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Development of new analytical methods for selenium speciation in selenium-enriched yeast material

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Abstract

A sequential extraction allowing the discrimination of water-soluble and non-soluble selenium fractions has been developed to evaluate the availability of selenium (Se) in an Se-enriched yeast candidate reference material. The fractionation of selenium-containing compounds in the extracts was achieved on preparative grade Superdex 75 and 200 columns. It showed that water-soluble selenium is present in several fractions with a large mass distribution. Low-molecular-($\leq$10 000) and high-molecular-mass selenocompounds (range 10 000–100 000) were considered separately for further experiments. The analytical approach for low-molecular-mass selenocompounds was based on anion-exchange HPLC with on-line inductively coupled plasma (ICP) MS for quantitative analysis. Selenocystine, selenomethionine, selenite and selenate were quantified in the fractions isolated in preparative chromatography. The study revealed the existence of various unidentified Se species in yeast material. The Se-containing proteins in the yeast material have been further separated and selenium quantified by the combination of gel electrophoresis and electrothermal vaporization–ICP-MS. This new approach allows the separation of the proteins with high resolution by sodium dodecylsulfate–polyacrylamide gel electrophoresis and the sensitive determination of selenium in the protein bands.

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1. Introduction

The safety of food and the health of European citizens are now recognized as high priority issues. Consequently there is increased dependence on monitoring trace elements in the environment and food. Selenium (Se) is a micronutrient for human and animals and their main source of Se is food. However, Se is both essential and toxic depending on its chemical form and concentration and it has the narrowest tolerance band of any element. Evidence is growing that Se-enriched derivatives prevent Se deficiencies and also provide protection against various forms of cancers [1–3]. The form in which Se is absorbed by the body is of fundamental importance.

Since the bioavailability and the toxicity of Se are closely correlated with its chemical forms, information on speciation [4,5] in natural products is vital. For these reasons the demand for accurate and sensitive methods for Se speciation in nutritional supplements, e.g. Se-enriched yeast, has rapidly increased. There is substantial evidence of the complexity of selenium speciation in yeast and of the

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importance of the selective determination of the particular species of this element in order to understand its metabolism and biological significance [6].

Under appropriate conditions, yeasts (*Saccharomyces cerevisiae*) are capable of accumulating large amount of trace elements, such as selenium, and incorporating them into organic compounds [7]. It has been found that the introduction of water-soluble selenium salt (as selenite) as a component of the culture medium for yeasts produced by conventional batch processing results in a substantial amount of selenium being absorbed by the yeast [7]. Using a culture medium supplemented with 30 μg/ml selenite added during the exponential growth phase results in selenium accumulation in the range of 1200–1400 μg/g dried yeast material. A candidate reference material produced by a pharmaceutical company is used to develop methods for selenium speciation, in particular to enable accurate measurement of the beneficial selenomethionine and toxic inorganic selenium compounds and for the investigation of unknown selenium species.

The objective of this work is to develop and to evaluate a sequential extraction procedure for the complete solubility and the recovery of selenium present in the yeast candidate reference material. The fractionation of selenium compounds in yeast extracts is achieved by preparative size-exclusion chromatography. Anion-exchange chromatography with online inductively coupled plasma (ICP) MS detection is developed to monitor the speciation of selenium in low-molecular-mass selenium fractions. Further separation and determination of Se-containing proteins in yeast has been recently applied [8]. This approach includes protein separation by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and sensitive and precise determination of selenium in the protein bands by electrothermal vaporization (ETV) ICP-MS.

2. Experimental

2.1. Samples

A sample of industrially produced selenium-enriched yeast (*S. cerevisiae* grown in the presence of selenite) material was used for feasibility studies (Pharma Nord, Vejle, Denmark). The total selenium concentration in the lyophilized material given by Pharma Nord is 1300 μg/g.

2.2. Instrumentation

2.2.1. Electrothermal atomic absorption spectrometry (ET-AAS)

A Perkin-Elmer (Uberlingen, Germany) model 5100 ET-AAS instrument was used for selenium quantification. The ET-AAS system includes the concept of transversely heated pyrolytic graphite-coated platform-equipped graphite tubes and incorporates Zeeman effect background correction. An electrodeless discharge lamp (EDL) for selenium was used as an element specific line source at 196 nm.

2.2.2. Preparative chromatography

Size-exclusion preparative chromatography was carried out using an Econo System (Bio-Rad Labs, Richmond, CA, USA) composed of a compact biocompatible gradient pump (Econo Pump model EP-1), a UV monitor model EM-1 and a model 2110 fraction collector. Injections were made using an auto-injection valve model MV-6-6 with a 1.0 ml injection loop. Chromatographic separations were carried out using two different size-exclusion preparative columns, a Superdex 75 HiLoad 16/60 (60 cm×1.6 cm I.D.) and a Superdex 200 HiLoad 16/60 (60 cm×1.6 cm I.D.) (Amersham Biosciences, Sweden).

2.2.3. HPLC–ICP-MS coupling

The ICP-MS (inductively coupled plasma mass spectrometry) instrument used was a Perkin-Elmer ELAN 6000 (PE Sciex, Canada). The ICP-MS was mass tuned with the Perkin-Elmer multielemental tuning solution.

The sample introduction system included a Cinnabar cyclotronic spray chamber fitted with a Micromist nebulizer (Glass Expansion, Romainmotier, Switzerland). For chromatographic experiments a Model Dionex GP40 HPLC pump was used as the sample delivery system (Dionex, Sunnyvale, CA, USA). All the connections were made of polyether ether ketone (PEEK) tubing (0.17 mm I.D.). Anion-exchange chromatography was carried out using a

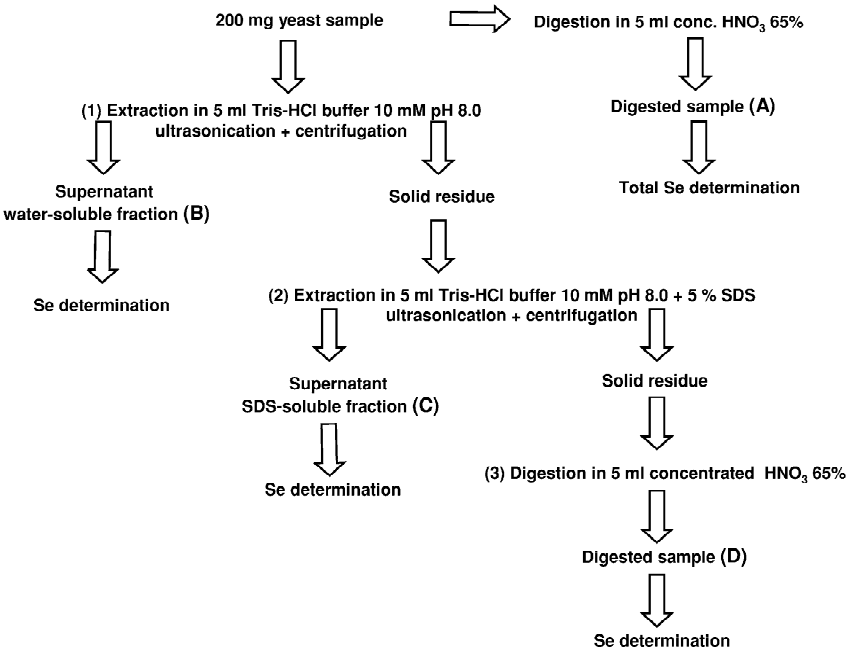

Fig. 1. Total selenium determination and sequential extraction procedure for selenocompounds in yeast material.

microbore Dionex AS10 column (25 cm×2 mm I.D.) with a AG10 guard column (5 cm×2 mm I.D.)

2.2.4. SDS–PAGE

SDS–PAGE was carried out using the following equipment: a horizontal electrophoresis system (Multiphor II Electrophoresis Unit) for running precast polyacrylamide gel (250×110×0.5 mm, T=15%, C=2%)[1], a Hoefer Automated Gel Staining System (Amersham Biosciences). The gels were left to dry and scanned with a densitometry imaging system (Bio-Rad Labs) before quantifying Se in the bands with ETV–ICP-MS.

2.2.5. ETV–ICP-MS

The ICP-MS instrument was a Perkin-Elmer ELAN 5000 (PE Sciex) with modifications described in Chéry et al. [8]. For solid sampling the electrothermal vaporisation system HGA 600 MS was equipped with an autosampler AS-60 (Perkin-Elmer, Uberlingen, Germany) for solid sampling.

[1] T=[g acrylamide+g N,N'-methylenebisacrylamide (Bis)]/100 ml solution; C=g Bis/%T.

2.3. Procedures

2.3.1. Se quantification and sequential extraction of selenocompounds

For total selenium determination in yeast, a sample of ~200 mg (accurately weighed) was digested in 5 ml concentrated HNO_3 for 24 h. A total of ten independent sample digestions were made in parallel. After digestion, the resulting solutions (fraction A in Fig. 1) were diluted for selenium determination by ET-AAS.

An extraction procedure was developed to provide information on selenium solubility in yeast material (protocol shown in Fig. 1). Yeast sample was fractionated by sequential extraction using (1) Tris–HCl buffer (water-soluble fraction), (2) Tris–HCl buffer with SDS (solubilization of the protein fraction) and (3) concentrated HNO_3 (dissolution of the resulting solid residue to recover all the Se present in yeast).

A sample of 200 mg (accurately weighed) was placed in a centrifuge tube and extracted sequentially with:

1. 5 ml of Tris–HCl buffer, 10 mM, pH 8.0. The sample was sonicated by ten 5 s pulses at maximum energy and incubated at room tempera-

ture for 5–10 min. Selenium was determined in the supernatant remaining after centrifugation at 10 000 *g* for 30 min (fraction B in Fig. 1). The final residue was subject to further extraction (2).
2. 5 ml Tris–HCl buffer, 10 m*M*, pH 8.0 containing 5% SDS. Selenium was determined in the supernatant remaining after centrifugation at 10 000 *g* for 30 min (fraction C in Fig. 1). The final residue was subject to the final analysis (3).
3. 5 ml concentrated HNO_3 (65%). The solid residue was completely solubilized after 24 h. Selenium was determined in the obtained solution (fraction D in Fig. 1).

Ten independent extractions were made from the same material and an aliquot of each extract was kept for Se measurement in ET-AAS (ten replicates for each measurement). The ten extracts were pooled and homogenised and aliquots were stored at −22 °C for experiments in preparative chromatography.

2.3.2. Isolation of Se-containing fractions by preparative chromatography

Size-exclusion preparative chromatography was chosen as the technique for preliminary screening of the extracts. Compared to previous work [9], high capacity columns were used for preparative step. Isolation of selenium-containing fractions was performed on Superdex 75 and 200 HiLoad columns. Tris–HCl buffer, 10 m*M*, pH 8.0 was used as an eluent (flow-rate of 0.5 ml/min). An aliquot of 1.0 ml of supernatant obtained after sequential extraction (extracts B and C in Fig. 1) was injected on each column. Fractions of 2.5 ml (time per fraction: 5 min) were collected for a total volume of 225 ml (90 fractions). The absorption of the eluates was monitored on-line at 210 and 280 nm. Selenium concentration was further measured off-line in the fractions by ET-AAS. The recovery of selenium in preparative chromatography was determined using the peak area quantification mode.

The molecular masses of the selenium fractions were evaluated on the columns by comparison with the following markers: M_r=1300 (vitamin B_{12}), 17 000 (myoglobulin), 44 000 (ovalbumin), 158 000 (globulin) and 670 000 (thyroglobulin). The Se-containing proteins ($M_r \geq 10\,000$) and low-molecular-mass fractions ($M_r \leq 5000$) isolated from the Superdex 75 column were pooled separately, frozen and preconcentrated by lyophilization. The resulting lyophilized proteins were stored at −22 °C before analysis.

2.3.3. Separation and detection of Se-containing low-molecular-mass compounds by anion-exchange HPLC–ICP-MS

Experimental conditions for anion-exchange chromatography of selenium species were adapted from our previous works [10,11]. Separation of selenium species was performed on Dionex AS10 column using ammonium phosphate buffer, pH 8.0 as an eluent (flow-rate of 0.25 ml/min). A sample of 10 μl of yeast extract was injected on the column. The eluents used were: (A) 5 m*M* ammonium phosphate buffer, pH 8.0 and (B) 50 m*M* ammonium acetate buffer, pH 8.0. The gradient used to achieve the separation was: 0–7 min: 100% A; 7–8 min: 0–100% B; 8–25 min: 100% B.

The ICP-MS was operated at 1150 W and the ionic lens voltage was adjusted to 9.20 V. The optimal nebulizer gas flow was 0.9 l/min. Se response was monitored for all experiments at masses 78 and 82 with a dwell time of 100 ms, and ^{82}Se was used for the results and calculations.

One of the main difficulties related to Se determination by quadrupole ICP-MS is the formation of monoatomic and polyatomic ions in the argon plasma [12,13]. The major interferences are the formation of the dimers $^{38}Ar^{40}Ar$ and $^{40}Ar_2$ which will overlap with the two most abundant Se isotopes ^{78}Se (23.8% abundance) and ^{80}Se (49.6% abundance), respectively. Interference from $^{38}Ar_2$ also makes it impossible to determine ^{76}Se (9.4% abundance). Additionally, the combination of major constituents of the plasma (argon, hydrogen and oxygen) and elements present in the sample matrix (such as carbon and chloride in the case of a Tris–HCl eluent) may generate polyatomic species with a resulting mass equal to that of the various Se isotopes. The isotopes ^{77}Se and ^{78}Se may be subject to interferences such as $^{40}Ar^{37}Cl$ and $^{40}Ar_2H_2$, respectively. The formation of $C^{35}Cl_2$, due to the presence of C and Cl (from the Tris–HCl) could affect the detection of ^{82}Se. However, this combination is unlikely to occur since an excess of carbon would be combined with oxygen to form CO or

CO_2. The two isotopes ^{78}Se and ^{82}Se have been simultaneously monitored during the chromatographic experiments and the ratio $^{78}Se/^{82}Se$ has been observed for the chromatogram of the blank (acquisition of the numeric signal). The relative sensitivity observed for the two selected isotopes was stable and in agreement with their relative natural abundance, leading to the conclusion that the interference due to the combination of C with another element is negligible.

The standards used for Se quantification in HPLC–ICP-MS were selenomethionine (Se-Met), selenocystine (Se-Cys), selenite (Se^{IV}) and selenate (Se^{VI}) in the concentration range 20–100 ng/ml. The quantification of selenocompounds in low-molecular-mass yeast fractions was carried out by external calibration as peak area measurements.

2.3.4. Protein separation by SDS–PAGE and selenium determination by ETV–ICP-MS

For water- and SDS-soluble high-molecular-mass fractions, protein separation was achieved by SDS–PAGE. The sample consisted of all fractions collected from HPLC with a molecular mass higher than 5000, pooled together, freeze-dried and reconstituted in the application buffer. To protect selenocysteine (likely to be present in the selenium-containing proteins) from oxidation, derivatization with iodoacetic acid was used [14,15].

On a 250×110×0.5 mm SDS–polyacrylamide gel (homogeneous 15: in the separating gel $T=15\%$ and $C=2\%$; separation range 20 000–170 000), 18 lanes were loaded with 10 μl samples further diluted in the application buffer (62.5 m*M* Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue). The approximate molecular masses of the proteins in the gel were estimated by comparison with the following low-molecular-mass protein markers: 14 000, 20 000, 30 000, 43 000, 67 000 and 94 000. To visualize the bands, proteins were silver-stained using the automated staining system [16].

For selenium quantification, the lane of interest was excised every ~5 mm in the direction of migration. The resulting samples (~1 mg dry gel) were put in the oven of the ETV–ICP-MS. An external calibration with internal spike was used. Optimization of the ETV and ICP-MS parameters and calculation of the amount of Se are described in detail elsewhere [8]. The ICP-MS was operated at 1000 W. The coolant and auxiliary argon flow-rates were 15 and 1.2 l/min, respectively. The optimal carrier gas flow was 1.0 l/min. The multi-step program of the solid sampler was: (1) drying step: 10 s ramp to 130 °C, 20 s hold at 130 °C; (2) pyrolysis step: 10 s ramp to 800 °C, 20 s hold at 800 °C; (3) vaporization step at 2500 °C, 10 s hold at 2500 °C; (4) cleaning step: 1 s ramp to 2700 °C, 3 s hold at 2700 °C. Se response was monitored for the isotope ^{82}Se. The sensitivity and spectral interferences in ICP-MS due to the matrix (gel) and the argon carrier gas flow (for solid sampling) are described elsewhere [8].

3. Results and discussion

3.1. Measurement of the solubility of selenium in yeast after sequential extraction

A calibration curve (not shown) was established for the determination of selenium by ET-AAS (investigated range: 0–100 ng/ml Se). The sensitivity (slope of the calibration curve) was $9.49\cdot10^{-4}$ AU per ng/ml and the regression coefficient (R^2) was 0.9990. The detection limits calculated for Se were 3.3±0.2 ng/ml. The results obtained for total selenium determination in yeast sample and quantification after each extraction step are shown in Table 1. The total selenium concentration determined in yeast (sample A in Fig. 1) was 1580±5 μg/g (1300 μg/g given by Pharma Nord).

Only 15.2±1.0% of the selenium present in yeast was extracted in the first extraction step (fraction B in Fig. 1). The compounds recovered included possibly globular selenoproteins (water-soluble) and the weakly bound and water-soluble low-molecular-mass species (selenite (Se^{IV}), selenate (Se^{VI}), seleno-amino acids (Se-Met, Se-Cys), and possibly seleno-glutathion). The recovery of the second extraction step using SDS was 36.8±1.5% of the total Se present in yeast (fraction C in Fig. 1). The recovered quantity obtained after digestion of the final solid residue with HNO_3 (fraction D in Fig. 1) accounts for 39.5±2.0% of the total Se present in yeast. The calculated recovery obtained for the complete sequential extraction was 91.5±1.5% (Table 1).

Table 1
Total Se determination in yeast material and Se quantification after the extraction procedure by ET-AAS

	Se concentration in the extract dil. $\times 100$[a] (ng/ml)	Concentration SD	Se concentration in the material (μg/g)	Concentration SD	Selenium recovery[b] (%)
Digestion with HNO_3	431.30	5.02	1580.05	5.69	–
(1) Extraction with Tris–HCl buffer	63.92	4.29	240.56	16.82	15.2±1.0
(2) Extraction with SDS	154.77	6.43	582.35	23.72	36.8±1.5
(3) Extraction digestion with HNO_3	165.70	8.41	623.51	31.64	39.5±2.0
(1)+(2)+(3)			1446.42	20.53	91.5±1.5

[a] Results for ten independent extractions, ten replicates for each measurement.
[b] Se recovery in the various extracts is calculated taking into account the total Se concentration determined after digestion with HNO_3 (A in Fig. 1).

A number of strong extraction and hydrolysis (using different enzymes) sample treatment procedures were tried in previous studies [17,18]. However none of these approach can be used to assess solubility and consequent availability of a mineral micronutrient in a food supplement. The achievement of this objective turned out to depend critically on the extraction procedure to keep the integrity of the chemical forms present in the yeast material. The gentle sequential extraction developed in our work is a systematic approach to evaluate the solubility and recovery of selenium expected for a nutritional supplement.

3.2. Fractionation of selenium-containing compounds in yeast extracts by preparative chromatography

The fractionation of selenium-containing compounds in the water and SDS extracts was first investigated on preparative grade Superdex 75 column for an evaluation of the mass range.

Fig. 2a shows the chromatographic profile with UV detection and selenium determination by ET-AAS for the injection of 1.0 ml water-soluble extract (B in Fig. 1) (6.39 μg Se injected). It shows that water-soluble selenium is distributed in several fractions with a large mass distribution. High- (fractions 17–33, $100\,000 \geq M_r \geq 10\,000$) and low-molecular-mass compounds (fractions 34–53, $M_r \leq 10\,000$) account, respectively, for 17.7±2.5 and 48.7±2.0% of the Se amount injected on the column, giving a recovery of 66.4±2.2% (HMW and LMW, respectively, in Fig. 2a) for Se after the preparative step (cf. Table 2). Two maximums are observed for selenium in high-molecular-mass fractions with approximate masses of 20 000 and 100 000.

A size-exclusion chromatogram obtained for the injection of 1.0 ml leachate in SDS (extract C in Fig. 1) (15.47 μg Se injected) is shown in Fig. 2b. In this case 57.3±2.1% of Se was found associated with higher molecular mass selenocompounds. These HMW compounds have a narrower mass distribution ($100\,000 \geq M_r \geq 40\,000$) compared to that observed for the water extract (HMW in Fig. 2a). Unresolved peaks are observed with maximums at about 45 000 and 100 000. The chromatographic pattern of the low-molecular-mass fraction was similar to that obtained for the aqueous extract (with a lower intensity) (LMW in Fig. 2a) but corresponded to only 6.2±3.4% of the Se quantity injected. A recovery of 63.6±2.5% (HMW+LMW in Fig. 2b) was obtained for Se after the preparative step (cf. Table 2).

The same fractionation of Se (from extracts B and C in Fig. 1) was further investigated by using a preparative grade Superdex 200 column dedicated to larger molecular masses for a better evaluation of the protein mass range (Fig. 3). Fig. 3a shows a chromatogram obtained for the injection of 1.0 ml of water-soluble extract (B in Fig. 1). For high-molecular-mass Se-containing compounds, fraction proteins are observed in the range 10 000–100 000. An incomplete resolution is obtained and two maximums are observed corresponding to ~20 000 and 100 000. For small compounds the Se profile is similar to that

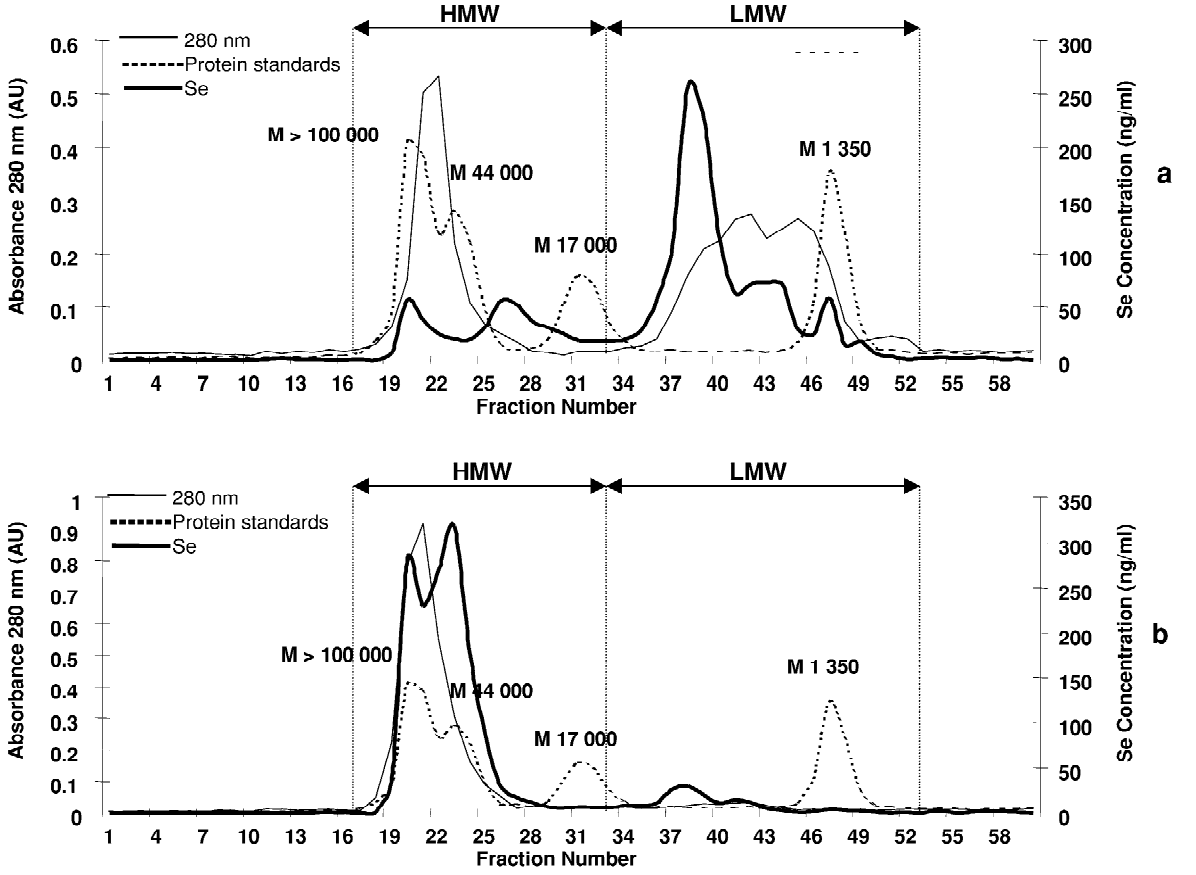

Fig. 2. Fractionation of selenium compounds in yeast extracts by size-exclusion preparative chromatography. Superdex 75 HiLoad column 60 cm×1.6 cm I.D. Injection: 1.0 ml. Mobile phase: 10 m*M* Tris–HCl buffer, pH 8.0. Flow–rate: 0.5 ml/min. Number of collected fractions: 80, time/fraction: 5 min. Fractions measured with UV (280 nm) and ET-AAS detections for Se. (a) Leaching with Tris–HCl (B in Fig. 1) (6.39 μg/ml Se), (b) leaching of the solid residue with SDS (C in Fig. 1) (15.47 μg/ml Se).

Table 2
Se recovery after size-exclusion preparative chromatography of yeast extracts on HiLoad Superdex 75 and 200 columns

Selenium extract	Amount of Se injected (μg)	Se (μg) in fractions 17–33	Se recovery (%)	Se (μg) in fractions 34–53	Se recovery (%)	Total Se recovery (%)
Se fractionation on HiLoad Superdex 75 column (in Fig. 2)						
Fraction in Tris buffer	6.39	1.13	17.7±2.5	3.11	48.7±2.0	66.4±2.2
Fraction dissolved in SDS	15.48	8.88	57.3±2.1	0.97	6.2±3.4	63.6±2.8
Se fractionation on HiLoad Superdex 200 column (in Fig. 3)						
Fraction in Tris buffer	6.39	1.11	17.4±3.2	3.30	50.1±2.2	67.5±2.5
Fraction dissolved in SDS	15.48	8.50	54.8±2.5	0.98	3.6±4.8	61.2±3.1

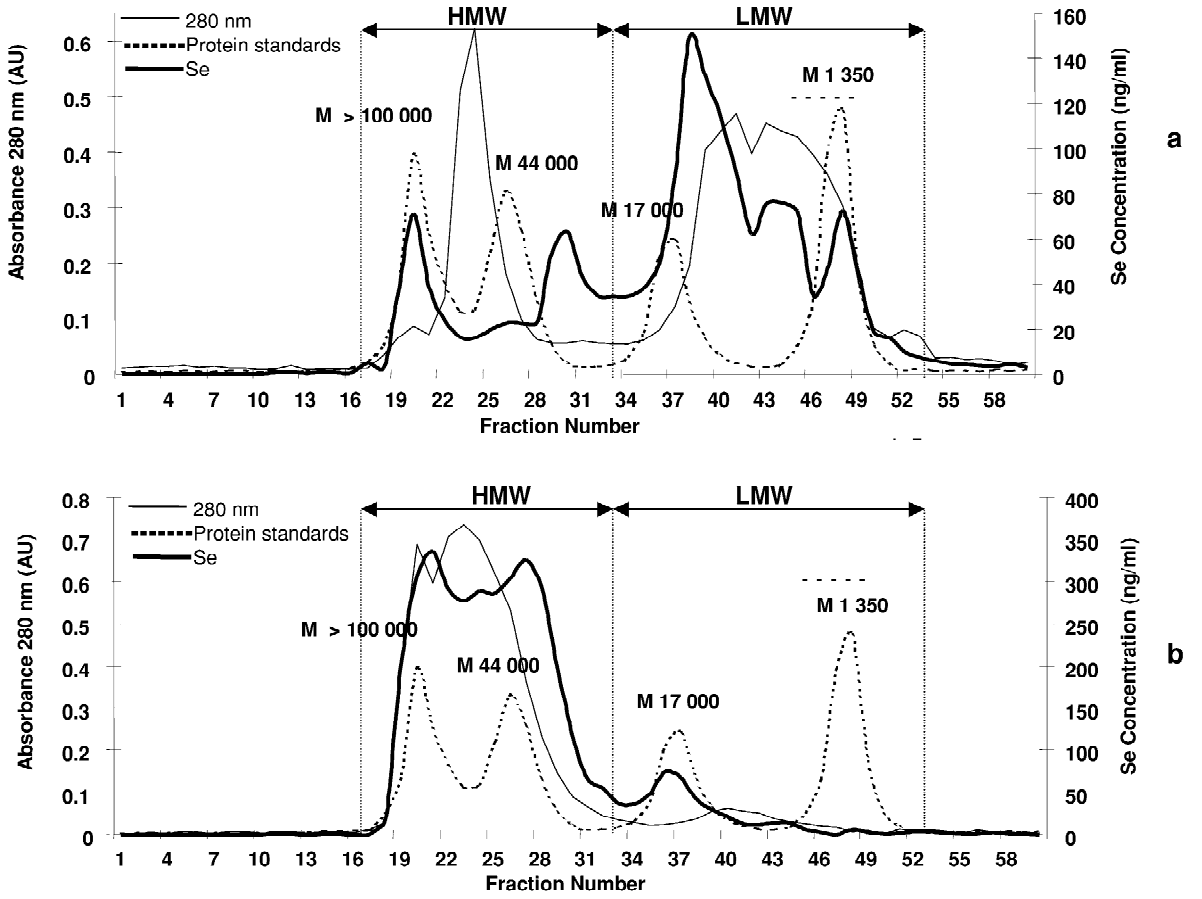

Fig. 3. Fractionation of selenium compounds in yeast extracts by size-exclusion preparative chromatography. Superdex 200 HiLoad column 60 cm×1.6 cm I.D. Injection: 1.0 ml. Mobile phase: 10 m*M* Tris–HCl buffer, pH 8.0. Flow-rate: 0.5 ml/min. Number of collected fractions: 80, time/fraction: 5 min. Fractions measured with UV (280 nm) and ET-AAS detections for Se. (a) Leaching with Tris–HCl (B in Fig. 1) (6.39 μg/ml Se), (b) leaching of the solid residue with SDS (C in Fig. 1) (15.47 μg/ml Se).

obtained on Superdex 75 (cf. LMW in Fig. 2a). A typical chromatogram obtained for the SDS extract (C in Fig. 1) on the column is presented in Fig. 3b. Compared to Fig. 2b a large distribution of Se compounds (unresolved peaks with three maximums) is observed in the mass range 40 000–100 000. The recoveries obtained for Se on Superdex 75 and 200 columns were identical and are summarised in Table 2.

For low-molecular-mass selenocompounds, the fractions 34–53 (LMW in Fig. 2a and b) were collected, pooled, preconcentrated by freeze-drying and stored at −22 °C. The objective is the further analysis by anion-exchange HPLC with element-specific detection technique to enable accurate measurement of selenomethionine, selenocystine and inorganic selenium compounds. For high-molecular-mass species, the fractions 17–33 (HMW in Fig. 2a and b) are combined, freeze-dried and stored at −22 °C for further experiments in SDS–PAGE.

Selenium fractionation in yeast material was carried out in previous studies [9,17,19] using low-capacity size-exclusion columns. However no reliable information was given concerning the expected mass range of selenoproteins. Additionally the method proposed a further enzymatic degradation (proteolysis) of the high- and medium-molecular-mass Se fractions prior to measurements by capillary

zone electrophoresis (CZE) ICP-MS [19]. In our work a real preparative step is developed to separate and isolate a group of proteins from a complex mixture. However this type of technique suffers from low resolution for protein separation [20]. In addition, a large volume of effluent used during separation dilutes the samples, making the subsequent steps difficult.

3.3. Monitoring of Se-containing low-molecular-mass compounds by anion-exchange HPLC–ICP-MS

Experimental conditions in anion-exchange HPLC were first optimized for the selenium standards. At a concentration of 5 m*M* ammonium phosphate buffer, Se-Met, Se-Cys and Se^{IV} were baseline separated on the anion-exchange column. However, an increase in the phosphate buffer concentration is required to elute the doubly charged Se^{VI}. Fig. 4 shows a typical chromatogram obtained from a 10 μl injection of an aqueous standards solution (100 ng Se/ml as Se-Met, Se-Cys, Se^{IV} and Se^{VI}). The accuracy of the retention times for each species (standard deviation) and the peak area reproducibility (%) (calculated for five independent injections of the 100 ng Se/ml standard solution) are shown in Table 3.

The calibration curves (not shown) were established for five independent injections of five different concentrations (investigated range: 0–100 ng Se/ml). The sensitivity (slope of the calibration curve), detection limit and regression coefficient (R^2) obtained for each selenium species are summarised in Table 3. The limits of detection were estimated as the concentration (or mass of Se) necessary to produce a net signal equivalent to three times the

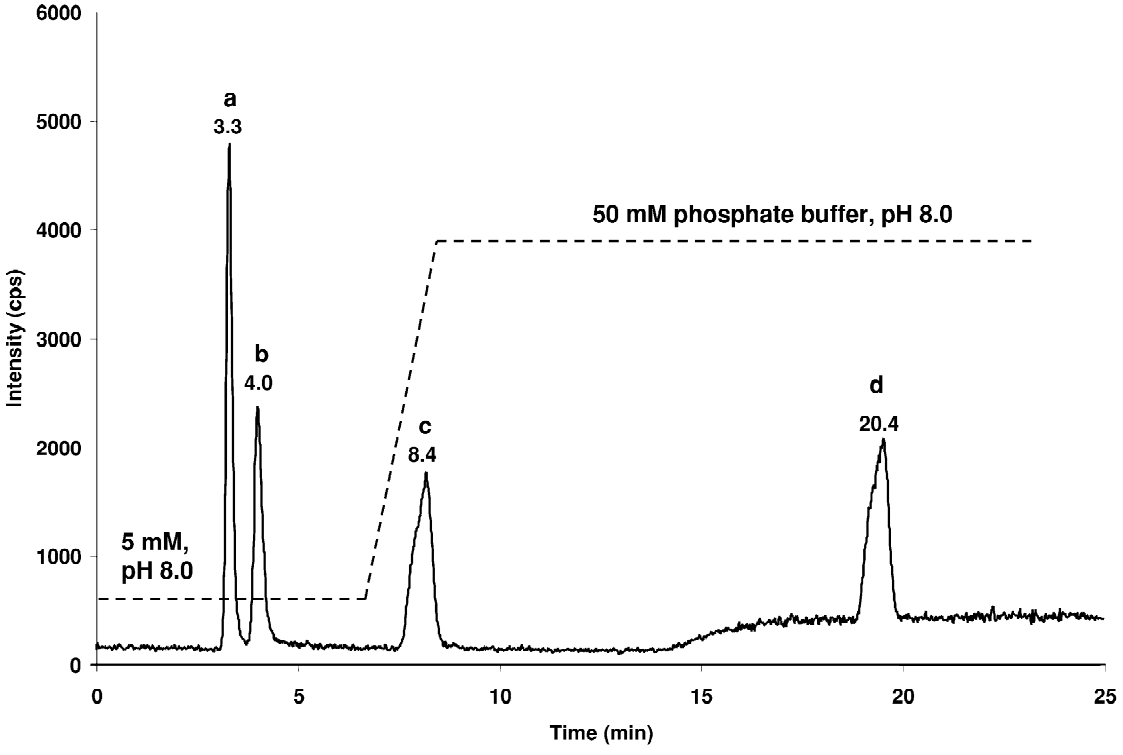

Fig. 4. Anion-exchange HPLC–ICP-MS chromatogram obtained from a 10 μl injection of an aqueous standard solution, 100 ng/ml Se as (a) selenomethionine, (b) selenocystine, (c) selenite, (d) selenate. Dionex Ion-Pac AS10 column 250 mm×2 mm I.D. with AG10 guard column 50 mm×2 mm I.D. Mobile phase: A: 5 m*M* ammonium phosphate buffer, pH 8.0; B: 50 m*M* ammonium acetate buffer, pH 8.0. Program: 0–7 min: 100% A; 7–8 min: 0–100% B; 8–25 min: 100% B. Flow-rate: 0.25 ml/min. Pressure: 136 bar. Data are shown for ^{82}Se. Numbers above peaks correspond to the respective retention times.

Table 3
Figures of merit in anion-exchange HPLC–ICP-MS analysis for selenium standards (concentration range: 0–100 ng Se/ml)

Compound	Retention time[a] (min)	Peak area reproducibility[b] (%)	Slope (AU per ng/ml)	Correlation coefficient R^2	Detection limit[c] DL (ng/ml)	RSD (%)
Selenomethionine (Se-Met)	3.3±0.1	3.5	6.44	0.9986	6.5	2.5
Selenocystine (Se-Cys)	4.0±0.1	5.2	4.64	0.9972	5.3	4.8
Selenite (Se)	8.4±0.2	4.6	10.53	0.9984	7.0	3.7
Selenate (Se)	20.4±0.2	10.5	9.75	0.9990	5.5	4.2

[a] Results for five independent measurements for the selenium standards solution 100 ng Se/ml.
[b] Results for five independent measurements for the selenium standards solution 100 ng Se/ml, signal for the isotope ^{82}Se.
[c] Calculated as three times the fluctuation of the base line noise of the chromatogram of the blank ($n=4$) (3DL, where DL is the standard deviation of the signal).

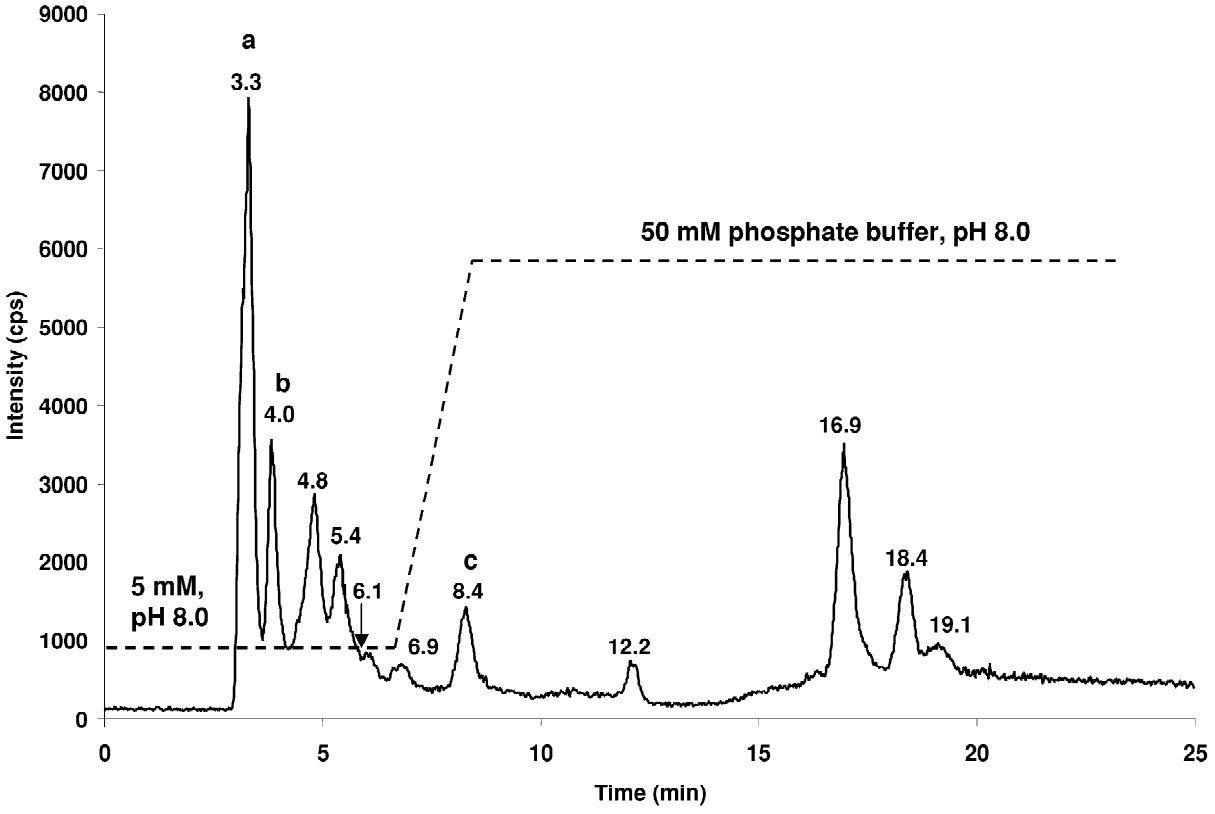

Fig. 5. Anion-exchange HPLC–ICP-MS chromatogram of water-soluble low-molecular mass Se-containing fraction (LMW in Fig. 2a, 3.79 μg/ml Se). Injection: 10 μl. Dionex Ion-Pac AS10 column 250 mm×2 mm I.D. with AG10 guard column 50 mm×2 mm I.D. Mobile phase: A: 5 m*M* ammonium phosphate buffer, pH 8.0; B: 50 m*M* ammonium acetate buffer, pH 8.0. Program: 0–7 min: 100% A; 7–8 min: 0–100% B; 8–25 min: 100% B. Flow-rate: 0.25 ml/min. Pressure: 136 bar. (a) Selenomethionine, (b) selenocystine, (c) selenite. Data are shown for ^{82}Se. Numbers above peaks correspond to the respective retention times.

Table 4
Determination and quantification of Se species in low-molecular-mass Se fractions by anion-exchange HPLC–ICP-MS

Compound	LMW in Tris–HCl buffer[a]			LMW in SDS[b]		
	Se concentration[c] (ng/ml)	Concentration SD	Se recovery (%)	Se concentration[c] (ng/ml)	Concentration SD	Se recovery (%)
Se-Met	390.79	7.88	10.3	104.23	8.67	11.5
Se-Cys	180.44	4.31	4.7	–	–	–
Se^{IV}	93.40	8.84	2.5	67.38	3.67	7.5
Total			17.5			19.0

[a] LMW in Fig. 2a (total Se concentration: 3795 ng Se/ml).
[b] LMW in Fig. 2b (total Se concentration: 900.44 Se/ml).
[c] Results for five independent injections.

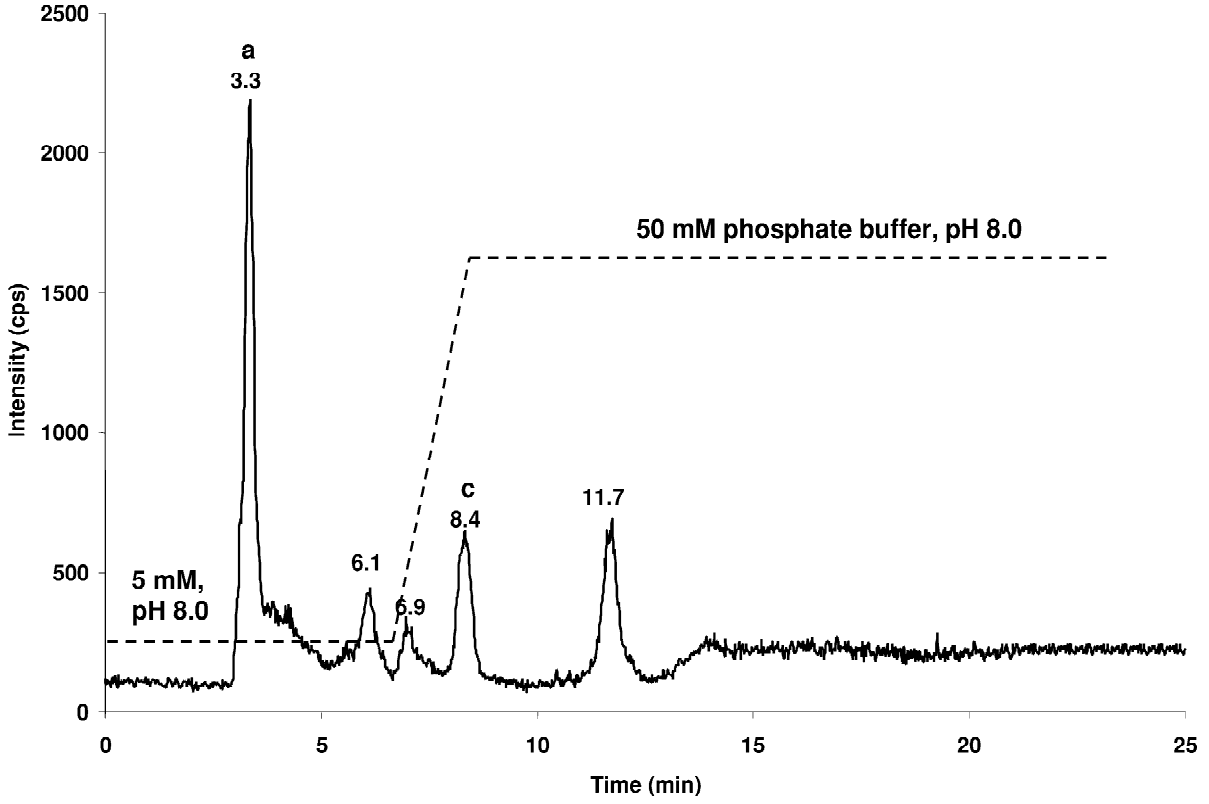

Fig. 6. Anion-exchange HPLC–ICP-MS chromatogram of SDS-soluble low-molecular mass Se-containing fraction (LMW in Fig. 2b, 0.90 μg/ml Se). Injection: 10 μl. Dionex Ion-Pac AS10 column 250 mm×2 mm I.D. with AG10 guard column 50 mm×2 mm I.D. Mobile phase: A: 5 m*M* ammonium phosphate buffer, pH 8.0; B: 50 m*M* ammonium acetate buffer, pH 8.0. Program: 0–7 min: 100% A; 7–8 min: 0–100% B; 8–25 min: 100% B. Flow-rate: 0.25 ml/min. Pressure: 136 bar. a, Selenomethionine, c, selenite. Data are shown for ^{82}Se. Numbers above peaks correspond to the respective retention times.

fluctuation of the base line noise (3DL, where DL is the standard deviation of the signal for the analysis of four blanks) of the chromatogram of the blank. Absolute detection limits ranged from 53 to 70 pg Se, or 5.3–7.0 ng Se/ml of original solution injected (10-μl injection), for the different selenium compounds. The standard deviation (SD) on the DLs are specified in Table 3. These results are similar with those reported earlier using HPLC–ICP-MS [18]. A regression coefficient >0.99 was obtained for each curve.

The optimized chromatographic conditions were applied to the separation of selenium species from the low-molecular-mass fractions collected in the size-exclusion chromatograms (on Superdex 75) of yeast extracts (cf. LMW in Fig. 2a and b). A chromatogram obtained for water-soluble low-molecular mass Se-containing fraction (LMW in Fig. 2a) is shown in Fig. 5 whereas Fig. 6 shows a chromatogram of SDS-soluble low-molecular mass Se-containing fraction (LMW in Fig. 2b). For both cases the chromatograms show one intense peak at 3.3 min (peak a) identified as Se-Met and a smaller one at 8.4 min (peak c) identified as Se^{IV}. In Fig. 5 the peak at 4.0 min (peak b) was attributed to Se-Cys which elutes just after Se-Met. No selenate (Se^{VI}) was detected in the yeast samples.

Table 4 shows the results for quantification of selenium compounds in selenized fractions by anion-exchange HPLC–ICP-MS. The qualitative and quantitative analysis of selenium species was based on the retention time of the selenium standards and the intensity of ICP-MS detection. The average concentration found and the calculated standard deviations were based on five independent injections for each fraction. For water-soluble Se fraction (cf. Fig. 5), Se-Met, Se-Cys and Se^{IV} were detected and account for only 17.5% of the Se amount injected on the column. For the fraction in SDS (cf. Fig. 6) Se-Met and Se^{IV} represent 19.0% of the Se amount injected on the column. The study revealed the existence of various unidentified Se species separated in chromatography. The corresponding HPLC peaks should be isolated and purified for further identification by, for example, electrospray tandem mass spectrometry (ESI–MS-MS).

Many studies aimed at evaluate the potential of CZE–ICP-MS [19,21] or HPLC–ICP-MS [18] for mapping of water-soluble Se compounds in yeast material. In contrast, our approach is to develop a reliable and reproducible method to enable accurate measurement of selenomethionine and inorganic selenium compounds in low-molecular-mass Se compounds. This information is of major importance in the context of mineral supplementation.

3.4. Investigation of selenium-containing proteins by SDS–PAGE

SDS–PAGE aimed at the detection of the selenium-containing proteins in yeast material. Typical scans of the silver stained gel of SDS-soluble (HMW in Fig. 2b) and water-soluble (HMW in Fig. 2a) proteins after SDS–PAGE are shown in Fig. 7a

Fig. 7. Silver stained gel after one-dimensional SDS–PAGE separation of water-soluble and SDS-soluble proteins (10 μl loaded on the gel). (a) SDS-soluble sample (HMW in Fig. 2b), (b) molecular mass protein markers: 14 000, 20 000, 30 000, 43 000, 67 000 and 94 000, (c) water-soluble sample (HMW in Fig. 2a).

sensitivity of the ICP-MS detection technique. Seleno amino-acids (selenocystine and selenomethionine) and inorganic selenium (selenite and selenate) can be rapidly identified and quantified in the low-molecular-mass selenium-containing fractions. The sensitivity of ICP-MS provides detection limits in the range 5–7 ng/ml Se. By combining SDS–PAGE and selenium determination in detected bands by ETV–ICP-MS detection, the distribution of Se-containing proteins in Se-enriched yeast has been refined. For SDS-soluble proteins the selenium distribution was determined in several bands between 10 000 and 100 000.

In the case of low-molecular-mass fractions three species were identified and quantified. However most of the selenium species are unknown. A better insight into selenium speciation will be investigated in the future by the isolation of unknown species and their characterization. However, the use of ESI-MS–MS to characterize compounds eluting from an anion-exchange column encountered a number of problems due to the low concentration of the compounds and the presence of phosphate buffer in the collected fractions. For the protein fractions the perspectives are the development of a method in two-dimensional gel electrophoresis to resolve all the proteins present in yeast extracts. The selenium determination in protein bands will be improved by using more sensitive techniques without the need for sample excision procedure (solid sampling), e.g. laser ablation with online ICP-MS detection.

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