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# Alternative approach to post column online isotope dilution ICP-MS

Claudia Swart*, Olaf Rienitz, Detlef Schiel

*Physikalisch-Technische Bundesanstalt, Metrologie in der Chemie, Bundesallee 100, 38116 Braunschweig, Niedersachsen, Germany*

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ABSTRACT

An alternative post column online double isotope dilution inductively coupled plasma mass spectrometry (ID-ICP-MS) method was developed. The resulting equation allows a straightforward calculation of the mass concentration of the analyte in the sample from the measured isotope ratio chromatogram. The use of a balance to determine and monitor the mass flow of the spike and a solution of the species under investigation as the reference are the two core components of this new method. Changes in the viscosity of the system eluent–analyte–spike will not affect the results due to the direct determination of the mass flow rate. The use of the species under investigation as the reference makes the method independent of the injected volume. To simplify matters, the integration of the isotope ratio chromatogram was done with Excel using Simpson's rule instead of sophisticated programs for transformation and integration. The advantages of the new approach were demonstrated with the help of the determination of selenomethionine in the selenized yeast reference material SELM-1 with liquid chromatography coupled to ICP-MS (HPLC ID-ICP-MS) applying the new online double IDMS method.



## 1. Introduction

Isotope dilution mass spectrometry (IDMS) is a well established method for the accurate determination of the content of certain elements in a sample [1,2]. It is based on the measurement of isotope ratios in a sample after its isotopic composition has been altered by the addition of a known amount of an isotopically enriched spike. One prerequisite for the successful application of isotope dilution analysis is that the element under investigation has at least two stable or long-lived isotopes which are free of interferences in mass spectrometry.

IDMS has several advantages compared to the determination of an element via external calibration [3]. Ideally, the spike is added prior to any sample treatment and therefore sample losses will not alter the isotope ratio and thus the analysis result will not be affected, given that spike and sample are properly equilibrated. No matrix effects occur in mass spectrometry as analyte and spike are in the same matrix.

For total elemental analysis the method is well documented and isotopically enriched materials are available [4]. Isotope dilution analysis in elemental speciation is a quite different matter. Two approaches are documented in the literature. The first is the species specific IDMS analysis [5]. Here the elemental species under investigation is synthesized [6,7] using the isotopically enriched element, e.g. selenomethionine enriched in $^{76}$Se or $^{77}$Se for the determination of selenomethionine (Se-Met) in selenium enriched yeast [8,9] or in human serum [10]. The application of this kind of IDMS is limited as isotopically enriched elemental species are hardly commercially available.

The second approach is the species unspecific spiking method first published by Rottmann and Heumann [11,12]. In case the structure of the species is not known or the isotopically enriched species is not available the isotopically enriched element is added independent of its chemical form after the separation. This approach, however, lacks some of the advantages of species specific IDMS. As the spike is added after sample preparation and separation took place, it is essential to make sure that no loss or conversion of species occurs before then. Furthermore, it must be ensured that the species under investigation and the spike result in the same elemental response of the detector. With ICP-MS this is usually the case except for special nebulizers or complex matrices [13]. The spike is added to the effluent of the chromatographic separation via T-piece. The flow rate of the spike is determined by inverse isotope dilution introducing a standard solution of known isotopic composition via an injection loop while the spike is pumped continuously through the system. The flow rate is then determined by measuring the isotope ratio. This approach assumes that the flow rate of the spike is constant throughout the whole experiment even when gradients are used that may result in different viscosities and thus back pressures of the eluent in chromatographic separation.

In this paper we present an alternative approach that allows determining the mass flow directly and continuously without the use of an additional standard solution and without the subsequent

* Corresponding author. Tel.: +49 531 592 3317; fax: +49 531 592 3015.
*E-mail address:* Claudia.Swart@ptb.de (C. Swart).

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conversion of volume flow rates to mass flow rates. Selenium speciation in yeast serves as an example.

Selenium (Se) is an essential element for humans found in enzymes like glutathione peroxidase which play an important role in cancer prevention, while others like iodothyronine-5′-deiodonase are necessary in hormone balance [14]. However, the range between deficiency (<30 μg daily intake for adults) and toxicity (>700 μg daily intake for adults) is rather narrow. The limit to toxicity is still discussed in the literature [15]. An intake of 30–70 μg/d is recommended by D-A-CH for Germany, Austria and Switzerland [16]. Moreover, not only the dose but also the chemical form of the element is crucial for the uptake and physiological effectiveness of Se in the body. Therefore, precise and traceable elemental speciation analysis is necessary to ensure a sufficient, non-toxic Se-provision of healthy people as well as cancer patients, where Se is used as a complementary therapy [17].

An important goal of the project T2J10 of the European Metrology Research Programme (EMRP) is the development of a *primary method of measurement* [18] for the determination of Se species in biological samples ensuring the metrological traceability of the results in Se speciation analysis. IDMS is one important approach to achieve this goal [19].

**Table 1**
Instrumental operating conditions.

| | |
|---|---|
| ICP-MS | |
| RF power | 1500 W |
| Sample depth | 8.1 mm |
| Torch-H | 0.4 mm |
| Torch-V | 0.1 mm |
| Plasma gas flow | 15 L/min |
| Carrier gas flow | 0.81 L/min |
| Makeup gas flow | 0.1 L/min |
| Spraying chamber temp. | 2 °C |
| Isotopes monitored | 76, 77, 78, 82 |
| Reaction gas | 2.3 mL/min $H_2$ + 2 mL/min He |
| Cell entrance | −40 V |
| Cell exit | −50 V |
| Quadrupole focus | −11 V |
| Quadrupole bias | −18 V |
| Octopole bias | −16 V |
| HPLC | |
| Autosampler temp. | 20 °C |
| Injection volume | 10 μL |
| Flow rate | 0.5 mL/min |
| Column | Zorbax SB-$C_8$ |
| Eluent | 0.1% TFA in 2% MeOH |
| Column temp. | 35 °C |

## 2. Materials and methods

### 2.1. Experimental

#### 2.1.1. Chemicals

The purity of seleno-DL-methionine (Sigma–Aldrich, St. Louis, USA) was determined to be $w = (99.6 \pm 0.3)\%$ by quantitative high resolution NMR at the Federal Institute of Materials Research and Testing (BAM), Germany, using Na-3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionate (TSP) as internal calibration standard and methanol-$d_4$ as solvent. The quantification was performed with a BRUKER DMX 400 at 400.14 MHz, with a sweep width of 3205.1 Hz, an acquisition time of 10.2 s, a relaxation delay of 30 s, 30° pulse and 80 scans [20]. Protease XIV from streptomyces griseus (5.9 U/mg = $9.83 \times 10^{-5}$ kat/g), lipase from candida rugosa type VII (1449 U/mg = $2.415 \times 10^{-2}$ kat/g) and driselase from basidiomycetes sp. (≥10%) (all Sigma–Aldrich) were used for the digestion of the yeast reference material SELM-1 (NRC-CNRC, Ottawa, Canada). The buffer used for the enzymatic digestion was degassed 30 mmol/L tris(hydroxymethyl)-aminomethane hydrochloride (Tris–HCl) buffer (ultra, ≥99.0%, Sigma–Aldrich). Methanol (HPLC gradient grade, Baker, Phillipsburg, USA) and trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) were used for the chromatographic separation. For the preparation of the spike solution $^{76}$Se enriched Se metal (Se-76, lot number 34-01-36-3383, Chemotrade, Leipzig, Germany) was dissolved in subboiling distilled 65% nitric acid (p.a., Merck) and diluted appropriately.

#### 2.1.2. Instrumentation

The enzymatic digestion was performed with an orbital shaker (KS 260 basic, IKA, Staufen, Germany) and an incubation hood (Unihood 650, UniEquip, Martinsried, Germany). Prior to the separation the solutions were centrifuged (SIGMA 3-16PK, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), filtered through a 0.45 μm teflon filter (DigiFILTER) using the DigiFILTER manifold (both SCP Science, Baie D'Urfé, Canada). The chromatographic separation was accomplished on an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a degasser, a binary pump, a thermostated autosampler and a thermostated column compartment. The isocratic separation was performed on an Agilent Zorbax SB-C8 (5 μm × 3 mm i.d. × 250 mm) column using 0.1% TFA in 2% methanol as eluent. Se isotopes were detected with a quadrupole ICP-MS (Agilent 7500cx) equipped with Scott type spray chamber, microconcentric nebulizer, a shielded torch with an injector with 1 mm i.d., a 7500cs lens stack and an octopole collision/reaction cell. The optimal cell conditions were found to be a mixture of 2.3 mL/min $H_2$ and 2 mL/min He. Argon was used as plasma, nebulizer and make-up gas. All gases were of 5.0 quality (99.9990%, Air Liquide, Paris, France). The other instrumental conditions are summarized in Table 1. The parameters for the lense system were daily optimized for sensitivity. The mass flow of the isotopically enriched spike was monitored using the balance Genius ME215S (Sartorius, Göttingen, Germany). With a frequency of 0.1 Hz the balance sent the mass reading to the data acquisition software SartoConnect provided by the manufacturer of the balance. The acquisition both of the ICP-MS data and the balance readings was performed using the same computer to ensure a consistent time basis. From the mass readings and the time the mass flow was calculated.

The HPLC was coupled to the ICP-MS via a PEEK capillary (i.d. 0.13 mm).

#### 2.1.3. Enzymatic digestion

The digestion method which was used by LGC [9] in the international intercomparison CCQM-P86 was also applied here. To 300 mg of the yeast sample 60 mg protease, 30 mg lipase and 10 mL of 30 mmol/L Tris–HCl were added and the mixture was then incubated at 37 °C for 18 h. Afterwards the solution was decanted and the procedure was repeated with the residue using the same amount of protease and lipase and an additional 100 mg driselase in the solution. The resulting solution was centrifuged, the solutions of both steps were combined, filtered and transferred to an HPLC vial.

#### 2.1.4. Double ID-ICP-MS method

For the determination of Se-methionine in yeast samples using the double IDMS method a solution of Se-methionine with a mass concentration comparable to the one expected in the sample was prepared as reference solution. To ensure that both sample and reference have a comparable matrix the reference was treated in the same way as the yeast sample. Sample and reference were then injected onto the HPLC column alternately and separated using the same chromatographic conditions.

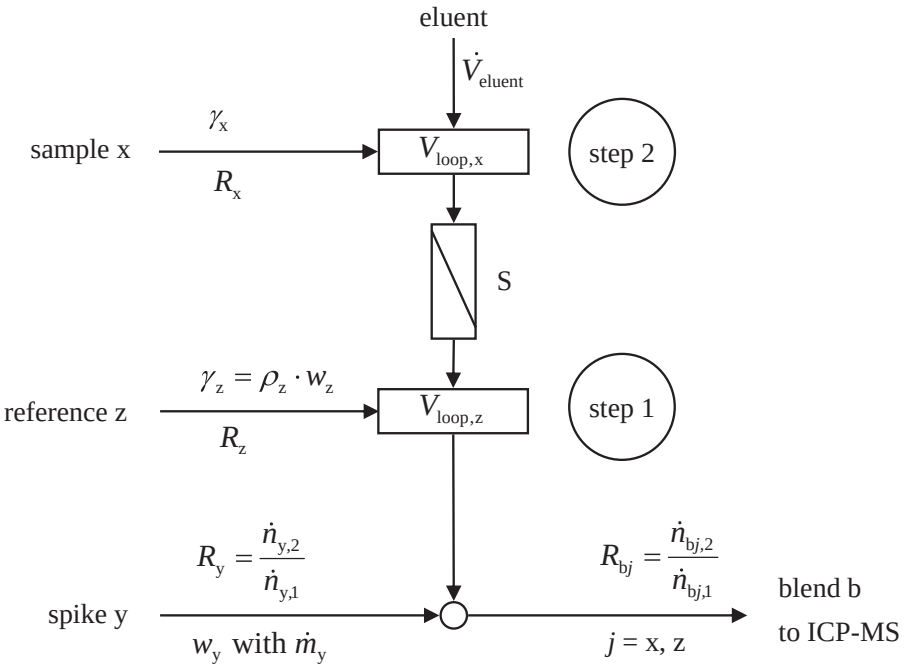


**Fig. 1.** Schematic diagram of the set-up used by Rottman and Heumann for the post column ID-ICP-MS [11].

### 2.2. Conventional post column ID-ICP-MS method

In post column online ID-ICP-MS the isotopically enriched spike is added continuously with a peristaltic pump via a T-piece after the separation S of the species (Fig. 1). In the spike solution the element may exist in any chemical form as the high temperature in the argon plasma of the ICP is able to atomize and ionize the compounds virtually independent of the form of the element. For the quantitative determination of the different species it is necessary to plot the mass flow $\dot{m}(\mathrm{E}) = \dot{m}_x \cdot w_x$ of the element E under investigation versus time $t$ throughout the whole chromatographic run. This mass flow is equal to the mass flow of the sample solution $\dot{m}_x$ times the mass fraction of the element in the sample solution $w_x$. In the literature, the flow rate of the spike was usually transformed from the isotope ratio chromatogram in a first step to the volume flow rate $\dot{V}_y$. In order to gain a mass flow rate, the flow rate of the spike solution has to be calibrated by reverse isotope dilution [21]. Therefore, a standard solution usually containing the element under investigation in its natural isotopic composition is injected in step 1 via an additional sample loop and a T-piece. The volume of this loop $V_{loop,z}$ must be large enough to reach a steady state, which means that the calibration standard flows through the system without mixing with the eluent at the same flow rate $\dot{V}_{eluent}$ as the eluent so that only calibrant is blended with the spike. The instrumental set-up first used by Rottmann and Heumann [11] is shown in Fig. 1. All variables are compiled in Table A1 (Appendix A).

Afterwards, different equations were used by different authors to calculate the mass flow chromatogram from the obtained chromatogram of the isotope ratio $R_{bx}$ [11,22]. The intensities for all measured isotopes have to be dead time corrected if this is not done automatically by the software of the instrument. If isobaric interferences occur on the measured masses and no interference free isotopes are available for the certain element, mathematical corrections are required (for example $^{79}BrH^+$ on $^{80}Se$). All isotope ratios need to be corrected for mass bias. The isotope ratio chromatogram is then mathematically converted into a mass flow chromatogram. Dependent if the blank is taken into account or not, different equations can be found in the literature. The derivation of the different equations always starts with a consideration of the total number of atoms of the different isotopes. Taking into account the density $\rho_y$ and the flow rate $\dot{V}_y$ of the spike y as well as the isotopic abundances in sample $x_{x,1}$ and spike $x_{y,2}$ solutions yields Eq. (1) given by Rodríguez-Gonzáles et al. [22] (quantities renamed to follow the IUPAC recommendations, definition of $R$ according to Eq. (3)) [23]:

$$\dot{m}_x w_x = w_y \cdot \rho_y \cdot \dot{V}_y \cdot \frac{M_x}{M_y} \cdot \frac{x_{y,2}}{x_{x,1}} \cdot \left( \frac{(1/R_{bx}) - (1/R_y)}{1 - (1/R_{bx})R_x} \right) \quad (1)$$

After calibration of $\dot{V}_y$ using an inverse isotope dilution step and integration and some rearrangements results the mass concentration of Se in the sample $\gamma_x$ as:

$$\gamma_x = \frac{\gamma_z \cdot \dot{V}_{eluent}}{V_{loop,x}} \cdot \frac{R_{bz} \cdot x_{z,1} - x_{z,2}}{x_{y,2} - R_{bz} \cdot x_{y,1}} \cdot \int_{t_1}^{t_2} \left( \frac{x_{y,2} - R_{bx} \cdot x_{y,1}}{R_{bx} \cdot x_{x,1} - x_{x,2}} \right) dt \quad (2)$$

### 2.3. Alternative approach

In our method the mass flow rate of the spike introduced after the separation was determined directly using a balance (Fig. 2). The mass flow of the spike solution was recorded continuously over the whole time of the chromatogram using the computer program SartoConnect provided by the manufacturer of the balance. As will be discussed in the following, this was done merely to ensure the stability of the mass flow of the spike. The value of this flow rate cancels from the final Eq. (18) used for the evaluation. All variables are compilled in Table A1 (Appendix A).

After separation and addition of the spike the resulting isotope ratio of the blend $R_{bx}$ is given by:

$$R_{bx} = \frac{\dot{n}_{bx,2}}{\dot{n}_{bx,1}} = \frac{\dot{n}_{x,2} + \dot{n}_{y,2}}{\dot{n}_{x,1} + \dot{n}_{y,1}} \quad (3)$$

$\dot{n}_{bx,1}$, $\dot{n}_{bx,2}$ are the amount-of-substance flow rates of isotope 1 and 2, respectively, in the blend, while $\dot{n}_{x,i}$ ($i$ = 1, 2) and $\dot{n}_{y,i}$ are the amount-of-substance flow rates of the isotopes in the sample x and the spike y, respectively. Isotope 1 represents the isotope with the highest abundance in the sample (provided it is not interfered

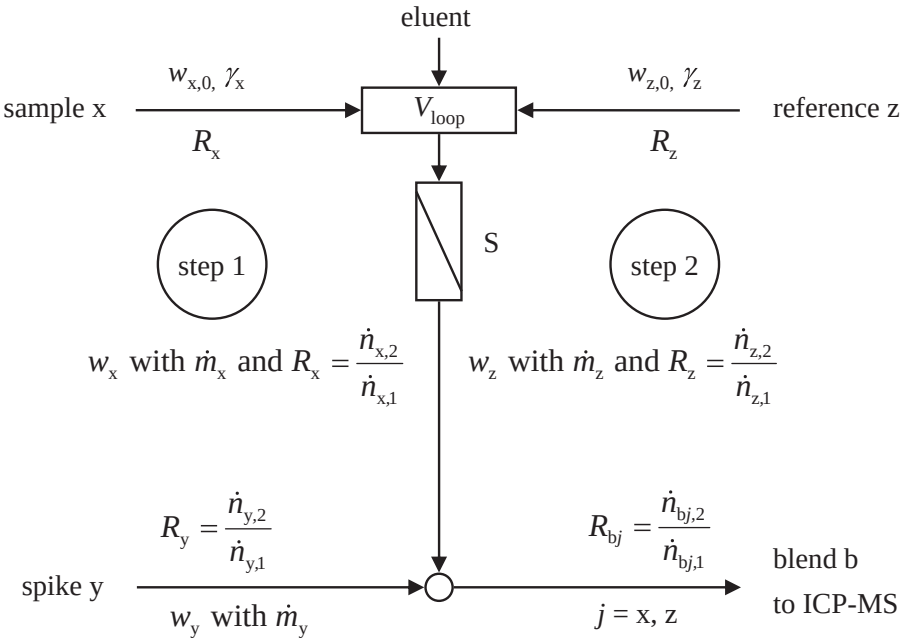


**Fig. 2.** Schematic diagram of the set-up for the alternative post column double ID-ICP-MS.

and stable), in the following called *reference isotope*, while isotope 2 is the isotope enriched in the spike, in the following called *spike isotope*. All isotope ratios $R$ are defined analogue to Eq. (3) with the reference isotope in the denominator and the spike isotope in the numerator.

The amount-of-substance flow rate can be replaced by the mass flow rate using the amount-of-substance fraction $x$ of the isotope, the molar mass $M$ and the mass fraction $w$ of the element in sample or spike.

$$\dot{n}_{x,1} = x_{x,1} \cdot \dot{n}_x = x_{x,1} \frac{\dot{m}_x w_x}{M_x} \tag{4}$$

Replacing all amounts of substances in accordance with Eq. (4) the isotope ratio is given as

$$R_{bx} = \frac{x_{x,2}(\dot{m}_x w_x/M_x) + x_{y,2}(\dot{m}_y w_y/M_y)}{x_{x,1}(\dot{m}_x w_x/M_x) + x_{y,1}(\dot{m}_y w_y/M_y)} \tag{5}$$

The amount-of-substance fractions can be substituted by the isotope ratios

$$x_i \equiv \frac{n_i}{\sum n_i} = \frac{R_i}{\sum R_i} \tag{6}$$

$$R_{bx} = \frac{(R_x/\sum_i R_{x,i})(\dot{m}_x w_x/M_x) + (R_y/\sum_i R_{y,i})(\dot{m}_y w_y/M_y)}{(1/\sum_i R_{x,i})(\dot{m}_x w_x/M_x) + (1/\sum_i R_{y,i})(\dot{m}_y w_y/M_y)} \tag{7}$$

After rearranging Eq. (7) the mass flow rate of the element in the sample can be calculated:

$$\dot{m}_x w_x = \dot{m}_y w_y \cdot \frac{M_x}{M_y} \cdot \frac{(R_y - R_{bx})}{(R_{bx} - R_x)} \cdot \frac{\sum_i R_{x,i}}{\sum_i R_{y,i}} \tag{8}$$

In this equation only $R_{bx}$ and $\dot{m}_y$ are functions of time. $R_{bx}$ is determined with ICP-MS while $\dot{m}_y$ is monitored using a balance.

To avoid the necessity to determine all isotope ratios in the spike as well as its exact concentration a double IDMS method was developed. In this case, the mass flow of the analyte in the reference solution can be calculated analogue to Eq. (8):

$$\dot{m}_z w_z = \dot{m}_y w_y \cdot \frac{M_z}{M_y} \cdot \frac{(R_y - R_{bz})}{(R_{bz} - R_z)} \cdot \frac{\sum_i R_{z,i}}{\sum_i R_{y,i}} \tag{9}$$

As long as the flow rate of the spike is held constant, only the isotope ratios $R_{bx}$ and $R_{bz}$, respectively, are time-dependent on the right side of Eqs. (8) and (9). Therefore, a new constant $k_{y,j}$ can be defined and all the isotope ratios can be combined in new variables $\hat{R}_{bj}$ ($j$ = x, z):

$$k_{y,x} \equiv \dot{m}_y w_y \cdot \frac{M_x}{M_y} \cdot \frac{\sum_i R_{x,i}}{\sum_i R_{y,i}} \tag{10}$$

$$k_{y,z} \equiv \dot{m}_y w_y \cdot \frac{M_z}{M_y} \cdot \frac{\sum_i R_{z,i}}{\sum_i R_{y,i}} \tag{11}$$

$$\hat{R}_{bx} \equiv \frac{(R_y - R_{bx})}{(R_{bx} - R_x)} \tag{12}$$

$$\hat{R}_{bz} \equiv \frac{(R_y - R_{bz})}{(R_{bz} - R_z)} \tag{13}$$

Assuming that all isotope ratios, and therefore the molar masses, in sample and reference are identical: $k_{y,x} = k_{y,z} = k_y$.

To obtain the mass of the element $m$(E) in sample and reference, respectively, the peaks in the mass flow chromatogram of the analytes in sample and reference have to be integrated.

$$m_x(\mathrm{E}) = \int_{t_1}^{t_2} (\dot{m}_x w_x) dt = w_{x,0} \cdot \rho_x \cdot V_{loop} = \gamma_x \cdot V_{loop}$$

$$= k_y \cdot \int_{t_1}^{t_2} \hat{R}_{bx} dt \tag{14}$$

$$m_z(\mathrm{E}) = \int_{t_3}^{t_4} (\dot{m}_z w_z) dt = w_{z,0} \cdot \rho_z \cdot V_{loop} = \gamma_z \cdot V_{loop}$$

$$= k_y \cdot \int_{t_3}^{t_4} \hat{R}_{bz} dt \tag{15}$$

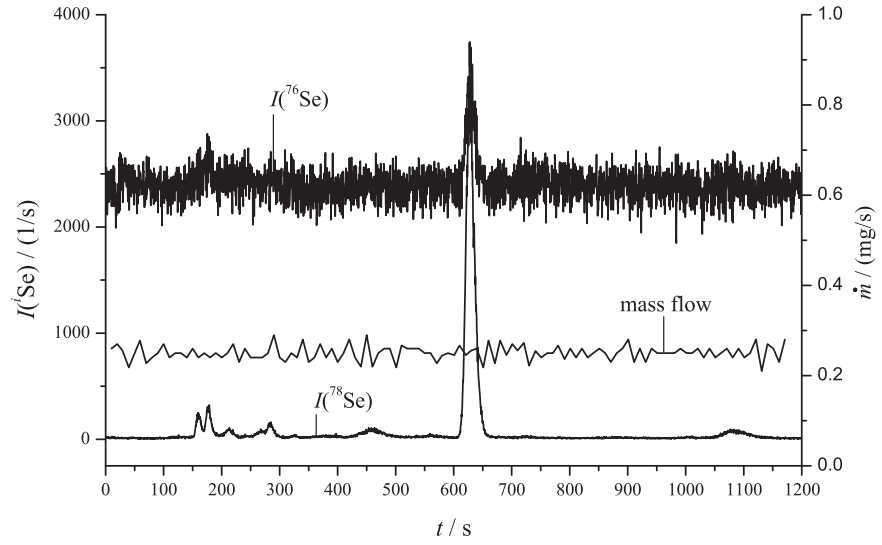


**Fig. 3.** Chromatogram of a yeast sample extract: $^{76}$Se serves as the spike and $^{78}$Se as the reference isotope. Additionally, the mass flow of the spike solution is shown.

Now the mass concentration of the species in the sample $\gamma_x$ can be calculated. From Eq. (15) results

$$k_y = \frac{\gamma_z \cdot V_{loop}}{\int_{t_3}^{t_4} \hat{R}_{bz} dt} \tag{16}$$

Introducing Eq. (16) into Eq. (14)

$$\gamma_x \cdot V_{loop} = \frac{\gamma_z \cdot V_{loop}}{\int_{t_3}^{t_4} \hat{R}_{bz} dt} \cdot \int_{t_1}^{t_2} \hat{R}_{bx} dt \tag{17}$$

As can be seen in Eq. (17) the volume of the sample loop $V_{loop}$ of the injector of the HPLC can be canceled as long as the volume is identical for both sample and reference. The resulting final Eq. (18) merely contains quantities that can be measured directly.

$$\gamma_x = \gamma_z \cdot \frac{\int_{t_1}^{t_2} \hat{R}_{bx} dt}{\int_{t_3}^{t_4} \hat{R}_{bz} dt} \tag{18}$$

The mass concentration of the reference $\gamma_z$ is well-known as the reference solution was prepared gravimetrically [24]. $\hat{R}_{bx}$ and $\hat{R}_{bz}$ can be calculated as $R_x$, $R_y$ and $R_z$ are known and $R_{bx}$ and $R_{bz}$ were measured with ICP-MS. In our case, the IUPAC values [25] were used for $R_x$ and $R_z$ as selenium shows only small natural variations in its isotopic composition. The isotope ratio $R_y$ in the spike was taken from the certificate of the isotopically enriched material.

The use of the temperature-independent mass fraction $w$ instead of mass concentration is preferable: taking the density $\rho_j$ into account the mass concentration $\gamma_j$ can easily be transformed into mass fraction $w_j$:

$$\rho_x \cdot w_x = \rho_z \cdot w_z \cdot \frac{\int_{t_1}^{t_2} \hat{R}_{bx} dt}{\int_{t_3}^{t_4} \hat{R}_{bz} dt} \tag{19}$$

### 2.3.1. Integration

The integration necessary to fulfill Eqs. (18) and (19), respectively, was done using Simpson's rule [26]

$$A = \int_a^b f(x) \approx \sum_{i=1}^{n} A_i = \frac{h}{3} \cdot (S_0 + 4 \cdot S_1 + 2 \cdot S_2) \tag{20}$$

with

$$h = \frac{b-a}{2n} \tag{21}$$

$$S_0 = y_0 + y_{2n} \tag{22}$$

$$S_1 = y_1 + y_3 + y_5 + \ldots + y_{2n-1} \tag{23}$$

$$S_2 = y_2 + y_4 + y_6 + \ldots + y_{2n-2} \tag{24}$$

To integrate the chromatogram, each peak was regarded as an interval $[a,b]$ (with $a = t_1$ and $b = t_2$ and $a = t_3$ and $b = t_4$, respectively), which was divided into an even number of $2n$ equal subintervals $h$ as required by Eqs. (21)–(24). The number of subintervals of around 100 was dictated by the data acquisition rate and the peak width. The different sums $S_0$–$S_2$ were then calculated using directly the "isotope ratios" $\hat{R}_{bx}$ and $\hat{R}_{bz}$ from the chromatogram as $y_0$ to $y_{2n}$. As only the values from the chromatogram were used to calculate the results straightforward without further complicated (e.g. numerical) manipulations, this kind of integration can easily be done with Excel (Microsoft, Redmond, USA) without any programming.

## 3. Results

### 3.1. Determination of Se-Met in yeast as an example

To demonstrate the potential of this method the selenized yeast reference material SELM-1 was analyzed. The resulting separation is shown in Fig. 3. Then $\hat{R}_{bj}$ was calculated and the according chromatograms $\hat{R}_{bj}$ versus $t$ for the Se-Met peak of samples and reference were plotted (Fig. 4).

The precondition of exact matching was clearly fulfilled as the concentration of Se-Met in reference and sample were equal within a range of 1.5%, meaning that the impact of systematic influences such as blanks and mass discrimination effects changed the results less than 0.1%, which was well within the claimed uncertainty [27]. The peaks were fitted with Origin 8 (OriginLab Corporation, Northhampton, USA) using a modified Gauss function (Eq. (25)):

$$f(x) = \frac{A}{t_0} \cdot e^{(1/2)\cdot(w/t_0)^2 - (x - x_c/t_0)} \cdot \int_{-\infty}^{z} \frac{1}{\sqrt{2\pi}} \cdot e^{-(y^2/2)} dy \tag{25}$$

where $z = (x - x_c/w) - (w/t_0)$, $x_c$ is $x$ (in this case the time) at the peak maximum, $w$ is related to the peak width, $A$ is a fit parame-

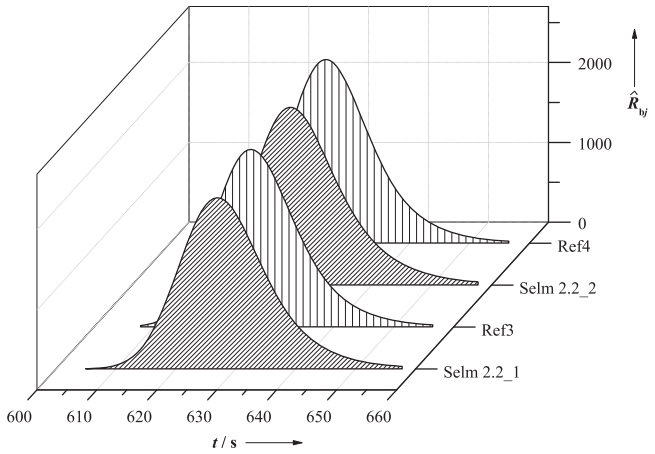


**Fig. 4.** Se-Met peak of repeated measurements of extracts of the yeast reference material SELM-1 and a reference solution.

ter related to the peak height and defines the asymmetry of the peak. The fit parameters were determined with the chi-square-minimization method using the Levenberg–Marquardt algorithm.

For an example of the fitting a Se-Met peak for a sample is shown in Fig. 5. The integration of $\hat{R}_{bx}$ and $\hat{R}_{bz}$ was done both with Origin after fitting of the peaks and with Excel directly from the raw data. The results showed a difference of less than 0.005%. Within the limits of uncertainty (3.4%) this difference is negligible. Therefore, the proposed Excel method for integration was independently verified.

The result for SELM-1 of $w = (3.25 \pm 0.11) \times 10^3$ mg/kg was within the window defined by the revised certificate of $w = (3.389 \pm 0.173) \times 10^3$ mg/kg and in good agreement with values published in [9].

### 3.2. Uncertainty estimation

For the calculation of the expanded uncertainty $U$ according to the *Guide to the Expression of Uncertainty in Measurement* (GUM) [28] with a coverage factor of $k = 2$ different aspects have to be taken into account.

The major part of the uncertainty is caused by the uncertainty in the integration of $\hat{R}_{bx}$ and $\hat{R}_{bz}$, respectively (Table 2). The factors $K$ used to correct the measured isotope ratios for mass discrimination [29] vary within ±2%. As the uncertainty of these so-called $K$-factors influences the uncertainty of $\hat{R}_{bx}$ and $\hat{R}_{bz}$, these quantities should at least vary accordingly. In fact, an overall uncertainty associated with $\hat{R}_{bx}$ and $\hat{R}_{bz}$ of 2.7% was determined experimentally,

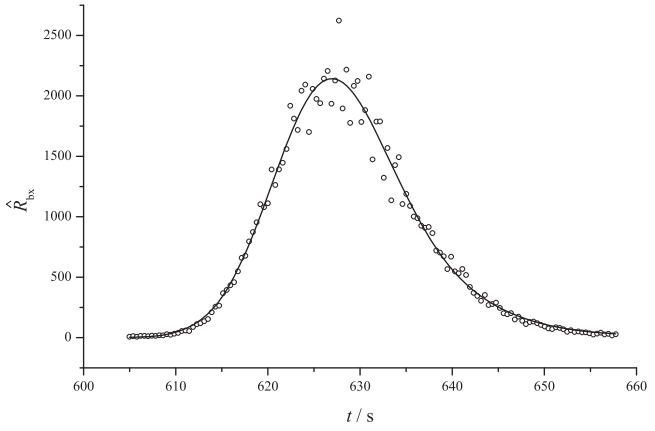


**Fig. 5.** Fitting of the selenomethionine peak in a yeast sample with Origin using a modified Gauss function.

**Table 2**
Major contributions to the combined uncertainty $U$.

| Variable | Uncertainty contribution |
|---|---|
| $f_{exp}$ | 2.9% |
| $\int \hat{R}_{bx}$ | 36.2% |
| $\int \hat{R}_{bz}$ | 36.2% |
| $w_{z,stock}$ | 15.2% |
| $m_i$ | 9.5% |

reflecting the other input quantities contributing to the uncertainty of $\hat{R}_{bx}$ and $\hat{R}_{bz}$ (sample preparation, injection and separation procedure as well as the detection itself). As the $K$-factors used for the calculation of $\hat{R}_{bx}$ and $\hat{R}_{bz}$ are the same and the alternative approach presented here features several advantages like the same sample preparation, the use of the same sample loop and column for the separation for both sample and reference, this procedure comes close to an exact matching method (well known from total element determination with IDMS), meaning that $\hat{R}_{bx}$ and $\hat{R}_{bz}$ should be correlated strongly resulting in a decreased combined uncertainty of the final result. A correlation coefficient of $r(\hat{R}_{bx}, \hat{R}_{bz}) = 89\%$ was calculated from the experimental data. Due to the limited number of measurements a correlation of 75% was estimated conservatively. Additional uncertainty arises from preparation of the reference stock solution $w_{z,stock}$ (including the purity of the reference material used), the sample preparation $f_{exp}$ (enzymatic digestion, filtering, etc.) and the dilution of sample and reference $f_{dil}$. Also the uncertainty of the balance used for weighing of sample and reference and for further dilution of sample and reference contributes to the overall uncertainty ($m_3$–$m_{10}$). In Table 2 all contributions of the different weighings were summarized to the uncertainty of the "mass" $m_i$.

To achieve the model equation used for the calculation of the uncertainty with the GUM workbench (version 1.2, Metrodata GmbH, Grenzach-Wyhlen, Germany), Eq. (19) was adapted.

$$w_x = f_{exp} \cdot f_{dil} \cdot w_{z,stock} \cdot \frac{\int \hat{R}_{bx} dt}{\int \hat{R}_{bz} dt} \tag{26}$$

with $f_{dil} = (m_6/m_5) \cdot (m_4/m_3) \cdot (m_7/m_8) \cdot (m_9/m_{10})$

## 4. Discussion

To see the advantages of our approach compared to the conventional method, it is necessary to further transform Eq. (2) as it still contains some quantities that cannot be determined by measurements:

$$\gamma_x = \frac{\gamma_z \cdot \dot{V}_{eluent}}{V_{loop,x}} \cdot \frac{R_{bz} - R_y}{R_y - R_{bz}} \cdot \int_{t_1}^{t_2} \left( \frac{R_y - R_{bx}}{R_{bx} - R_y} \right) dt \tag{27}$$

Even Eq. (27) still contains quantities that cannot be measured directly without any detours like the determination of the flow rate via an additional calibration of the system. In contrast, our approach allows the easy and direct calculation of the required mass concentration of the analyte in the sample without any further transformation of the results.

The advantage of using the mass flow rate instead of the volume flow rate $\dot{V}_{eluent}$ taken from the HPLC software is that a mass flow rate can be measured more accurately than a volume flow rate as the latter depends on temperature and density (and their changes during the experiment). Using mass flow rates, those variations do not influence the result. If a gradient (especially in the organic fraction of the eluent) is used for the separation of the species, the density and through it the viscosity of the system eluent–analyte–spike can vary, thus influencing the flow rate of

the eluent and the spike solution. Even if temperature and density are carefully controlled, fluctuations in the pump rate of the peristaltic pump can still occur. In case the system is only calibrated once at the beginning of an experiment, such changes cannot be corrected for. In our set-up the balance can be used to control the flow rate of the peristaltic pump and to adjust the flow rate accordingly. As we used an isocratic separation method in our experiment, a constant mass flow rate was observed and thus adjusting was not necessary. Holding the mass flow rate of the spike approximately constant admits to cancel it completely from the calculation (Eqs. (14)–(17)).

The mass concentration of the reference $\gamma_z$ is given by the density of the reference solution at the beginning of the experiment, which can be measured with a pycnometer, and the mass fraction of the reference in the solution, which can easily be determined using a balance. The integration of the quantities $\hat{R}_{bx}$ and $\hat{R}_{bz}$ can be done from the isotope ratio chromatogram using the above described method in Excel.

It is also noteworthy that in the equation used in the conventional method the volume of the sample loop $V_{loop,x}$ in the injector of the HPLC must be known (Eq. (27)). As it is difficult to determine this volume the values given by the manufacturer have to be taken for granted. This renders a real metrological evaluation of the measurement virtually impossible.

The use of the Se species as reference that undergoes the same procedure from sample preparation to separation as the sample possible losses and species conversions are accounted for to some degree. As another advantageous consequence the densities of sample $\rho_x$ and reference $\rho_z$ solution after all sample preparation steps were found to be approximately equal so that they could be canceled from Eq. (19) to yield Eq. (26).

The double ID-ICP-MS method described in this paper results in an equation that allows the direct calculation of the mass concentration of the analyte in the sample from the monitored isotope ratio chromatogram. This was possible because mass flow rates were measured directly instead of the conventional calibration of the volume flow rate of the spike and subsequent transformation of volume flow rate to mass flow rate. Our approach eliminates the problem that the volume flow rate of the spike is dependent of the density of the eluent in the HPLC and thus can vary throughout a chromatographic run using a gradient and under different temperature conditions. Now the changes in the mass flow rate can either be mathematically corrected for or a balance can be used to control the peristaltic pump that delivers the spike.

The additional sample loop used to introduce the reference solution to calibrate the spike mass flow becomes dispensable and thus simplifies the experimental set-up. It is also superfluous to know the exact volume of the sample loop of the HPLC injection system as long as the injected volume is the same for sample and reference. As a reference the species under investigation was used which was treated as the sample so that potential species changes and losses that can occur during sample preparation can partly be accounted for. Additionally, the simple use of Excel instead of sophisticated software solutions enables this method to be used by a wider range of users.

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**Table A1**
Meaning of the symbols used in Eqs. (1)–(19).

| Symbol | Unit | Quantity |
|---|---|---|
| $x_{x,1}, x_{y,1}, x_{z,1}$ | mol/mol | Isotope abundance of the reference isotope (1) in sample x, spike y, and reference z, respectively |
| $x_{x,2}, x_{y,2}, x_{z,2}$ | mol/mol | Isotope abundance of the spike isotope (2) in sample x, spike y, and reference z, respectively |
| $w_x, w_y$ | g/g | Mass fraction of Se in sample x and spike y, respectively |
| $\gamma_x, \gamma_z$ | g/L | Mass concentration of Se in sample x and reference z, respectively |
| $M_x, M_y, M_z$ | g/mol | Molar mass of sample x, spike y, and reference z, respectively |
| $R_x, R_y, R_z$ | mol/mol | Isotope amount ratio of spike and reference isotope (2/1) in sample x, spike y, and reference z, respectively |
| $R_{bx}, R_{bz}$ | mol/mol | Isotope amount ratio of spike and reference isotope (2/1) in the blend of sample and spike (bx) as well as reference and spike (bz) |
| $\dot{m}_x, \dot{m}_y, \dot{m}_z$ | g/s | Mass flow of sample x, spike y, and reference z, respectively |
| $\rho_x, \rho_y, \rho_z$ | g/L | Density of sample x, spike y, and reference solution z, respectively |
| $\dot{V}_y, \dot{V}_{eluent}$ | L/s | Volume flow of spike solution and eluent, respectively |
| $V_{loop,x}$ | L | Volume of the sample loop of HPLC |

## Appendix A.

All variables used in Eqs. (1)–(19) are summarized in Table A1.

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