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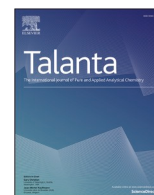

Towards single cell ICP-MS normalized quantitative experiments using certified selenized yeast

J.S.F. Pereira [a,b], R. Álvarez-Fernández García [b], M. Corte-Rodríguez [b], A. Manteca [c], J. Bettmer [b], K.L. LeBlanc [d], Z. Mester [d], M. Montes-Bayón [b,*]

[a] Instituto de Química, Universidade Federal Do Rio Grande Do Sul, 91501-970, Porto Alegre, RS, Brazil
[b] University of Oviedo, Faculty of Chemistry, Dept. of Physical and Analytical Chemistry and Instituto de Investigación Sanitaria Del Principado de Asturias (ISPA), C/ Julián Clavería 8, E-33006, Oviedo, Spain
[c] University of Oviedo, Faculty of Biology, Dept. Functional Biology and Instituto de Investigación Sanitaria Del Principado de Asturias (ISPA), C/Julián Clavería 8, E-33006, Oviedo, Spain
[d] National Research Council Canada, 1200 Montreal Road, K1A OR6, Ottawa, Ontario, Canada

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ABSTRACT

In the search for a normalized procedure to replicate and compare single cell-inductively coupled plasma-mass spectrometry (SC-ICP-MS) experiments, SELM-1, a certified reference material containing selenium enriched yeast cells has been used. Selenium concentrations (both, intra- and extracellular) have been measured using either sequential or simultaneous procedures. Regarding quantitative results, the sequential procedure involving cell washing followed by freeze drying of the washed material and intracellular Se quantification using SC-ICP-MS provided best results. In this case, intracellular Se accounted for 1304 ± 48 mg kg^{-1} (corresponding to 64% of the certified Se content). The average mass of Se per yeast cell was 41.6 fg Se with a dispersion of 1.6–279 fg Se/cell. In the isolated extracellular Se fraction, the Se concentration accounted for 412 ± 48 mg kg^{-1} (about 21% of the total Se). Thus, the sequential procedure provided a total Se recovery of about 85% with respect to the certified value. The direct dilution and simultaneous measurement of intra- and extracellular Se by SC-ICP-MS provided results of 1024 ± 42 mg kg^{-1} for intracellular and 316 ± 30 mg kg^{-1} for extracellular Se representing a total recovery of about 66%. In both cases, an initial thorough characterization of the cell density per solid weighed material was conducted by flow cytometry and the cell integrity ensured using confocal microscopy. These results clearly demonstrated that with appropriate sample preparation, SC-ICP-MS is a unique tool, which is capable of providing quantitative information about intracellular and extracellular Se. In addition, SELM-1 seems the ideal tool to enable data normalization at the single cell level to replicate, benchmark, and improve new SC-ICP-MS studies by using the same material for data validation.

1. Introduction

Selenium enriched yeast or selenized yeast has been widely used as a nutritional supplement in the case of Se deficiency [1]. In the year 1996, when its potential as nutraceutical product against colon and prostate cancer was indicated, the investigations on this material raised dramatically [2,3]. *Saccharomyces cerevisiae* (baker's yeast) is, most frequently, the microorganism used for selenium enrichment using various inorganic Se sources (mainly Se (IV)) [4,5]. *S. cerevisiae* is able to proliferate under either anaerobic or aerobic conditions and can use a wide variety of compounds as carbon sources, but glucose is the preferred source for fermentative metabolism [6]. The process of intracellular accumulation of Se occurs through active transport inside the cell of the Se added to the growing media which seems to be more efficient in the early logarithmic growth phase of yeasts [7]. The metabolic transformation process of SeO_3^{2-} within the cell occurs with the participation of glutathione (GSH) in a similar route to the sulfur analogue [8–10]. As a result, selenodiglutathione (GS-Se-SG) and the oxidized form of glutathione (GSSG) are formed. In a further step, intracellular selenodiglutathione is converted into glutathionyselenol (GS-Se-H), and then to hydrogen selenide (H_2Se/HSe^-), with simultaneous formation of the oxidized form of glutathione (glutathione

* Corresponding author.
E-mail addresses: montesmaria@uniovi.es (J.S.F. Pereira), montesmaria@uniovi.es (M. Montes-Bayón).

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disulfide, GSSG) [10–12]. Apparently one of the main terminal products of this biosynthetic process is the non-canonical amino acid selenomethionine which could be non-specifically incorporated into proteins, displacing methionine on a massive scale [13]. In addition, during this biotransformation pathway the formation of Se-nanoparticles can occur, as documented by some authors [14,15]. Hydrogen selenide can, also, pass cell walls in a passive way, and therefore be exported into the yeast growing media [16].

The evaluation of the selenium species produced in yeast cells has been approached through different analytical methods that tried to achieve a quantitative mass balance with respect to the total incorporated Se [17]. In fact, certified reference materials have been brought to the market with the aim of validating analytical procedures for the quantitative characterization of the total Se content in selenized yeast [18,19]. SELM-1, produced by the National Research Council Canada (NRC) [20], is certified for total Se as well as Se-methionine content, the most abundant Se metabolite [19]. However, little is known regarding the characterization of the yeast material at the individual cell level [21]. For instance, few studies refer to the intracellular/extracellular levels of Se due to possible diffusion of the volatile Se-forms during cell metabolism or to the weakly adsorbed Se from the growing media into the yeast cells. Nowadays, due to the widespread use of the single cell analytical techniques, this study can be feasible to achieve a full characterization of the selenized yeast material.

Single cell analysis combined to inductively coupled plasma mass spectrometry (ICP-MS), can be conceptually derived from the single particle analysis ICP-MS experiments [22,23]. Basically, once the cells are individually introduced into the ICP, the plume of ions generated can be directly measured using fast scanning mass analyzers in a sequential (quadrupole instruments) [24] or quasi-simultaneous (time of flight instruments) [25] way. However, cells are heterogeneous and fragile entities in comparison to nanoparticles. These characteristics increase the complexity of sample handling required to obtain reliable results from these experiments. In addition, data validation in the case of nanoparticles (e.g. transport efficiency, particle number concentration and particle size results) can be directly obtained using the commercially available certified reference materials of gold nanoparticles [26]. However, single cell experiments are still limited in terms of validation and, frequently, data from nanoparticles are often used as an approximation. In this work, we propose the use of the certified reference material SELM-1 as a mean to validate, similarly to single particle, the single cell ICP-MS experiments. For this aim, various sample preparation strategies will be applied to SELM-1 to obtain mass balance in terms of intracellular and extracellular Se content. In addition, evaluation of the possible data normalization to the cell number concentration provided by flow cytometry together with the mass of yeast will be addressed. These data could be extremely useful towards the normalization of the analytical measurements based on single cell experiments. The use of a publicly accessible reference material (SELM-1) for these experiments enables the validation of our findings and fine-tuning of these methodologies by others in the future by the virtue of using the exact same study material we have employed in this study.

2. Experimental

2.1. Instrumentation

A microwave oven (Ethos-1, Milestone, Sorisole, Italy), equipped with 10 TFM vessels (rotor type HPR-1000/10) with internal volumes of 100 mL, was used for microwave-assisted digestion (MW-AD) of SELM-1 for subsequent Se determination by ICP-MS. The temperature was monitored in real time during sample digestion in the reference vessel using a probe.

All measurements were performed on the inductively coupled plasma mass spectrometer iCAP™ TQ (Thermo Fisher Scientific, Bremen, Germany). The instrument was used in the triple quadrupole mode using oxygen as reaction gas for the measurement of $^{80}Se^+$ and $^{31}P^+$ as their respective oxides ($^{80}Se^{16}O^+$ and $^{31}P^{16}O^+$). For the determination of the total Se content, the ICP-TQ-MS instrument was equipped with a conventional nebulization system composed of a concentric MicroMist nebulizer and a cyclonic spray chamber (both from ESI Elemental Service & Instruments GmbH, Mainz, Germany).

For single cell experiments, the instrument was fitted with the Single Cell Sample Introduction System (SC–SIS, Glass Expansion, Weilburg, Germany) that consists of a high efficiency, low uptake, concentric glass nebulizer and a low volume, on-axis spray chamber that uses a sheath gas flow to increase the transport efficiency of the nebulized cell suspension by reducing cell deposition. The operational conditions used for Se determination by TQ-ICP-MS are shown in Table 1.

A chromatographic system was also used to identify the dissolved Se species extracted from SELM-1. Separations were carried out using a chromatographic system Agilent 1260 Series (Agilent Technologies, Tokyo, Japan) at a flow rate of 0.7 mL min^{-1} with a mobile phase containing a 50 mmol L^{-1} ammonium acetate buffer (pH 6.5). A size exclusion column (Superdex™ Peptide 10/300 GL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used. Under these conditions, peptides and other small biomolecules followed a size exclusion mechanism and were eluted according to their size (exclusion molecular weights between 100 and 7000 Da). A sample volume of 20 μL was injected in each analysis. The HPLC was coupled to a concentric MicroMist nebulizer and a cyclonic spray chamber nebulization system of the ICP mass spectrometer, as previously described.

Yeast cell integrity after washing was analyzed using fluorescence microscopy and yeast cell counting by flow cytometry. Intact yeast cells were stained with SYTO9 and PI (Invitrogen, 3.34 μM and 20 mM, respectively) and observed under a Leica TCS-SP8 confocal laser-scanning microscope at wavelengths of 488/530 and 568/640 excitation/emission for SYTO 9 and PI respectively. Membrane-intact cells are stained with SYTO 9 (green), whereas membrane-broken cells are stained with PI (red). Yeast cells were quantified in the stained samples using a CytoFLEX S Flow Cytometer (Beckman Coulter). Raw data were processed using the CytExpert 2.3 software. Unstained samples were used as controls.

Other instrumentation included a freeze dryer (Heto Lyolab 3000, Thermo Fisher Scientific), centrifuges (Biofuge Stratos, Thermo Fisher Scientific and MiniSpin Plus, Eppendorf, Hamburgo, Germany), vortex (Reax Top, Heidolph Instruments, Schwabach, Germany) and ultrasonic bath (Ultrasons 30000514, J.P. Selecta, Barcelona, Spain). An analytical balance (MSD205DU/M, Mettler Toledo) with an accuracy of 0.0001 g was used to prepare the sample suspension, analytical standards and to perform all dilutions.

Table 1
Optimized conditions for Se determination by ICP-MS in the triple quadrupole mode.

Parameter	Value
Radiofrequency power	1550 W
Plasma gas flow rate	14 L min^{-1}
Auxiliar gas flow rate	0.8 L min^{-1}
Nebulizer gas flow rate	[a]0.8 L min^{-1} and [b]0.5169 L min^{-1}
Sheath gas flow rate	[b]0.650 L min^{-1}
Cell gas flow rate (O_2)	[a,b]0.2775 mL min^{-1}
Sample flow rate	[a]0.33 mL min^{-1} and [b]0.01 mL min^{-1}
Q_1 bias	−1.64 V
Q_{cell} bias	−6.86 V
Q_3 bias	−12 V
Isotopes measured	m/z 96 ($^{80}Se^{16}O^+$) and m/z 47 ($^{31}P^{16}O^+$)
Dwell time	[a]0.1 s and [b]0.005 s
Run time	[b]120 s

[a] Conventional nebulization system coupled to ICP-MS and.
[b] SC-ICP-MS analysis for Se determination.

2.2. Sample, reagents and standards

The cells analyzed in the present work were from a selenium enriched yeast CRM (SELM-1). This CRM is provided by the National Research Council Canada (Ottawa, Canada) and establishes certified values for the mass fraction of selenium, selenomethionine (SeMet), and methionine (Met) [19,20].

Water, used for preparation of all solutions and standards, was purified in a Purelab flex 3 system (ELGA VEOLIA, Lone End, United Kingdom) with a minimum resistivity of 18.2 MΩ cm at 25 °C. Concentrated nitric acid 65% (m/m) (Acros Organics, Thermo Fisher Scientific) was distilled in a sub-boiling system (DTS-1000 Acid Purification System, Savillex, Eden Prairie, USA) and was used for SELM-1 digestion.

For Se quantification by ICP-MS and SC-ICP-MS, standard solutions were prepared daily by sequential dilution of a multi-element stock solution containing 1000 mg L^{-1} of Se (Sigma Aldrich, St. Louis, USA) in water in the range of 0–50 μg L^{-1}. For total Se determination in the samples obtained after MW-AD, standard solutions were prepared in 2% (m/m) HNO_3 using the same multi-element stock solution.

Argon (99.999% of purity) and oxygen (99.995% of purity), both from Air Liquide (Valladolid, Spain) were used for ICP-MS operation and for the reaction cell, respectively. Argon was also used for SC-ICP-MS as an additional gas to prevent cell deposition in the spray chamber and to minimize damages in the cell membranes/walls [27]. Transport efficiency of calibration standards was calculated by analyzing a citrate-stabilized gold nanoparticle standard (RM 8012, nominal diameter of 30 nm) obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, USA).

Sodium selenite ≥98% (Sigma-Aldrich), SeMet (L-(+)-selenomethionine 97%, Sigma-Aldrich), seleno-L-cystine 95% (Sigma-Aldrich), dimethyldiselenide (96%, Sigma-Aldrich) and diethyldiselenide 97% (Strem Chemicals, Boston, USA) were used for the characterization of the dissolved Se species in the SELM-1 CRM by HPLC-ICP-MS.

2.3. Sample digestion using microwave-assisted acid digestion for subsequent determination of total Se content

The total amount of Se in the CRM was determined by ICP-MS after MW acid digestion. About 60 mg aliquots of the CRM were weighed and transferred directly to the vessels together with 8 mL of 7 M HNO_3. After addition of the reagents, the vessels were closed and fixed in the rotor which was then positioned inside the microwave cavity. The heating program was started as follows: 1200 W for 15 min (ramp of 10 min) and 0 W for 60 min (cooling step). The maximum temperature was set at 220 °C. After cooling, final solutions were quantitatively transferred to polypropylene vessels with water for further determinations.

2.4. Sample treatments

In this work, two strategies were evaluated for quantification of Se in the selenized yeast cells by SC-ICP-MS: *i)* the cell suspensions were directly measured without any previous treatment (just after suspending the SELM-1 CRM in water), and *ii)* the cells were washed to remove the dissolved Se content and then, they were freeze dried to obtain washed dried cells. All the procedures adopted for cells and Se analysis are shown in Fig. 1.

For the direct SC-ICP-MS studies, about 20 mg of SELM-1 were suspended in 10 mL of water. The suspension was homogenized in the vortex for 1 min and directly used to quantify the amount of Se inside the cells and outside (as the background signal) within the same run.

On the other hand, an aliquot of the cell suspension previously prepared was centrifuged for 5 min at 600 *g* and the supernatant was collected for further analysis. The sample pellet was suspended in 5 mL of water, homogenized in the vortex for 1 min at the maximum speed and the supernatant was again collected. This procedure of cell washing was repeated another three times to ensure an effective elimination of extracellular Se and all supernatants obtained in each washing step were combined in the same vial for further analysis. The washed cells were lyophilized by freeze-drying and the resultant sample pellet was suspended in water and used for further SC-ICP-MS and flow cytometry analysis. To validate the SC-ICP-MS results, an aliquot of the dried pellet was also digested under MW-AD for subsequent determination of the total amount of Se by ICP-MS.

The supernatant obtained from the cell washing steps was also analyzed by ICP-MS in order to determine the total content of Se (using the conventional nebulization system). Additionally, the speciation of the supernatant was conducted by HPLC-ICP-MS to identify the Se species presented in the extracellular fraction of SELM-1.

To validate the results obtained for dissolved Se content in the unwashed cells, a suspension, prepared as mentioned before, was filtered using a syringe filter (hydrophilic PVDF filter, pore size 0.22 μm, 25 mm in diameter, Filter-Lab, Filtros Anoia, Barcelona, Spain) to retain the cells and the filtrate was also analyzed by both, conventional ICP-MS

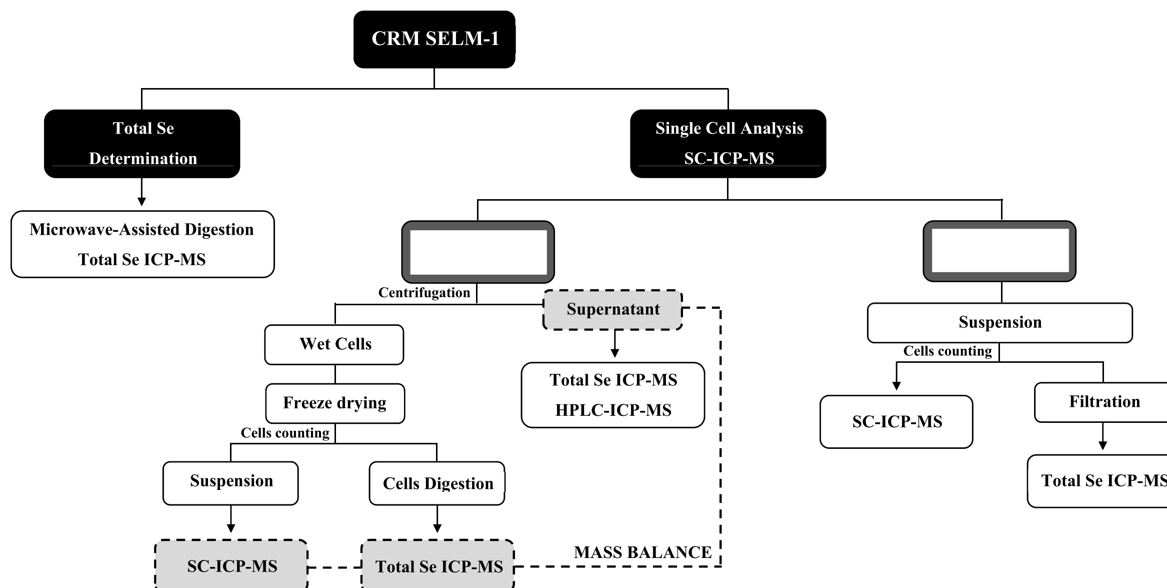

Fig. 1. Strategies adopted for Se quantification in the selenized yeast CRM by ICP-MS and SC-ICP-MS.

and SC-ICP-MS. The results were compared with those obtained by the measurement of cells and background in unwashed cell suspensions in the same run.

2.5. Se determination by SC-ICP-MS

For single cell analysis, solutions and cell suspensions were introduced to the ICP-MS at a flow rate of 10 μL min^{-1} using a syringe pump (model 100, Fisher Scientific, Thermo Fisher Scientific) fitted with a 1 mL Hamilton syringe (Hamilton, Reno, USA). Measurements were acquired using time resolved analysis mode during 120 s with a dwell time of 5 ms and by monitoring both the *m/z* 96 ($^{80}Se^{16}O^+$) and *m/z* 47 ($^{31}P^{16}O^+$) in a separate run since phosphorous can be used as an internal cell marker as being present in all cells as a constitutive element.

The acquired data was further processed using Excel 365 (Microsoft) and OriginPro 8 (OriginLab, Northampton, MA, USA). Cell events were filtered by selecting all data points whose intensity was in the range of 3–5 times the standard deviation above the mean of the entire data set. The remaining data set was rearranged and subjected again to the same procedure. This procedure was repeated until there were no data points higher than the value of mean plus 3σ or 5σ, and that value was used as threshold for cell events. The amount of Se in individual cells was determined by using an external calibration with selenium elemental standards and taking into account their transport efficiency (estimated by using AuNPs), the sample flow rate and dwell time, as previously described [24,27].

2.6. Statistical analysis

All the results were evaluated using statistical tests (confidence interval and *t*-test) considering 95% of confidence level. Results obtained after SC-ICP-MS analysis were always validated by MW-AD and total Se determination by ICP-MS.

3. Results and discussion

Before the analysis of SELM-1 by SC-ICP-MS, the CRM was subjected to MW-AD to evaluate the total content of Se. After digestion, the obtained solutions were completely clear, without solid residue, indicating complete digestion of the sample. Final solutions were analyzed by ICP-MS and the total Se concentration found in the CRM was 1934 ± 54 mg kg^{-1} (n = 3), which was comparable to the certified value for total Se (2031 ± 70 mg kg^{-1}). To prove if the differences found between obtained and certified data were statistically significant, a t-student test was performed. The obtained results revealed that at 95% confidence level both sets of data do not show statistically significant differences. With this result, a mean recovery of approx. 95% was achieved.

As often claimed, single cell ICP-MS experiments permit the quantification of the intracellular content of a given element (proportional to the height of the cell event as observed in the mass spectrometer) and simultaneously, the extracellular content (as indicated by the intensity of the background signal). To further prove this possibility as well as provide a normalized procedure to validate constantly developed SC-ICP-MS experiments, different strategies were applied.

3.1. Quantification of intracellular Se in individual cells after separation of extracellular components

Various strategies have been adopted in the literature to remove dissolved species to further conduct single particle analysis. Among them, cloud point extraction has been used for Ag nanoparticles determination [28] or ultracentrifugation to remove dissolved Te species for subsequent determination of Te nanoparticles [29]. In both cases, the dissolved analyte was removed to reduce the background and improve accuracy of the single particle experiments. The procedure adopted here for isolation of yeast cells was relatively simple involving only the addition of MilliQ water to the yeast powder, followed by homogenization and centrifugation. This procedure was conducted using 5 mL of Milli-Q water (three times) as described in the experimental section. The supernatants containing dissolved Se species of each washing step were combined and stored for subsequent Se quantification by ICP-MS. The remaining wet pellet was submitted to freeze drying.

The dried cells were first counted by flow cytometry, which also served to prove cellular integrity through the handling procedure. The obtained results could be used to calculate the number of cells per mass of CRM (cell number per mg of material) and so, to estimate transport efficiency into the ICP-MS. The flow cytometry results (Fig. 2A) indicated that after the washing/drying procedure, the number of cells per mass of material was 21,173 ± 1299 cells $μg^{-1}$ CRM. In order to ensure cell integrity throughout the washing/freeze drying procedure, cells were stained and measured by confocal microscopy as demonstrated in Fig. 2B. For this aim, yeast cells were stained with the SYTO 9/PI vital fluorescent stains. Membrane-intact cells are stained with SYTO 9 (green), whereas membrane-broken leaking cells are stained with PI (red). Cells stained in green (seen in Fig. 2B) confirmed the stability of the yeast cells through this procedure and the unlike event of Se-leaking through cell walls.

Further, dried cells were divided in two aliquots for the following procedures: *i)* MW-AD with subsequent total Se determination by ICP-MS and *ii)* suspension preparation and further analysis by SC-ICP-MS to quantify the mass of Se per individual cell. In both cases, the results obtained corresponded to the intracellular Se content. The acid digestion of the dried cells and further analysis by ICP-MS provided a Se concentration (intracellular level) of 1449 ± 192 mg kg^{-1} (n = 8), which corresponded to approximately 71% of the certified Se content in SELM-1. For the single cell analysis, a cell suspension was prepared using the dried cells. An initial optimization of the ICP-MS system was performed for this kind of measurements. It is expected that cells were not disrupted during the nebulization, taking into account that yeast have a high mechanically resistant cell wall that facilitates the transport along the nebulization system in the ICP-MS equipment [22]. Therefore, the crucial point is to optimize the cell density introduced into the plasma in order to isolate individual cells for analysis. This procedure aims to avoid more than one cell at a time entering the mass analyzer and being detected within the same event. Thus, in the first step several suspensions with variable cell density were prepared and analyzed by SC-ICP-MS in order to find a suitable sample dilution. These experiments were performed under time resolved analysis mode during 120 s with a dwell time of 5 ms. Together with $^{80}Se^{16}O^+$, $^{31}P^{16}O^+$ was also monitored as an internal cell marker. Sample dilution of about 300 times (from the initial suspension) was suitable for SC-ICP-MS analysis providing similar height for P events and minimizing the presence of double cell events.

The time resolved signals obtained for Se and P in the cell suspension after washing by SC-ICP-MS are shown in Fig. 3 together with the corresponding histograms.

It is important to mention that the background signal for Se is almost negligible after cell washing (Figure S1 A). The mass of Se per event was calculated using the 5-σ criterion to filtrate the data and after an external calibration with inorganic selenium standards measured under the same conditions [24]. An average mass of Se of 41.6 ± 0.6 fg per cell was obtained. The range of Se content varied from 1.6 to 279.6 fg per cell (see Fig. 3 for the corresponding histogram). To compare the results obtained with the SELM-1 certified value (expressed in mg Se/kg SELM-1), the average mass of Se per cell was transformed in mass of Se (mg) per mass of the CRM (kg) using the results previously obtained by flow cytometry in the same aliquot (cells per mg of CRM) but also transport efficiency (calculated considering the P events in comparison to the number of cells obtained by flow cytometry) and Se-containing cells with respect to the total number of cells. Table 2 shows the results obtained by SC-ICP-MS after cell washing and all the calculations conducted to obtain such results.

As can be seen, after cell washing, the obtained results of

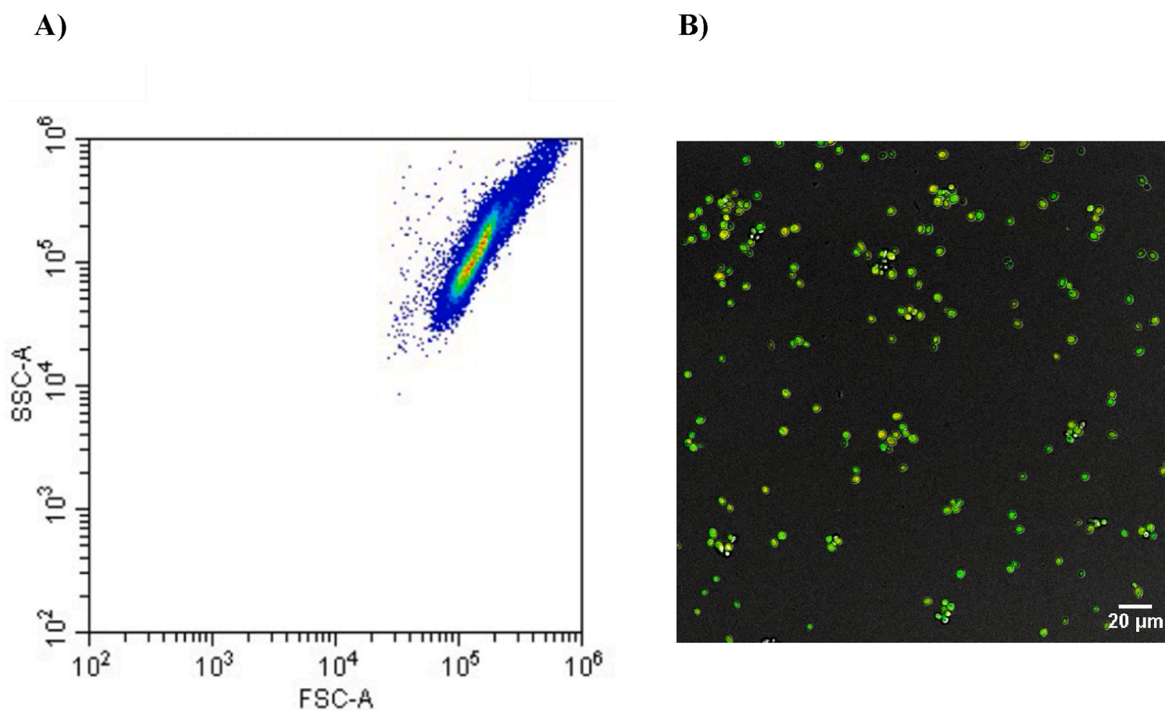

Fig. 2. A) Flow cytometry plot for cell counting and B) fluorescence microscopy photograph of cells stained with SYTO 9 showing intact cells with round shapes and regular borders.

intracellular Se turned out to be 1304 ± 48 mg kg^{-1} Se which compares favorably to the result obtained after the acid digestion of the dried cells (1449 ± 192 mg kg^{-1} Se). Therefore, it can be concluded that in the absence of soluble selenium species that increase the Se background, the SC-ICP-MS experiment provided quantitative Se results in the yeast material.

3.2. *Quantification of extracellular Se*

The concentration of Se found in washed and dried cells by acid digestion revealed that just about 71% of the total Se in the yeast material was present inside of the yeast cells. Therefore, to obtain the complete mass balance, total selenium determination was also performed in the supernatants obtained after all washing steps. This procedure was repeated several times to estimate the level of dissolved Se. The value obtained was 412 ± 48 mg kg^{-1} (n = 8) and correspond to about 20% of the total Se in the CRM. The presence of extracellular Se can be ascribed to unremoved Se from the enrichment procedure but also to the cell metabolism producing Se-methylated species that can be excreted by the cells.

Considering the sum of the Se concentration found in the digested cells after washing and in the supernatant, total Se concentration was 1861 ± 192 mg kg^{-1} (n = 10). This value was statistically similar with the certified value for total Se in the CRM investigated (92% recovery). If considering the total intracellular Se content obtained by SC-ICP-MS, the sum with the supernatant provides a total Se of 1716 ± 100 mg kg^{-1}, which corresponds to a recovery of about 85% of the certified value. The inaccuracy of the latest result is ascribed to cell losses during the centrifugation procedure that could be seen by directly introducing the cell washing supernatant in the ICP-MS (see Fig. S2). The presence of some P and Se events reveal that some of the yeast cells were mobilized from the solid fraction during the isolation process.

To the best of our knowledge, this is the first time that a CRM is used to validate the quantitative data obtained in individual cells by using SC-ICP-MS and could represent the first step towards data normalization among SC-ICP-MS experiments.

As complementary evaluation, SEC-ICP-MS analysis was performed in the supernatant obtained after cell washing as screening of the Se species present extracellularly. The Se chromatogram obtained for the cells supernatant are shown in Fig. S3 and, as can be seen, they basically belong to low molecular weight species. The signal for S was also acquired as a tool to give hint on the presence of water extractable species containing both, sulfur and selenium. Such low molecular weight compounds can penetrate cell walls by passive diffusion, and therefore be exported into the yeast growing media, being one of the observed Se-species in the chromatogram.

3.3. *Quantification of Se in individual cells in the presence of dissolved se*

Single-cell ICP-MS has become a powerful tool to evaluate elemental composition at the single cell level. Despite the many efforts that have been recently developed, some challenges remain, such as the loss of capability to discriminate the cell events in the presence of a high background baseline caused by dissolved ionic analyte species both for SC-ICP-MS and single particle-ICP-MS [14,28,30]. Consequently, some cell events can be covered and the intracellular selenium not effectively quantified in the presence of a high background.

Thus, the next step was to evaluate the suitability of SC-ICP-MS to achieve simultaneous quantitative information on the intra and extracellular Se species in SELM-1 CRM. In this case, both events and background were quantified to address the mass balance and compare the results obtained with the certified value of total Se provided by the CRM. The SC-ICP-MS analysis was performed using 5 ms of dwell time and the typical time-resolved measurements for Se are shown in Fig. 4.

In Fig. 4 is possible to see the presence of Se containing cell events although in lower abundance with respect to those observed in Fig. 3A. The background was high as result of the presence of dissolved Se in the sample (Figure S2 A) and, as expected, it changed according to the dilution factor used. By comparing the number of P (Fig. 4B) and Se (Fig. 4A) events for the same sample aliquot, it is possible to see that about of 80% of the cells contain Se, as previously observed in the case of the washed cells (see Table 2). However, the cell counting by flow

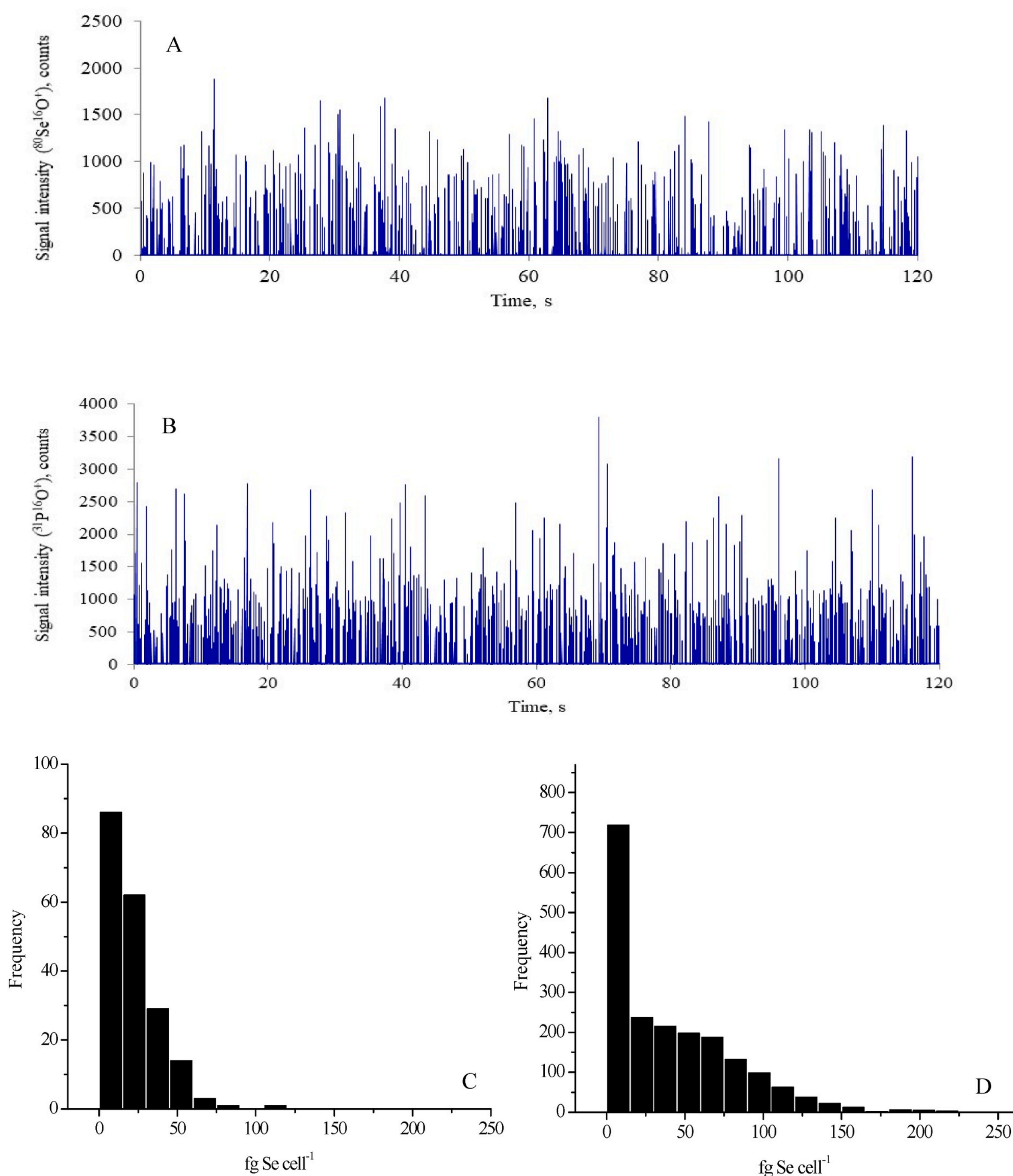

Fig. 3. (A) Time-resolved scan of $^{80}Se^{16}O^+$ and (B) $^{31}P^{16}O^+$ obtained from yeast (SELM-1) with a dwell time of 5 ms after cells washing and histograms obtained from SC-ICP-MS analysis for SELM-1 (C) without washing the cells and (D) after cells washing.

cytometry revealed, in this case, a value of 6320 ± 483 cells μg^{-1} CRM (n = 5) and the transport efficiency showed a significantly lower value of about 25%. Both results point out the presence of different matrix components remaining from the yeast preparation in the unwashed material that contribute to the weighed mass but do not correspond to yeast cells. Such components might provide some "matrix effects" during nebulization decreasing the transport efficiency from 75% obtained in the washed material to 25–30% in this case. It is noteworthy that such effects could be presumably minimized by dilution of the sample but, in that case, the background signal corresponding to dissolved Se would be too low to permit the accurate quantification of intra and extra cellular Se simultaneously.

The data were processed in a similar way than in the case of Fig. 3, using the 5-σ criterion and after external calibration with inorganic selenium standards that were measured under the same conditions [24]. In this case, an averaged mass of Se of 31.2 ± 1.3 fg per cell was obtained (approximately 30% lower of what was obtained when removing the extracellular species). The Se content in the cells ranged from 1.6 to 150.8 fg per cell and Fig. 3C shows the corresponding histogram.

To compare the results obtained with the SELM-1 certified value (expressed in mg (Se)/kg SELM-1), the mass of Se found per cell was converted in mass of Se (mg) per mass of the CRM (kg) using the result obtained by flow cytometry analysis after counting the cells in the same sample aliquot previously used for SC-ICP-MS, as shown in the formula of Table 2. The background signal of Se was used for quantification of the extracellular species, as previously described.

Table 2
Comparison of the results obtained by SC-ICP-MS and flow cytometry analysis after the two strategies investigated to treat the cells.

Parameter	Strategy Adopted for the Cells	
	Without Washing	Washing
[a,b]Average (median), fg cell^{-1}	31.2 ± 1.3 (22.5)	41.6 ± 0.6 (30.8)
[a,b]Range, fg cell^{-1}	1.6 to 150.8	1.6 to 279.6
[c]Cells μg^{-1} CRM	6320 ± 483	21,173 ± 1299
[d]Intracellular Se, mg kg^{-1}	1024 ± 42	1304 ± 48
[b]Extracellular Se, mg kg^{-1}	317 ± 31	412 ± 48
[e]Total Se, mg kg^{-1} (recovery)	1341 ± 72 (66%)	1716 ± 96 (85%)
[f]Transport efficiency	25%	75%
[g]Averaged number Se events	229	1588
[g]Averaged number P events	299	1764
[h]Cells containing Se	77%	90%

Results obtained by.

a) $m_c = \frac{\eta \times F \times t \times I}{b}$ where η is the transport efficiency of the inorganic standard solutions (calculated using NIST certified gold nanoparticles RM 8012 in the single particle mode and estimated to be 69%); F is the sample flow rate into the system, t is the dwell time (5 ms), I is the count rate of Se in the single cell and b is the slope of the Se calibration curve previously obtained with the inorganic standards.

b) SC-ICP-MS.

c) Flow cytometry analysis.

d) $$\frac{\text{Intracellular Se (mg)}}{\text{yeast mass (Kg)}} = \frac{\sum \text{fg Se}}{\text{n}^{\text{o}}\text{events} \times \frac{\text{detected cells}}{\text{total number of cells}} \times \frac{\text{cells with Se}}{\text{total number of cells}}} \times \frac{\text{number of cells}}{\text{microgram of yeast}} \times 10^{-3}$$

e) Sum of intracellular and extracellular Se content. Calculated recovery with respect to the certified value 2031 mg Se kg^{-1}.

f) Estimated according to the detected cell events (P) with respect to the number of cells counted by flow cytometry (b)

g) Average number of events measured in different days of different aliquots.

h) Calculated as. $\frac{Se\ events}{P\ events} \times 100$

Considering the results obtained without washing the cells and performing the calculations previously mentioned, total Se concentration found in the CRM was 1341 ± 72 mg kg^{-1} (n = 4). Out of this, 1024 ± 42 mg Se kg^{-1} corresponded to the intracellular Se and 317 ± 31 mg kg^{-1} to the extracellular Se. The overall Se recovery is, in this case, about 66% with respect to the certified Se concentration. When comparing these results with the ones previously obtained using the sequential quantification of intracellular and extracellular Se, several conclusions can be extracted. First, the extracellular Se concentration is comparable in both procedures (317 ± 31 mg Se kg^{-1} vs 412 ± 48 mg kg^{-1}) although slightly lower in the simultaneous measurements. For complementary evidence, the cell suspension was filtered using a syringe filter to retain the cells for further quantification of dissolved Se. The efficiency of the filtration process was confirmed by monitoring Se and P in the single cell mode with the absence of events for both elements, as shown in Fig. S4 in the supplementary material (no events were observed for P and also for Se). The time resolved measurements indicate that the presence of cells in the filtrate was negligible and the total Se concentration in the filtrate was 352 ± 23 mg kg^{-1} (n = 4) that corresponds to about of 17% of the certified value. This value was similar (confidence interval, 95% of confidence level) to that obtained for extracellular Se in the simultaneous quantification (without washing the cells) and similar to this obtained in section 3.2 (after cells washing).

The differences observed in the determination of the intracellular Se content could be ascribed to several causes. On the one hand, the high Se background in the unwashed material might occlude several low intensity events that will be filtrated out in the mathematical calculations. Other authors also reported some difficulties to determine events for analytes in the presence of a high background and to overcome such limitation, they reduced the dwell time to the microsecond range [14]. To test this hypothesis, the unwashed samples were measured also using a dwell time of 0.4 ms. Such dwell time allows the detection of more than one point per event and requires the use of specific software to integrate the originated peaks. Considering all these conditions, the obtained mass of Se per cell turned out to be: 26.8 ± 3.6 fg Se/cell (median 27.0 fg/cell) for the unwashed cells. This result is statistically indistinguishable from the data obtained with 5 ms and does not seem to be the cause of the inaccurate results in this case. It is important to mention that data processing using 5 ms, like here, is much simpler than in the microsecond range and does not require special software for data evaluation.

On the other hand, the origin of the discrepancy can be also due to the previously described matrix effects that might compromise Se ionization in the plasma and yield slight signal suppression.

3.4. *Figures of merit*

According to the results obtained by SC-ICP-MS analysis, some figures of merit can be addressed below. The limit of detection of the SC-ICP-MS approach for Se was 0.156 fg per cell. This value was calculated considering the following equation: 3σ/b, where the "σ" corresponds to the standard deviation of the blank (acquired during 120 s with a dwell time of 5 ms) and "b" is the slope of the calibration curve. The coefficient of determination (R^2) was 0.9997 for Se determination by SC-ICP-MS.

The transport efficiency of the sample introduction system was used to convert the analytical standards used for calibration to mass of Se and it was calculated by comparing the number of detected and injected AuNPs (30 nm) as explained in the experimental section. The transport efficiency was determined daily and was always in the range of 65–90%. The transport efficiency was also daily calculated for the yeast cells by comparing the number of P detected events (as constitutive element) with the number of injected cells, precisely measured by flow cytometry. As expected, slightly lower values were obtained (25–75%), but still much higher than those typically obtained with conventional sample introduction systems [22].

4. Conclusions

This study shows, for the first time, the application of SC-ICP-MS for quantifying intra- and extracellular Se in a certified reference material of selenized yeast. The separation and independent analysis of the two Se

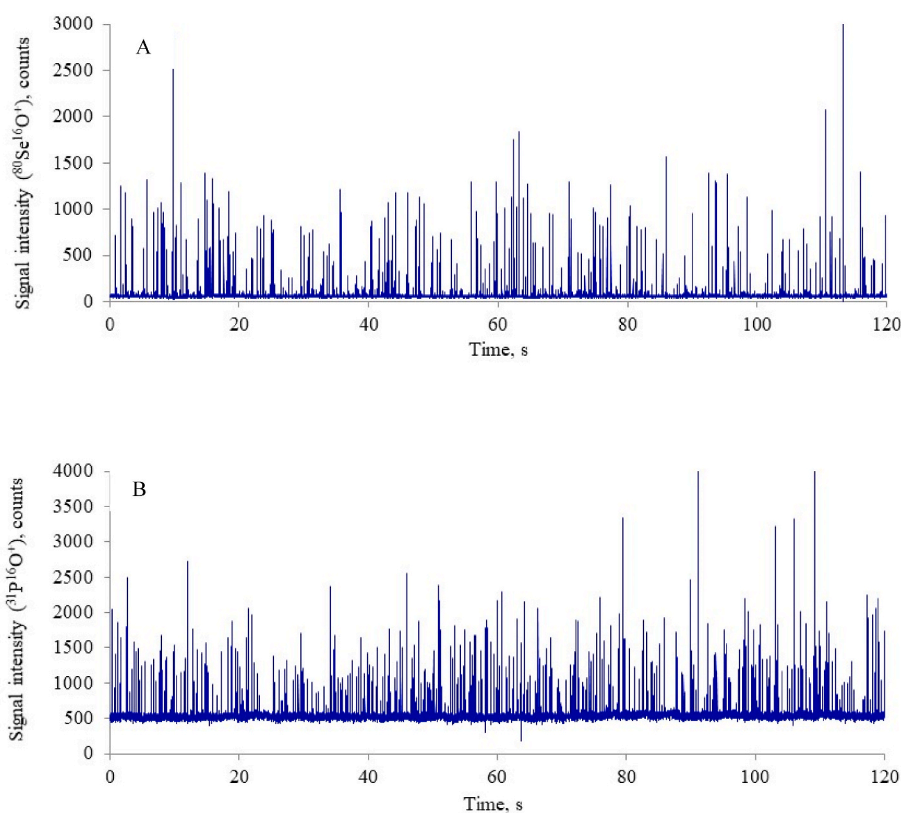

Fig. 4. Time-resolved scan of obtained for $^{80}Se^{16}O^+$ (A) and $^{31}P^{16}O^+$ (B) from yeast (SELM-1) with a dwell time of 5 ms in the presence of dissolved ionic Se. Dilution factor of about 100 times.

fractions revealed that about 64% of the total Se was incorporated inside the cells and the mass balance, considering also the dissolved Se content, reached an agreement of 85% with the reference value of SELM-1. These results obtained by SC-ICP-MS were validated by MW-AD of the cells and subsequent total Se determination by ICP-MS. Results obtained were statistically similar and both sets of data were inside the confidence interval of the certified value established for Se. Extracellular Se accounted for about 20% and is present in the form of low molecular weight species, some of them possibly containing S and Se atoms.

Regarding the attempt of the simultaneous determination of intra- and extracellular Se in a single run, using 5 ms dwell time, it was not possible to achieve quantitative results, in particular regarding the intracellular Se content. This is ascribed, in one hand, to the low Se-content events that might be neglected within the calculations as background noise and to some matrix effects, affecting at lower dilution levels. In any case, as established here, special care has to be taken when single cell ICP-MS data including high continuous background signals are being evaluated and the sequential determination of the extracellular and intracellular Se fractions is highly recommended for a more accurate determination.

Credit author statement

J.F.S Pereira: experimental work, writing and revision of the written text. R. Alvarez-Fernández: experimental work, revision of the written text. M. Corte Rodriguez: new experimental data, discussion of results, revision of the written text. A. Manteca: experimental results, discussion. Jörg Bettmer: capture of funding, discussion results, revision of the written text. Maria Montes Bayón: conceptual designed, capture of funding, writing of the manuscript, revisions. K. L. Le Blanc: discussion and revision of the written text. Z. Mester: discussion and revision of the written text.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123786.

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