographic column, were calculated by integration of the area of the peaks.

Again, this IPD strategy was validated in a urine reference material for quantitative speciation of Se. The sample, containing a certified amount of Se (with natural isotopic abundance) was spiked with the enriched ^{77}selenite (or, alternatively, ^{76}selenomethionine) before ICP-MS measurement. Applying the IPD model to the chromatograms for every Se isotope, the corresponding "natural" and "supplemented" mass flow chromatograms were obtained and from these the amounts ^{nat}Se-species and ^{77}Se-selenite (or ^{76}Se-methionine) in each fraction were calculated. The obtained results for total ^{nat}Se-species and ^{77}Se (or ^{76}Se-methionine) were in good agreement with the certified and spiked values, respectively [27].

After validation, this Se quantitative speciation methodology was applied to the determination of ^{nat}selencompounds and ^{77}Se-species in both the non-supplemented and supplemented group of rats. A peak eluting at the dead time of the column (2.5 min) for ^{nat}Se was detected in both groups of animals (see Fig. 7), although its intensity in the non-supplemented group turned out to be lower than that in the supplemented group. This may suggest that Se turnover is slow when dietary Se is low but it increases as dietary Se increases. It is interesting, though, that levels of this compound in the urine of lactating rats were higher than values reported previously by other authors [29] in adult rats, a fact which could be related with a differential bioavailability and minerals utilization by newborns as compared to adults

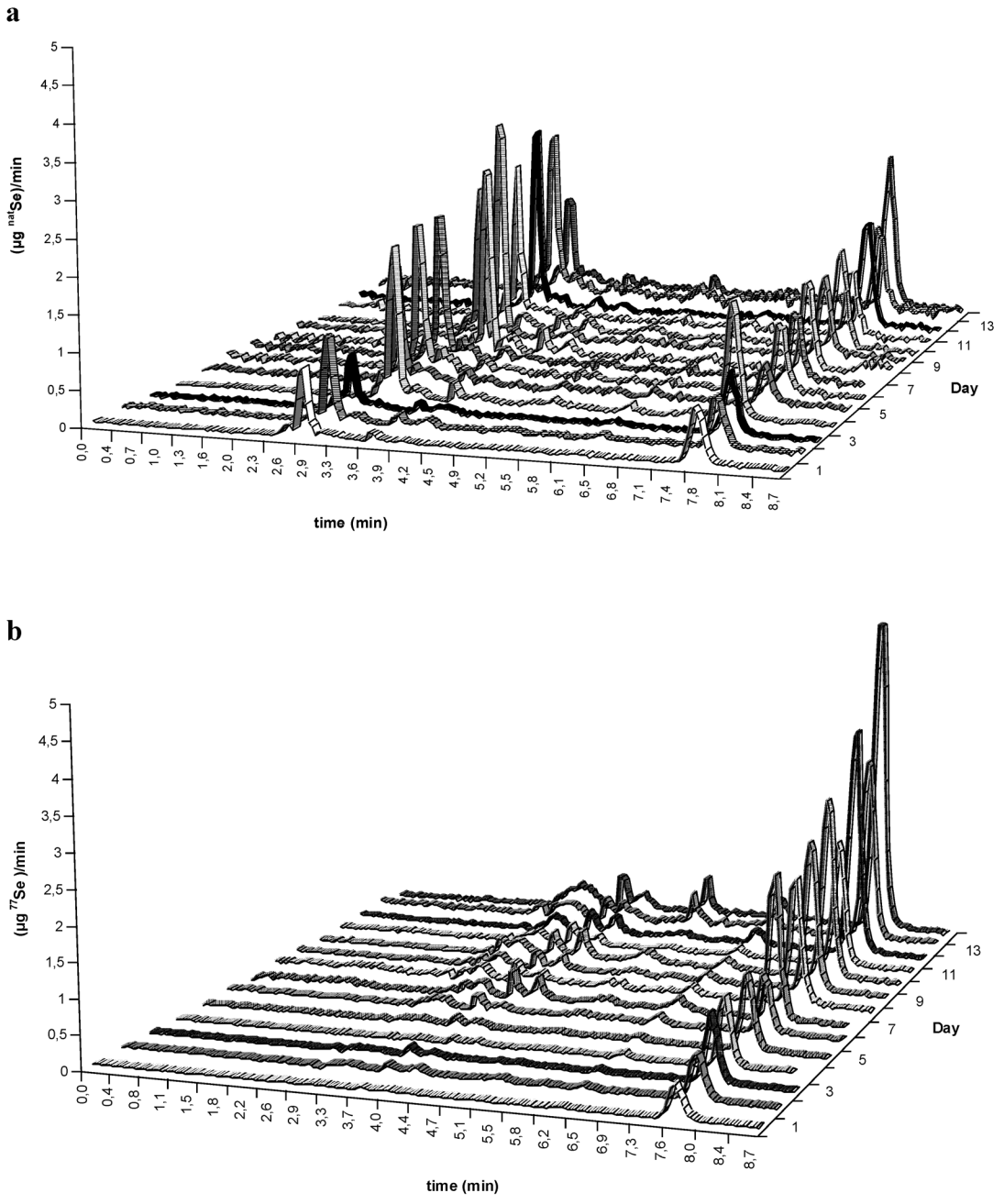

Fig. 7 (a) ^{nat}Se distribution in urine [µg ^{nat}Se/min vs. time (min)] for the supplemented group during the 14 days lactating period. (b) ^{77}Se distribution in urine [µg ^{77}Se/min vs. time (min)] for the supplemented group during the lactating period.

[30]. Figure 7 shows that ^{nat}Se in the supplemented animals is also excreted as a selenosugar [27] (t = 7.8 min).

Figure 7b shows the ^{77}Se mass flow chromatograms obtained for the same supplemented group: during the lactating period the main ^{77}Se peak occurred at 7.8 min as well. ESI-Q-TOF measurements

of this compound demonstrated that it is 1β-methylseleno-*N*-acetyl-D-galactosamine (a selenosugar), the main Se metabolite in human urine [27]. The daily amount of selenosugar excreted increased during the lactating period (the "inorganic Se", eluting at 2.5 min, was almost negligible for the first days). It was observed that after 6 days of supplementation, the amount of ^{77}selenosugar became higher than that of ^{nat}selenosugar (see Fig. 7a). As in blood serum, after 14 days of supplementation, the percentage of ^{77}Se in the selenosugar turned out to be around 60 %.

Cumulative urine excretion figures for the 14 days of the experiment, as obtained for every selenospecies containing natural and exogenous Se, are shown in Fig. 8. As can be seen, cumulative ^{nat}Se-sugar and ^{77}Se-sugar excretions increased with similar slope. This known selenosugar seems to be the main Se metabolite in urine in the supplemented group (interestingly, it was not detected in the non-supplemented group). Inorganic ^{77}Se excretion was very low during the supplementation period (or even not detected the first days) as Fig. 8 shows. This can indicate again the low turnover of Se (coming from some body tissues as brain, muscle, etc.).

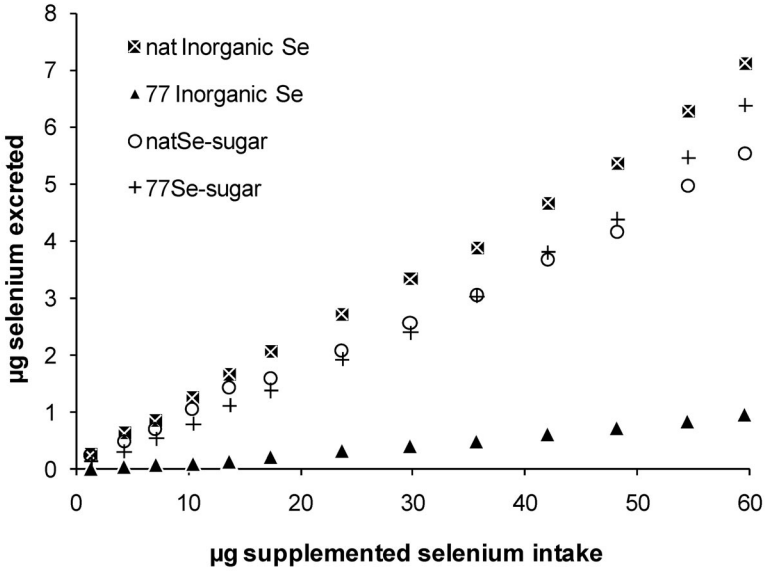

Fig. 8 Cumulative total inorganic Se and selenospecies amounts containing endogenous and exogenous Se, excreted in urine in the studied supplemented group.

From the above results, it appears that Se retention in the organism is mainly regulated by urinary excretion in such a way that at low dietary intakes Se seems to be used (not excreted), while at higher intakes the Se excess seems to be rapidly excreted, mainly as selenosugar in the urine. Thus, selenosugar synthesis and excretion are directly related to Se intake. Therefore, we believe that this selenosugar determination should be a better biomarker than "total" Se values to assess body Se status.

CONCLUSIONS

Chemical speciation is today mandatory to study scientifically the nutritional value of formulas and supplements used for baby nutrition. "Integrated" MS techniques (atomic and molecular ion sources) for state-of-the-art speciation of such compounds should be used to detect, identify, characterize, and finally quantify the desired species.

Moreover, ICP-MS ability to measure isotopes in combination with the IPD concept, offers a new non-radioactive analytical tool for quantitative nutritional and metabolism studies investigating total natural (endogenous) and exogenous (supplements) levels of the sought element.

Also, the use of stable isotopes in connection with IPD has proved to be a novel and powerful strategy to study quantitatively absorption and/or bioavailability of the particular species used in formulas by the manufacturers and to shed new light on Se metabolism. Of course, the accurate quantitation of endogenous and exogenous (supplemented) Se-species with time has proved also to be most useful to study kinetics of metabolism and may be exportable to other essential and toxic elements having several stable isotopes.

In brief, many fruitful applications of IPD in the future can be envisaged in quantitative kinetic studies of uptake and incorporation of (semi)metals in biomolecules (proteins) and through investigation of their elemental species metabolism in the body in a quantitative manner.

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