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FIGURE 1

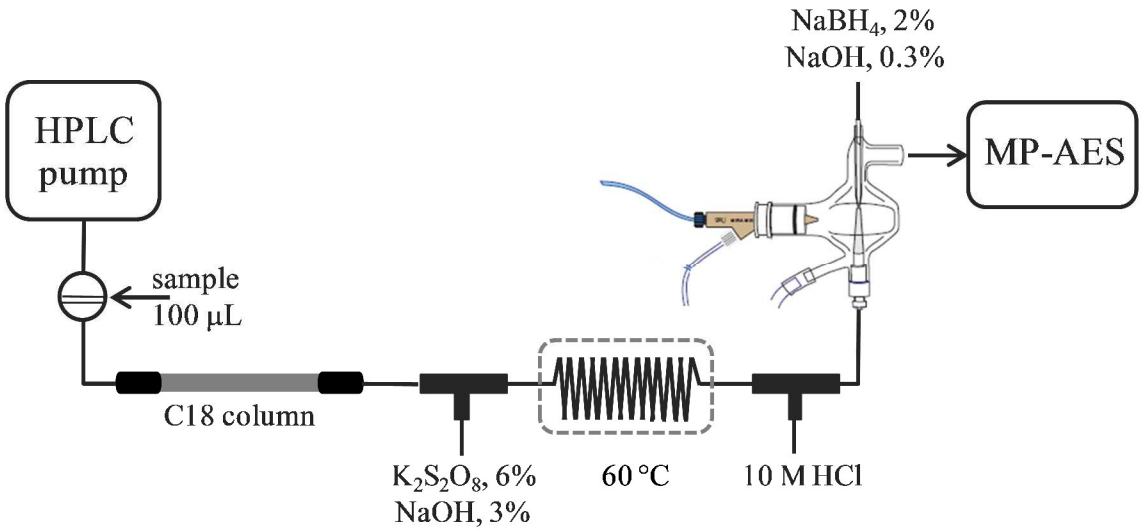
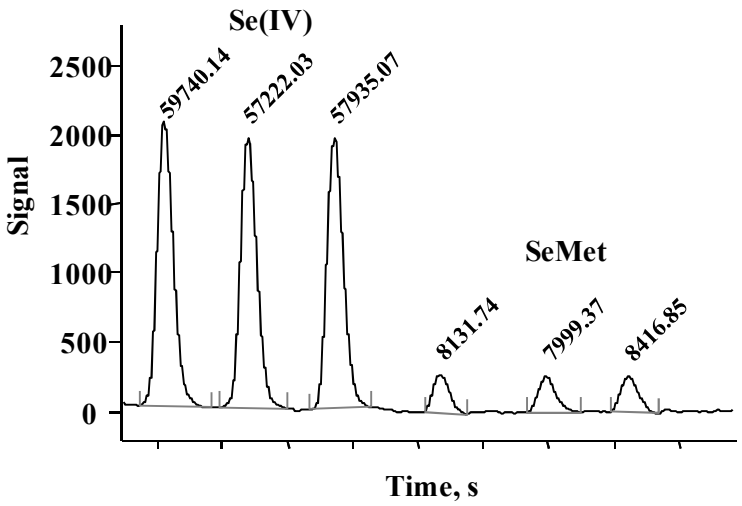


FIGURE 2



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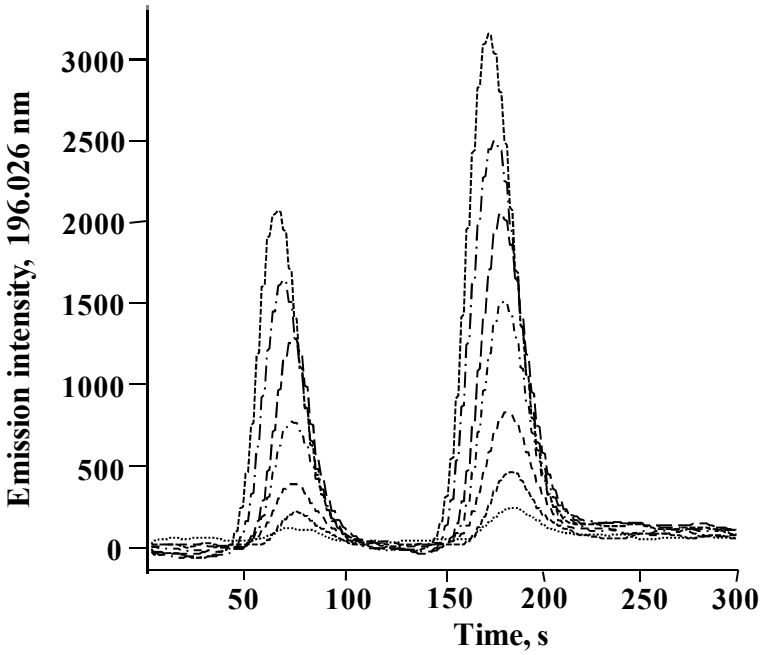


FIGURE 4

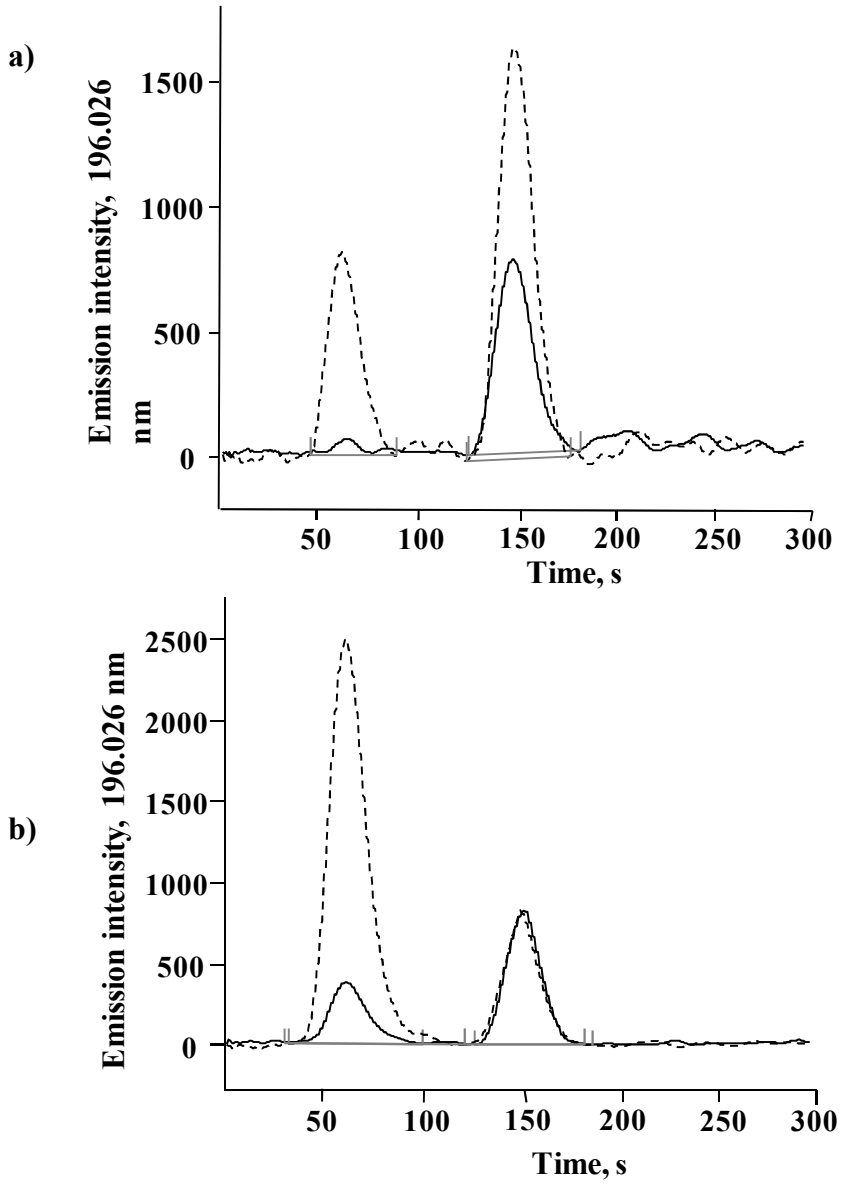


FIGURE 5

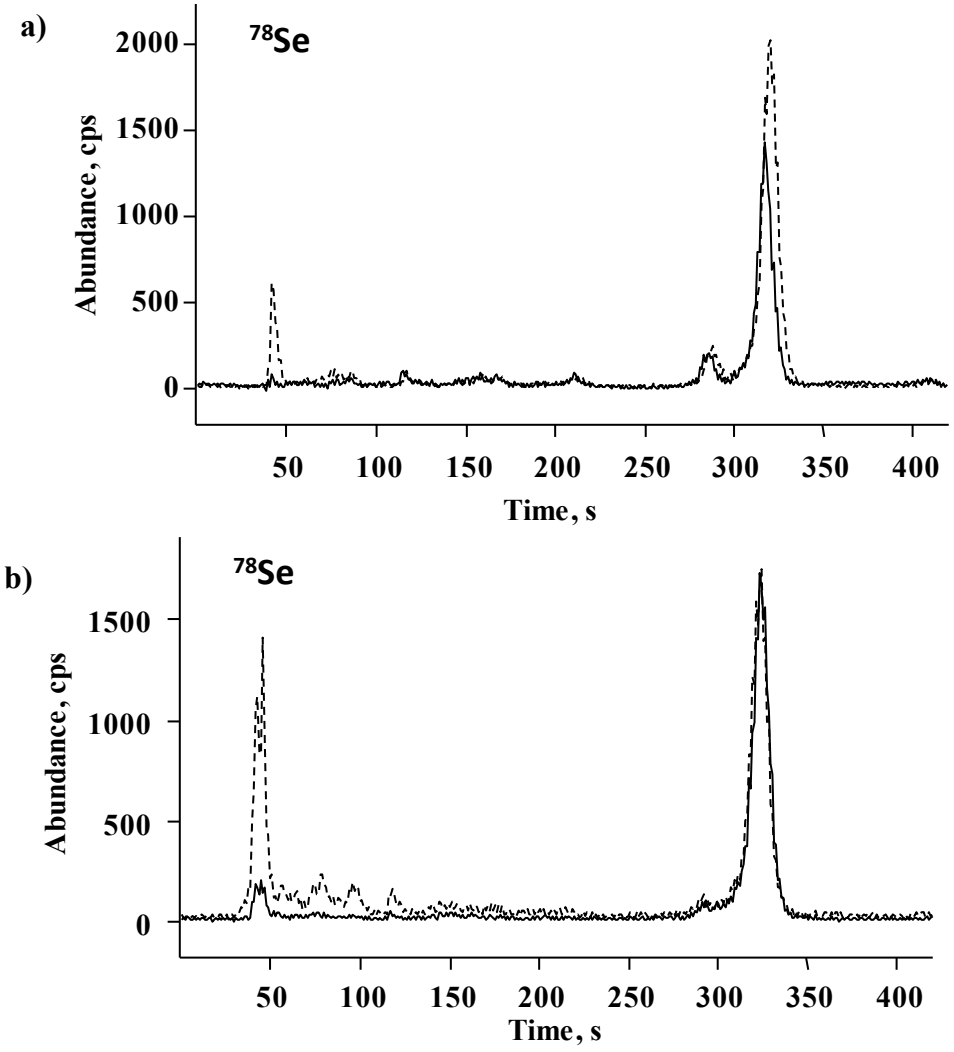
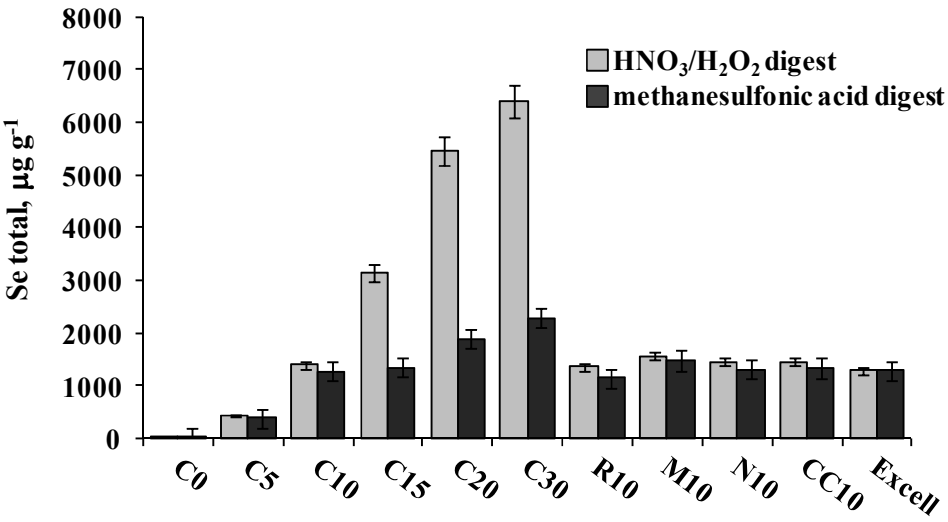
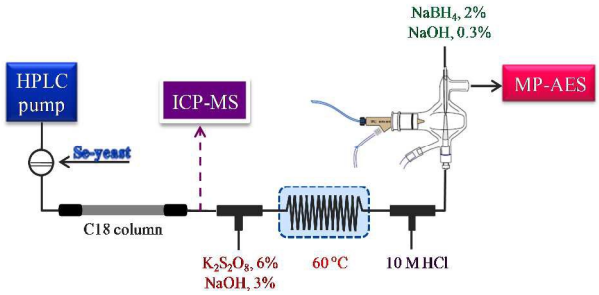


FIGURE 6





Liquid chromatography coupled to a nitrogen microwave plasma-atomic emission spectrometry via hydride generation for the first time. The procedure applied for the determination of Se(IV) and SeMet in Se-yeast. Good agreement with HPLC-ICP-MS results.

Determination of SeMet and Se(IV) in biofortified yeast by ion-pair reversed phase liquid chromatography - hydride generation - microwave induced nitrogen plasma atomic emission spectrometry (HPLC-HG-MP-AES)

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Abstract

Selenium biofortified yeast is the most common dietary Se supplement in human nutrition and in farm animals. Therefore, the production and routine quality control of the commercial products are highly demanded. In this work, a simple and cost-effective procedure is proposed for the determination of SeMet and Se(IV) in hydrolyzed yeast, consisting of an ion-pair reversed phase separation, post-column hydride generation and Se quantification by atomic emission spectrometry with microwave plasma sustained by nitrogen (HPLC-HG-MP-AES). Freeze-dried biomass was hydrolyzed with methanesulfonic acid; chromatographic separation was obtained with a mobile phase containing 0.08% v/v heptafluorobutyric and methanol (92:8) at a flow rate 1 mL min^{-1}; column effluent was on-line mixed with alkaline solution of potassium persulfate ($K_2S_2O_8$ 6% m/v, NaOH 3% m/v), passed through a reaction coil submerged in water bath at 60 °C, then 10 M hydrochloric acid was added prior to hydride generation in the MP-AES multimode sample introduction system ($NaBH_4$ 2% m/v, NaOH 0.3% m/v). Total chromatographic run was accomplished in 5 min and the evaluated on-column quantification limits were 59 ngSe mL^{-1} for Se(IV) and 0.52 μg mL^{-1} for SeMet. The procedure was tested using standardized Seleno Excell® high selenium yeast and then applied for the analysis of *Saccharomyces cerevisiae* biofortified at different fermentation and exposure conditions. The procedure was capable of detecting differences in selenium concentration among cultures and the results were consistent with those obtain while coupling HPLC separation directly to ICP-MS detection.

Keywords: selenomethionine, *Saccharomyces cerevisiae*, liquid chromatography, hydride generation, microwave induced nitrogen plasma - atomic emission spectrometry (MP-AES), ICP-MS

1. Introduction

The importance of selenium in human health and nutrition has long been recognized. Different Se-containing dietary supplements are in use and among them, biofortified yeast is the most common.[1, 2] Numerous experimental studies and clinical trials have proved chemopreventive and anti-tumor activity of selenized yeast.[3-5] Administration of Se-yeast in form of different dietary supplements or its incorporation into specific foods has been widely approved, and it was concluded that a daily element intake of about 100 μg does not present a safety hazard; at the same time, a Tolerable Upper Intake Level was set at 300 μg per day.[6, 7] In addition to the human supplementation, Se-yeast has been accepted as an additive for animal feed, so the overall market demand is quite large.[6] Commercial products are obtained from cultures of bakers´s yeast (*Saccharomyces cerevisiae*) that are grown in the presence of sodium selenite (Se(IV)), then are washed to remove residual inorganic selenium and other free minerals, and finally the biomass is pasteurized and dried to obtain homogenous powder.[6, 8] Depending on the strain type, fermentation conditions (medium, pH, temperature, aeration) and Se(IV) concentration, selenized yeast may contain up to 3 mg selenium per gram of the dry mass.[9] Speciation studies have often been undertaken with the aim to understand the incorporation pathways and to fully characterize all selenium compounds in biofortified yeast.[10-12] The reported species identity and their abundance have varied for different protocols applied in the fabrication and analysis of Se-yeast, indicating a need for the implementation of quality control schemes.[2, 13]

The primary organic, bioavailable and beneficial selenium compound in yeast is selenomethionine (SeMet) incorporated to proteins hence its reliable quantification has become the essential part of quality control; it should also be assured that the residual inorganic selenium has been efficiently eliminated. Specifically, Se-yeast of good quality does not contain more than 1% of inorganic Se whereas SeMet analyzed in enzymatic or acid digest accounts to 60 - 90% of the total selenium[9]; these specifications dictate analytical requirements that should be met by any procedure employed in the quality control.

Beyond any doubt, procedures based on the hyphenation of gas or liquid chromatography with species-specific or unspecific isotope dilution - inductively coupled plasma mass spectrometry provide accurate and precise quantification results[14-19]; however, they are still too demanding to be implemented in routine analysis. Simplification and miniaturization have been approached in

several studies with an emphasis mainly on the sample pretreatment. In this regard, microwave assisted acid- or enzymatic digestion as well as ultrasonic enzymatic hydrolysis have been explored[18, 20]; enzymatic hydrolysis was also carried out on a miniaturized microtiter plate.[21] On the other hand, less sophisticated and cost-effective techniques with respect to ICP-MS have been used as the detection systems for liquid chromatography.[22] To compensate for lower detection power of these techniques and to avoid matrix interferences, post-column hydride generation has been integrated while using atomic fluorescence, atomic emission or atomic absorption spectrometry.[22-26] In such approach, each selenium species eluting from the column has to be converted to Se(IV), because only this species forms hydrogen selenide in the reaction with sodium borohydride. For the oxidation of organic species, potassium persulfate in alkaline medium or the mixture of hydrogen bromide/potassium bromate can be used, the resulting Se(VI) is usually reduced to Se(IV) by heating with hydrochloric acid 6 -10 mol L^{-1}; alternatively, UV irradiation has often been employed with addition of alkaline potassium iodide for the reduction of Se(VI) to Se(IV).[24, 26-29]

Post-column hydride generation has proved to be a promising tool for efficient introduction of selenium species to a nitrogen microwave induced plasma with Okamoto cavity - mass spectrometry.[30] More recently, Hammer has developed a magnetically excited microwave plasma emission source[31], which is now used in a commercially available instrument (microwave nitrogen plasma – atomic emission spectrometry, MP-AES). In this system, nitrogen is supplied via air generator, which drastically lowers the operation cost as compared to any other atomic spectrometry technique. Robust diatomic gas (N_2) plasma of toroidal shape is generated with a conventional torch and the introduction of sample aerosol into the core of the plasma occurs through the central canal, in a similar way as in ICP nebulizer and spray chamber configuration. The feasibility of HPLC - MP-AES coupling for speciation analysis has recently been demonstrated[32, 33]; however poor detection power is an important limitation of this hyphenated technique. In line with many earlier studies[28, 34], post-column hydride generation might be helpful for enhanced transport efficiency of column effluent to the plasma and also for the elimination of chemical matrix, especially troublesome at the most intense selenium emission line (196.026 nm).

Due to the high demand on the market, many manufacturers produce Se-yeast and need suitable analytical tools that would be helpful during optimization of the production process and

could also be used for quality control purposes. Specifically, the determination of SeMet and Se(IV) by means of a simple and cost-effective procedure is required and for this purpose, ion-pair reversed phase liquid chromatography separation was coupled in this work with MP-AES detection via hydride generation for the first time (HPLC-HG-MP-AES). For comparative purposes, ICP-MS was used as an alternative element specific detection system, and the results obtained by the two procedures were in good agreement. The proposed procedure enabled for detecting differences in Se(IV) and SeMet concentrations among yeast biofortified at different fermentation conditions, which demonstrates its utility in the quality control schemes.

2. Experimental

2.1. Apparatus

An Agilent Series 1050 liquid chromatographic system controlled by Chemstation (Agilent Technologies) was used with Gemini C18 column (50 x 3 mm, 5μm) from Phenomenex. Column effluent was transported via PEEK® tubing and typical FIA tubing (0.5 mm i.d.) to the multimode sample introduction system (MSIS) of Agilent 4100 MP-AES nitrogen microwave plasma atomic emission spectrometer controlled by Agilent MP Expert Software (actualized from MP-AES 4200). Two T-joints were incorporated between column exit and MSIS for on-line introduction of the oxidizing agent and hydrochloric acid, respectively. After mixing with oxidizing agent, a reaction coil (100 cm) was integrated and it was submerged in a water bath (60 °C). The two reagents and the solution of sodium borohydride were pumped by MP-AES peristaltic pump, each of them at the flow rate 1 mL min^{-1}. Tygon peristaltic tubing (0.76 cm i.d.) was used. The above set-up is schematically presented in Figure 1.

An Agilent Series 1050 system was coupled directly to inductively coupled plasma mass spectrometry detection, via the short-length Teflon tubing. A model 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) with a Meinhard nebulizer and Peltier-cooled spray chamber (2 °C) was used. The analytical column was Polaris C18 column (100 x 3 mm, 3 μm) from Varian.

2.2. Reagents and samples

All chemicals were of analytical reagent grade. Deionized water (18.2 MΩ cm, Labconco, USA) and HPLC-grade methanol (Fisher Scientific, Pittsburgh, USA) were used throughout.

Stock solutions of selenomethionine (SeMet) and sodium selenite (Se(IV)) were prepared by dissolving respective Sigma standards in deionized water. Other Sigma reagents used in this work were: potassium persulfate, sodium hydroxide, methanesulfonic acid, hydrochloric acid, nitric acid, heptafluorobutyric acid (HFBA), sodium borohydride, β-mercaptoethanol and hydride peroxide. An Agilent Technology mixed internal standard solution was also used (4 mg L^{-1} In, 20 mg L^{-1} Li, 4 mg L^{-1} Y, 4 mg L^{-1} Bi, 10 mg L^{-1} Sc, 2 mg L^{-1} Rh).

Commercial high selenium yeast SelenoExcell®, containing 1255 μg g^{-1} Se, was from Cypress Systems, Inc.

Selenium biofortified yeast samples were obtained in the frame of on-going study performed in the Department of Biology, University of Guanajuato (Juan Carlos Torres Guzman, unpublished data). In brief, different cultures of *Saccharomyces cerevisiae* were obtained for a series of fermentation and exposure conditions. In the first approach $2x10^7$ cells mL^{-1} from *S. cerevisiae* strain (CC) were growth at 28 °C during 18 h with agitation, in an Erlenmeyer flask containing 50 mL of minimal medium (6.7 g L^{-1} Yeast Nitrogen Base without amino acids, Difco; 40 g L^{-1} Dextrose, BD Bioxon) in the presence of 0, 5, 10, 15, 20 and 30 mg L^{-1} Se, added in form of sodium selenite (Sigma-Aldrich). The cells were recovered by centrifugation and washed with deionized water, these samples were encoded as C0, C5, C10, C20, C30, respectively. The second series of samples was obtained from *S. cerevisiae* strains CC, S, M and R (the last three isolated from strain CC by directed evolution strategy) that were incubated in the same conditions in minimal medium in presence of 10 mg L^{-1} Se added in form of sodium selenite. The obtained yeast cultures were recovered by centrifugation and were washed with deionized water. These samples were denoted as CC10, S10, M10 and R10, respectively. Finally, all samples were lyophilized in a FreeZone 6 Freeze Dry System (Labconco) prior to analysis.

2.3. Determination of total selenium in yeast by ICP-MS

An aliquot (25 mg) of each biomass was placed in polypropylene eppendorf tube, 750 μL of internal standard mix and 250 μL of concentrated nitric acid were added, and the sample was heated at 110 °C during 1.5 h. After cooling to room temperature, 500 μL of hydrogen peroxide 30% m/m were added and the sample was kept at 110 °C during 1 h. Finally, the volume was adjusted to 1.5 mL, 25 μL were taken and diluted to 5 mL. The ICP-MS operating conditions were as follows: forward power 1500 W, plasma gas flow rate 15 L min^{-1}, carrier gas flow rate 0.89 L min^{-1}, make-up gas flow rate 0.15 L min^{-1}, sampling depth 8 mm, platinum sampling and skimmer cones, dwell time 100 ms per isotope, collision/reaction cell gas He, 4.5 mL min^{-1}. The isotopes ^{78}Se, ^{82}Se were monitored (^{115}In as IS). Five points external calibration was performed at element concentration levels 0 - 100 μg L^{-1} with the addition of the internal standard (4.0 μg L^{-1} In). The linear regression functions were obtained ($r^2 > 0.999$) and the evaluated instrumental detection limit for ^{78}Se was 25 ng L^{-1}. For accuracy checking, Seleno Excell® was analyzed.

2.4. Hydrolysis of yeast for speciation analysis

The procedure described previously was used.[35] To a 100 mg aliquot of the biomass, 2 mL of methanesulfonic acid 4 mol L^{-1} were added and the mixture was heated at reflux during 16 h (120 °C). The obtained hydrolyzate was evaporated and the residue was re-suspended in 10 mL of HFBA 0.08% m/v for MP-AES detection and in 10 mL of HFBA 0.1% m/v for ICP-MS. For each sample, hydrolysis was carried out in triplicate. Noteworthy, methanesulfonic acid hydrolysis was selected due to the efficient release of Se-methionine from proteins, as demonstrated elsewhere.[35, 36]

2.5. Selenium speciation by HPLC-HG-MP-AES

Separation was achieved on a reversed phase column using heptafluorobuturic acid as an ion-pair reagent. Prior to hydride generation, column effluent was mixed with alkaline solution of potassium persulfate, heated in a water bath at 60 °C and then hydrochloric acid was added, as shown in Fig. 1. In the MP-AES multimode sample introduction system, the column effluent was

introduced through the lower channel, sodium borohydride was pumped through the upper channel and nebulization gas was introduced perpendicularly. The chromatographic, hydride generation and MP-AES instrument operating conditions are given in Table 1. The chromatographic run and the detection system were started simultaneously; the raw data from continuous acquisition were saved as Microsoft Excel file for further treatment in GRAMS 5.0 (Thermo Scientific). For each chromatogram, binominal filtration and extrapolation (32 points) were applied, followed by Fourier filtration 97%. For external calibration, eight solutions containing Se(IV) 0; 0.25; 0.5; 1.0; 2.0; 3.0; 4.0; 5.0 mgSe $L^{-1}$and SeMet 0; 2.5; 5; 10; 20; 30; 40; 50 mgSe L^{-1} were used. The re-suspended yeast samples (10 mL) were filtered (0.22-µm Whatman filters) prior to their injection to the chromatographic system. For the recovery experiment, Seleno Excell® digest was evaporated and re-suspended in 5 mL of HHBA 0.08% m/v. Two aliquots of 1 mL were taken, one was diluted 1:1 with HFBA and the second was spiked with 500 µL of SeMet 40 mgSe L^{-1}, 100 µL of Se(IV) 40 mgSe L^{-1} and brought to 2 mL with HFBA. Addition of two standards corresponded to 200 µgSe g^{-1} of Se(IV) and 1000 µgSe g^{-1} of SeMet in the yeast biomass.

2.6. Selenium speciation by HPLC-ICP-MS

Separation was achieved on a reversed phase Polaris C18 column; isocratic elution with HFBA 0.1% m/v : methanol (95:5) was carried out at the flow rate 1 mL min^{-1} and the injection volume was 20 µL. ICP-MS operating conditions were these same as for total Se determination except for carrier gas flow rate 0.75 L min^{-1} and make-up gas flow rate 0.10 L min^{-1}. The isotopes ^{78}Se and ^{82}Se were monitored. For external calibration, a series of mixed Se(IV) and SeMet standard solutions was used, both species at the concentrations 0, 10, 20, 50, 100, 250, 500 µgSe L^{-1} . Linear regression functions were obtained for peak area measurements, yielding good linearity (r^2 > 0.999). The on-column detection limits (^{78}Se) were 0.76 µgSe L^{-1} for Se(IV) and 0.38 µgSe L^{-1} for SeMet, respectively. The re-suspended yeast samples were 40 times diluted and filtered (0.22-µm Whatman filters) prior to their injection to the chromatographic system. For the recovery experiment, 250 µL aliquot of the re-suspended Selen Excell® digest was taken, 500 µL of Se(IV) 750 µgSe L^{-1} and 500 µL of SeMet 2.5 mgSe L^{-1} were added and the volume was brought to 10 mL with HFBA; by so doing standard addition corresponded to 150 µgSe g^{-1} of

Se(IV) and 500 μgSe g^{-1} of SeMet in the biomass. All results are presented as μg of selenium per gram of the freeze-dried biomass.

3. Results and discussion

3.1. Method development

Direct introduction of Se(IV) or SeMet standard solution to MP-AES was tested and by so doing, selenium at concentration 5 mg L^{-1} was not even detected, which confirmed the need for post-column hydride generation. Se-yeast contain low concentration of Se(IV) which is efficiently converted to H_2Se in the presence of sodium borohydride, so a desired sensitivity enhancement was expected for this species in the proposed system. On the other part, SeMet is the most abundant Se compound and improving the detection power for this species was less critical. As briefly described in the Introduction, quantitative conversion of SeMet to Se(VI) and then to Se(IV) for hydrogen selenide generation is not straightforward and requires relatively harsh chemical conditions with prolonged heating, sonication and/or UV irradiation.[24, 26-29] To simplify this step, a direct oxidation of SeMet to Se(IV) was tested by using alkaline solution of sodium persulfate [29], expecting incomplete but reproducible conversion.

The conversion of SeMet to Se(IV) was examined using a set-up presented in Figure 1, but without chromatographic column. An aliquot (100 μL) of SeMet standard solution (5 mgSe L^{-1}) was repeatedly injected to the flow system, while varying the following parameters: concentration of potassium persulfate (2 - 8% m/v), length of the reaction coil (50 - 150 cm), temperature (20 - 80 °C), concentration of hydrochloric acid (6 - 10 mol L^{-1}) and concentration of sodium borohydride (1 - 4 % m/v). For comparative purposes, these same conditions were also tested for Se(IV), 5 mgSe L^{-1}. The selection criterions applied were: (1) as high as possible analytical signal and (2) as low as possible standard deviation obtained in replicate injections; in these experiments analytical signal was acquired as the peak area using manually selected MP-AES operating conditions listed in Table 1. As expected, the most important variables were those related with the oxidation step; in the absence of potassium persulfate, SeMet did not produce any signal whereas the highest and the most reproducible signal was obtained using 6% m/v persulfate. At this concentration, Se(IV) signal was 20.7% lower as compared to that obtained

without addition of persulfate. Noteworthy, when standard solution was introduced directly to MP-AES, Se(IV) 5 mg L^{-1} was not detected. Furthermore, analytical signal of SeMet gradually augmented when temperature and the length of reaction coil were increased; however the signal improvement was accompanied by peak broadening and also by sensitivity loss for Se(IV) so the compromise conditions had to be adopted. It should be stressed that the addition of oxidizing agent after elution of Se(IV) was not considered in order to keep the flow system as simple as possible (persulfate, hydrochloric acid and borohydride solutions were delivered by the MP-AES peristaltic pump). Other variables (hydrochloric acid and borohydride concentrations) were less critical and had similar effect on the signals of two Se species. The finally selected parameters are given in Table 1 and in Figure 2, typical records of three successive injections are presented for Se(IV) and SeMet. For Se(IV) 5 mg L^{-1}, average peak area was 58299 (RSD = 2.2%) and for this same concentration of SeMet average peak area was 8182 (RSD = 2.6%). For five between-day replicates the respective relative standard deviations were 2.6% and 3.1%. The signal for SeMet was about seven times lower than for Se(IV) but it presented acceptable repeatability both in successive injections and for between-days measurements.

Based on many earlier studies[12, 37], anion exchange and ion-pair reversed phase separations were considered and in the first approach, possible effect of the mobile phase composition on the analytical signal for SeMet and Se(IV) was examined in FIA system. Phosphate buffer at pH 6.8 (25 - 100 mmol L^{-1}), typically used in anion exchange separations, caused important depression of SeMet signal even at the lowest concentration tested (about 30% decrease). On the other part, heptafluorobutyric acid (0.05 – 0.2% m/v) did not affect selenium signal for any species, therefore it was decided to use a reversed phase column. It was also verified that up to 15% v/v of methanol could be safely added to the mobile phase without deterioration of analytical signals. Isocratic separation was carried out on Gemini C18 (50 x 3 mm, 5μm) column using the conditions listed in Table 1; Se(IV) eluted with the retention time 1.16 ± 0.05 min and SeMet 2.97 ± 0.07 min; total chromatographic run was 5 min.

3.2. Method validation

In Figure 3, typical chromatograms obtained for the calibration solutions are presented. For six independent calibration processes, linear regression functions were: $A_{Se} = 2862.8c_{Se(IV)} - 238.6$

and $A_{Se} = 451.78c_{SeMet} + 644.18$ with $r^2 > 0.998$ (A_{Se} - peak area; $c_{SeMet}/c_{Se(IV)}$ - concentrations as mgSe L^{-1}). The detection limits (DL) and quantification limits (QL) were evaluated based on the signal-to-noise ratio; the criterion of three and ten standard deviations was adopted, using the Se signal obtained for the lowest calibration standard for each selenium species.[38] The obtained calibration DLs were 18 μgSe L^{-1} for Se(IV) and 0.16 mgSe L^{-1} for SeMet; the respective QL values were 59 μgSe L^{-1} and 0.52 mgSe L^{-1}. To evaluate method DLs and QLs, baseline was acquired from the chromatogram of control biomass (C0) and this sample was analyzed after addition of the lowest calibration standard of the two species. The obtained DL and QL for Se(IV) were 3.7 μgSe g^{-1} and 11.9 μgSe g^{-1}, respectively, whereas for SeMet these values were 32 μgSe g^{-1} and 104 μgSe g^{-1}. For five between-day replicates, the lowest RSD for analytical signals of both species was obtained in the concentration range 10 - 40 mgSe L^{-1}; < 1.5% for SeMet and < 2.9% for Se(IV). The above analytical parameters were suitable for the application of the proposed procedure in the analysis of Se-yeast digests.

The procedure was tested by analyzing standardized Seleno Excell® yeast, which had been evaluated and determined to be Generally Recognized As Safe (GRAS) in accordance with the Federal Food, Drug, and Cosmetic Act.[7] This product was used by Clark et al. in the first clinical trial that demonstrated the potential of Se-yeast in cancer prevention.[39] Total Se determined in HNO_3/H_2O_2 digested biomass was 1289 ± 33 μgSe g^{-1} (n =3), in agreement with the 1255 μgSe g^{-1} reported by the manufacturer. The concentration found in methanesulfonic acid hydrolyzate was 1293 ± 39 μgSe g^{-1}, confirming efficient solubilization of all selenium forms in this sample. In Figure 4a two chromatograms are presented that were obtained for Seleno Excell® and for this same sample after standard addition, the respective quantification results are given in Table 2. Of note, more complex composition of yeast hydrolyzate as compared to the calibration solutions caused slight decrease of the retention times for both species (compare Fig. 3 and Fig. 4).

For further accuracy checking, methanesulfonic hydrolyzate of Seleno Excell® was also analyzed by HPLC-ICP-MS procedure as described in section 2.6. Two chromatograms corresponding to the spiked and non-spiked sample are shown in Figure 5a, whereas the quantification results are included in Table 2. The elution of several minor species, other than Se(IV) and SeMet is observed in Fig. 5a, in agreement with many earlier reports.[9, 10] Based on HPLC-ICP-MS results, the sum of SeMet and Se(IV) in non-spiked sample accounted for 81.6% of total acid-digested selenium, whereas for HPLC-HG-MP-AES this percentage 82.3%.

Previously reported data for Seleno Excell indicated 84% SeMet and below 1% of Se(IV) as refereed to total Se.[7, 9] Recovery results obtained for the two procedures after standard addition were in the range 98.7 -104.1% (Table 2); highly consistent speciation results obtained for the control sample by two different procedures demonstrates suitability of HPLC-HG-MP-AES system for the determination of SeMet and Se(IV) in Se-biofortified yeast.

3.3. Application for the analysis of different yeast cultures

A series of *S. cerevisiae* cultures were obtained in the Biology Department of the University of Guanajuato as shortly described in section 2.2. Results of Se determination in yeast digested with HNO_3/H_2O_2 and hydrolyzed with methanesulfonic acid are shown in Figure 6. For the first experiment (samples C0 - C30), total acid-digested Se increased accordingly to the increasing Se(IV) concentration added to the growth medium, whereas in methanesulfonic acid hydrolyzed samples this tendency was much less marked. Noteworthy, starting from Se(IV) concentration 15 mg L^{-1} (C15), yeast biomass was pink, likely revealing the presence of elemental selenium. Therefore, the difference observed between two treatments was indicative of the amount of Se^0 in the sample and Se concentration in methanesulfonic acid hydrolyzate corresponded to the total of other than Se^0 element forms incorporated to the biomass. In the second experiment, varying growth conditions were used while keeping the same concentration of Se(IV) in medium (10 mg L^{-1}), none of these cultures presented pink color. It can be observed that for the series of samples R10, M10, N10 and CC10, no important differences in Se concentrations were found between two treatments and the results were similar to those obtained for Seleno Excell® (Fig. 6), which indicates efficient incorporation of element in yeast. Speciation analysis in the yeast methanesulfonic acid hydrolyzates was carried out by HPLC-HG-MP-AES and by HPLC-ICP-MS; typical chromatograms obtained for two different cultures (C10, C20) are presented in Fig. 4b and in Fig. 5b, respectively. The concentration-dependent chromatographic signals of Se(IV) and SeMet are clearly observed in both systems; however, more sensitive HPLC-ICP-MS procedure allowed for the detection of several other, minor Se compounds (similarly as in Fig. 5a). Quantification results for SeMet and Se(IV) are summarized in Table 3, these data show good agreement between two different speciation systems which reaffirms validity of the proposed here procedure. It should be stressed that the percentage amount of individual species

and of their sum with respect to total HNO_3/H_2O_2 digested Se, were also consistent between the two procedures (Table 3). On the other part, the concentrations of SeMet and Se(IV) varied, depending on the growing and exposure conditions applied. In the first series, SeMet was relatively stable within a range 10 - 20 mgSe L^{-1} of Se(IV) in medium; however, increasing concentrations of Se(IV) in medium were accompanied by gradual buildup of this species in yeast. In the second series, CC10 was obtained using these same growth and exposure conditions as C10 in the first series; total Se concentrations and speciation results were consistent for these two samples, indicating good repeatability of microbiological experiment and also good repeatability of analytical results. The samples R10 and N10 corresponded to two different yeast strains, lower efficiency of Se incorporation was expected in these cultures and indeed the lowest total Se and SeMet were found (Table 3, Fig. 6). Finally, M10 sample contained the highest SeMet concentration, which was also expected because it was obtained from yeast grown by directed evolution strategy in minimal medium, deprived of amino acids. In this sample, the SeMet and total Se concentrations were slightly higher as compared to Seleno Excell®, yet respective percentages of SeMet in biomass were very similar in these two materials (81.5% versus 81.0% according to the proposed procedure and 81.9% versus 80.4% according to HPLC-ICP-MS; Table 3). Experimental data obtained in this work do not enable for any biological interpretation; however the above discussion proves the feasibility of HPLC-HG-MP-AES in refining experimental conditions for the production of Se-yeast of good quality. In particular, this procedure was capable of detecting differences of SeMet and Se(IV) concentrations among samples obtained from different yeast cultures, hence it meets the requirements of sensitivity and detection power dictated by this analytical task.

4. Conclusions

In this work, HPLC-HG-MP-AES system was used for the first time for the speciation analysis in the real-world samples. In particular, a procedure for the determination of SeMet and Se(IV) in biofortified yeast has been developed. Prior to the post-column hydride generation, relatively mild oxidation conditions ($K_2S_2O_8$ 6% m/v + NaOH 3% m/v, 100 cm reaction coil, 60 °C) were applied thus achieving H_2Se formation from both species; HG was necessary to enhance the detection power and to avoid the introduction of sample matrix to MP-AES that was tuned to the

emission line highly susceptible to interferences (196.026 nm). The compromise conditions applied allowed for the method quantification limits 11.9 μgSe g^{-1} and 104 μgSe g^{-1} for Se(IV) and SeMet, respectively. Therefore, the proposed procedure is well suited for the determination of the two species in Se-yeast of good quality that should contain 60-90% SeMet and below 1% of Se(IV) with respect to total Se content in biomass (1255 μgSe g^{-1} in Seleno Excell®). Furthermore, by analyzing two series of *S. cerevisiae* cultures biofortified at different growth and exposure conditions, the results were consistent with those obtained by HPLC-ICP-MS system. In summary, it was demonstrated that the proposed here simple and cost-effective HPLC-HG-MP-AES procedure can be helpful during optimization of the production process of selenized yeast and might be incorporated in the quality control scheme.

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Table 1 Instrumental operating conditions of the HPLC-HG-MP-AES system.

Chromatographic conditions	
Column	Gemini C18 (50 x 3 mm, 5μm), Phenomenex
Mobile phase	Heptafluorobutyric acid 0.08% m/v : methanol (92:8)
Flow rate	1 mL min^{-1}
Injection volume	100 μL
On-line treatment of the column effluent and hydride generation	
Oxidation agent[a]	$K_2S_2O_8$ 6% m/v + NaOH 3% m/v, 1 mL min^{-1}
Reaction coil	100 cm long, 0.4 mm i.d., 60 °C
Acidification[a]	HCl 10 mol L^{-1}, 1 mL min^{-1}
Hydride generation[a]	$NaBH_4$ 2% m/v + NaOH 0.3% m/v, 1 mL min^{-1}
MP-AES detection	
Nitrogen	140 psi
Air injection flow rate	Low
Nebulizer / spray chamber	MiraMist Teflon® / multimode sample introduction system
Nebulizer pressure	165 kPa
Viewing position	12
Acquisition mode	Continuum
Integration time	5s
Stop time / post time	20 min / 2 min
Detection wavelength	196.026 nm
Background correction	Off-peak left+right
Number of pixels	3

[a] – these solutions were carried via MP-AES peristaltic pump

Table 2 Determination of SeMet and Se(IV) in Seleno Excell® and percentage recoveries obtained in the method of standard addition by the proposed HPLC-HG-MP-AES procedure (1) and by HPLC-ICP-MS (2) (means and standard deviations based on n=3).

	SeMet			Se(IV)		
Procedure	Added, μgSe g^{-1}	Found ± SD, μgSe g^{-1}	Recovery, %	Added, μgSe g^{-1}	Found± SD, μgSe g^{-1}	Recovery, %
1	0	1044 ± 42		0	16.5 ± 1.5	
	1000	2085 ± 49	104.1	200	221 ± 18	102.2
2	0	1036 ± 37		0	15.5 ± 1.4	
	500	1529 ± 46	98.7	150	169 ± 15	102.6

Table 3 Determination of SeMet and Se(IV) in *S. cerevisiae* cultures obtained under different growth and exposure conditions by two different speciation procedures (HPLC-HG-MP-AES; HPLC-ICP-MS).

Yeast culture	SeMet		Se(IV)		SeMet+Se(IV)
	mean ± SD μgSe g^{-1}	% Se in biomass	mean ± SD μgSe g^{-1}	% Se in biomass	in hydrolyzate, % Se
HPLC-HG-MP-AES					
C0	nd	-	nd	-	-
C5	322 ± 25	73.3	58.1 ± 2.7	13.2	99.5
C10	1035 ± 41	73.5	98.1 ± 4.2	7.0	88.7
C15	1044 ± 37	33.1	202 ± 5	6.4	92.2
C20	1078 ± 42	81.0	675 ± 37	1.3	92.6
C30	969 ± 23	15.1	989 ± 39	15.5	85.5
R10	979 ± 21	71.7	108 ± 10	7.9	95.3
M10	1275 ± 31	81.5	129 ± 13	8.2	94.8
N10	791 ± 32	54.6	139 ± 18	9.6	70.7
CC10	1041 ± 39	71.7	114 ± 10	7.9	86.5
Excell	1044 ± 42	81.0	16.5 ± 1.5	1.3	82.0
HPLC-ICP-MS					
C0	7.3 ± 0.4	60.5	nd	-	62.6
C5	318 ± 23	72.5	59.7 ± 2.8	13.7	98.9
C10	1038 ± 34	73.7	96.7 ± 3.7	6.9	88.7
C15	1056 ± 42	33.5	210 ± 5	6.7	93.6
C20	1082 ± 33	19.8	663 ± 32	12.1	92.1
C30	976 ± 21	15.3	973 ± 41	15.2	85.1
R10	964 ± 24	70.6	105 ± 9	7.7	93.6
M10	1281 ± 38	81.9	121 ± 11	7.7	94.7
N10	802 ± 35	55.4	133 ± 16	9.2	71.1
CC10	1050 ± 42	72.4	110 ± 8	7.6	86.9
Excell	1036 ± 37	80.4	15.5 ± 1.4	1.2	81.4

Figure captions:

Fig.1. General scheme of HPLC-HG-MP-AES coupling.

Fig. 2. Successive FIA signals acquired for Se(IV) and SeMet (5 mgSe L^{-1} each) using hydride generation conditions listed in Table 1.

Fig. 3. Typical HPLC-HG-MP-AES chromatograms obtained for calibration solutions (0; 0.25; 0.5; 1.0; 2.0; 3.0; 4.0; 5.0 mgSe L^{-1} for Se(IV) and 2.5; 5; 10; 20; 30; 40; 50 mgSe L^{-1} for SeMet.

Fig. 4. HPLC-HG-MP-AES chromatograms of methanesulfonic acid hydrolyzates of yeast:
(a) (——) Seleno Excell and (----) this same sample after standard addition (details given in section 2.5)
(b) (——) sample C10, (----) sample C20.

Fig. 5. HPLC-ICP-MS chromatograms of yeast hydrolyzates (methanesulfonic acid):
(a) (——) Seleno Excell and (----) this same sample after standard addition (details given in section 2.5)
(b) (——) sample C10, (----) sample C20.

Fig. 6. Total selenium concentrations found in HNO_3/H_2O_2 digests and methanesulfonic acid hydrolyzates of different yeast cultures