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Evaluation of the Inorganic Selenium Biotransformation in Selenium-Enriched Yogurt by HPLC-ICP-MS

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Selenium is an essential element in the human diet. Interestingly, there has been an increased consumption of dietary supplements containing this element in the form of either inorganic or organic compounds. The effect of using selenium as a dietary supplement in yogurt has been evaluated. For this purpose, different concentrations of inorganic Se (ranging from 0.2 to 5000 μg g^{-1}) have been added to milk before the fermentation process. Biotransformation of inorganic Se into organic species has been carefully evaluated by ion-exchange, reversed-phase, or size-exclusion chromatography, coupled to inductively coupled plasma mass spectrometry (ICP-MS). Yogurt fermentation in the presence of up to 2 μg g^{-1} of Se(IV) produces a complete incorporation of this element into proteins as has been demonstrated applying a dialysis procedure. Analysis by SEC-ICP-MS showed that most of them have a molecular mass in the range of 30–70 kDa. Species determination after enzymatic hydrolysis has allowed the identification of Se-cystine using two different chromatographic systems. The biotransformation process that takes place during yogurt fermentation is very attractive because yogurt can act as a source of selenium supplementation.

KEYWORDS: Selenium speciation; selenium-enriched yogurt; biotransformation; dairy product; functional foods

INTRODUCTION

Although selenium is a well-established essential trace element for humans, it can also be toxic, and there are narrow margins between the doses needed to avoid selenium deficiency symptoms and those producing toxicity. Se deficiency diseases have been recognized since 1957, and there is well-established evidence that minor deficiencies may cause adverse health effects (*1*–*4*). Furthermore, supranutritional levels of Se may give additional protection against some diseases (*5*).

Food is the major source of Se for the human population. Se content in food varies from one country to another depending on the amount of the element in the soil and the capacity of plants to absorb it. The Se intake has been reviewed in several countries (*6*–*8*), and there is evidence to suggest that Se ingestion may be inadequate in many countries. Certain geographic zones are deficient in the element, and there is a need for an enriched diet. The human recommended intake is 55–70 μg/day, and the highest tolerable intake level has been set in 400 μg/day (Food and Nutrition Board, USA, 2000) (*8*). Regular adult intake of at least 40 μg/day is necessary to support a healthy development and perhaps as much as 300 μg/day to reduce the risk for certain types of cancer (*7*).

During the past decade, several strategies have been followed in order to supply sufficient Se to the population, and different types of food have been used as selenium supplements. They include Se-enriched fertilizers for the production of Se-rich plants, multimineral preparations containing inorganic Se, and "functional foods", obtained by adding Se supplements to growing cultures, such as the yeast *Saccharomices cerevisiae*, which can accumulate and biotransform large amounts of selenium (*6*, *9*–*12*).

Foodstuffs presenting naturally the highest concentration of selenium are, among others, offal, fish, broccoli, *Allium* plants, nuts, and eggs, followed by other meat products, bread, miscellaneous cereals, poultry, and milk (*13*, *14*). Nevertheless, selenium can be present in different chemical forms. As bioavailability of Se is highly dependent on the species present in the foods, the identification of this species has a primordial interest (*6*).

There are several methods that are very sensitive for the quantification of the total selenium content in food samples.

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Although ICP-MS exhibits very low detection limits (0.9 ng mL^{-1}), the method suffers from matrix interference that may produce a poor S/N ratio and low reliability (*15*). Atomic fluorescence spectroscopy (AFS) is routinely used for the analysis of Se in milk (*16*) due to its robustness, simplicity, good selectivity, and sensitivities (detection limits of 3.1 ng mL^{-1}). There are many publications devoted to the speciation of selenium (*17*) in different types of food such as mushroom (*18*), chives (*19*), green onions (*20*), dill (*21*), potatoes (*22*), garlic (*23*), alfalfa (*24*), and milk (*25*).

Selenium bioavailability has been studied by different authors by in vivo and in vitro studies (*26*). A recent study has been published by Muñoz-Naveiro et al. (*25*) showing that the content of selenium in cow's milk is higher for milk obtained after forage supplementation with organic selenium than for milk obtained after forage supplementation with inorganic selenium, suggesting a higher bioavailability of organic selenium. Schrauzer (*27*) indicates that selenium should ideally be supplemented in the same form than it occurs naturally in foods.

Yeast is well-known to biotransform Se(IV) into selenomethionine (*28*), and thus selenized yeast has already been commercialized for several years as a food supplement. *Lactobacillus* species are also known to incorporate selenite as selenocysteine (*29, 30*). This fact raised the possibility of using yogurt, a dairy product greatly consumed in most countries, as a Se-delivery system. We pursued the enrichment of yogurt by adding inorganic Se to the process of lactic fermentation. Moreover, the production of Se-enriched yogurt is more manageable than the production of Se-enriched plants. Selenium-enriched yogurt has the potential to be a complementary source to commercialized seleno yeast and inorganic seleno compounds.

The aim of this study was to investigate the distribution, nature, and amount of selenium species obtained when yogurt is produced in a medium with increasing amounts of Se(IV) and Se(VI). We have used the AFS technique to determine total selenium, size-exclusion chromatography (SEC) coupled to ICP-MS to estimate the molecular mass of aqueous-soluble selenium compounds, and HPLC using ion pairing reversed-phase (IP-RP), or anion-exchange columns, coupled to ICP-MS, for the speciation of low molecular mass seleno compounds, produced after enzymatic protein hydrolysis.

MATERIALS AND METHODS

Instrumentation. Samples were digested for total selenium determination in double-walled advanced composite vessels (ACV) using a 1000 W microwave oven (CEM, MSP 1000, Mattheus, NC).

For the determination of the total content of Se, an atomic fluorescence spectrometer (AFS), Excalibur (P.S. Analytical Ltd., Orpington, Kent, U.K.), was used.

Selenium hydride was generated in a system of flow injection with a peristaltic pump (Gilson HP4, Villiers-le-bel, France), a mixing and reaction coil (0.5 mm i.d. PTFE tubing), and a U-tube gas–liquid separator. The separator was connected to a commercial dryer membrane (Perma Pure Products, Farmingdale, NJ) to eliminate the moisture, and both together were used as an interface for the hydride generation (HG)−AFS system.

A Sonopuls ultrasonic homogenizer (Bandelin, Germany), fitted with a HF generator 2200, was used for the enzymatic hydrolysis. The ultrasonic homogenizer was equipped with a titanium microtip of 3 mm diameter, and the power applied was 20 W at a frequency of 20 kHz.

Aqueous extraction of yogurt was made with an ultrasonic bath, P-Selecta (Varian, Madrid, Spain), and/or a potter (Deltalab, Barcelona, Spain).

Chromatographic separations were carried out under isocratic conditions with a liquid chromatograph equipped with a high-pressure pump, Jasco PU-2089 (Crenella, Italy). The injection was done using a six-port Rheodyne model 7725i valve fitted with a 100 μL loop (Rheodyne, Rohner Park, CA). Detection by ICP-MS was carried out in a Thermo-X series (Thermo Instruments, U.K.), equipped with a conventional Meinhard nebulizer and a Peltier cooled spray chamber (2 °C). Chromatographic columns were coupled to the ICP-MS instrument by a 10 cm polytetrafluoroethylene capillary tubing (0.5 mm i.d.). For size-exclusion chromatography a BioSep SEC-2000 (300 × 7.8 mm), Phenomenex (Torrance, CA), with a effective separation range of 300–1 kDa was used. Anion-exchange chromatography was performed using a Hamilton PRP-X100 (Reno, NE) column (10 μm particle size, 250 mm × 4.1 mm i.d.). Reversed-phase chromatography was performed using a C-18 Gemini column (10 μm particle size, 150 mm × 2.0 mm i.d.), Phenomenex (Torrance, CA).

Reagents and Standards. Analytical grade reagents were used in all experiments. All solutions were prepared with deionized Milli-Q water (Millipore, Bedford, MA). Filters were 10 kDa cutoff (Millipore, Bedford, MA). An Eppendorf centrifuge 5804 F34-6-38 (Hamburg, Germany) was used for extract cleaning up before speciation. Dialysis was performed with 0.45 μm Cellu-Sep T1, nominal MWCO:3500 membrane filtration (Seguin, TX).

Selenocysteine ($SeCys_2$), selenomethionine (SeMet), and methylselenocysteine (MeSeCys) were purchased from Sigma (St. Louis, MO). Working solutions were prepared daily by appropriate dilution of concentrate standards in 3% HCl (Merck Suprapur, Damstadt, Germany). Inorganic selenium solutions were obtained by dissolving Na_2SeO_3 and Na_2SeO_4 (Merck, Darmstadt, Germany) in deionized water. Enzymatic hydrolysis was done using protease-type (XIV) *Streptomyces griseus* (Sigma).

For the determination of the total selenium content, working solutions were prepared daily by the appropriate dilution of a 1000 mg L^{-1} Se(IV) stock solution stored in the dark at 4 °C.

Heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), and ammonium citrate from Sigma Chemical Co. (St. Louis, MO) and methanol (Sharlab, Barcelona, Spain) were used in the different chromatographic mobile phases. The mobile phase for the SEC column was a 5 mmol L^{-1} phosphate buffer at pH 7.2 (Merck, Darmstadt, Germany). All of the HPLC solutions were filtered and degassed using 0.45 μm Millipore Nylon filters, except for SEC, where 0.22 μm Millipore Millex-HV filters were used.

The molecular mass standards for SEC were as follows: Blue dextran (2000 kDa); alcohol dehydrogenase (150 kDa); albumin (66 kDa); carbonic anhydrase (29 kDa); cytochrome *c* (12.4 kDa); aprotinin (6.5 kDa); coenzyme B12 (1.6 kDa); selenourea (123 Da) from Sigma Chemical Co. (St. Louis, MO). All of the standards were dissolved in a solution containing 50 mmol L^{-1} Tris-HCl, from Fluka (Neu Ulm, Germany) and 100 mmol L^{-1} KCl (Merck, Darmstadt, Germany) at pH 7.5.

A solution of 1% (w/v) sodium tetrahydroborate was prepared by dissolving $NaBH_4$ powder (Merck, Steinheim, Germany) in deionized water, which was stabilized in 0.1% (w/v) NaOH and filtered to eliminate turbidity. A 4 mol L^{-1} hydrochloric acid solution was prepared by diluting concentrated HCl (Merck, Suprapur). H_2O_2 (35%) from Panreac and HNO_3 (65%) distilled in our laboratory in a distilling system for acids (Model BSB 9391IR, Berghof, Germany) were used to digest the samples.

Nonfat milk powder NIST 1549 for total selenium was used as the certified reference material.

Fermentation Process. Sodium selenite or selenate from 7 μg to 350 mg (as Se) was added to 50 mL of reconstituted skimmed milk (containing 5 g of milk powder). The fermentation process was started by the addition of 20 g of commercial skimmed yogurt containing lactic ferments. Fermentation was allowed for 12, 24, and 36 h at temperatures between 37 and 42 °C.

Dialysis. About 15 g of unenriched and Se-enriched yogurt was dialyzed. The process of dialysis was performed for 20 h at 4 °C against Milli-Q water, using dialysis membranes with a molecular mass cutoff of 3.5 kDa. During this period two changes of the Milli-Q water were carried out.

Sample Preparation for Total Selenium Determination. *Protocol A*: Total selenium content was determined in fresh yogurt samples and

Table 1. Microwave Program Used for Digestion of Yogurt Samples (Pressure 150 psi)

step	time, min	power, W
1	1	250
2	1	0
3	5	250
4	5	400
5	5	650
6[a]	6	650

[a] This step requires the addition of 1.9 mL of 8 M HCl.

Table 2. Operating Conditions for Total Se Determination by HG-AFS

intensity of Se EDL lamp	16 mA
wavelength	196.06 nm
slit	2.0
HCl concn	4.0 mol L^{-1}
$NaBH_4$ concn	1% (w/v) in 0.1% (w/v) NaOH
sample flow rate	1.5 mL min^{-1}
acid flow rate	1.5 mL min^{-1}
$NaBH_4$ flow rate	1.5 mL min^{-1}
Ar flow rate	540 mL min^{-1}
H_2 flow rate	180 mL min^{-1}

in aqueous and enzymatic extracts. Three grams of sample was dissolved with 2 mL of $HNO_3-H_2O_2$ (3:1) using a microwave oven following the steps indicated in **Table 1**. Selenium(VI) was directly reduced to Se(IV) in the digestion mix by the addition of 1.9 mL of 8 M HCl and a microwave oven treatment of 6 min at 65% power (*31*). The reduced solutions were then diluted with 4 M HCl to a final volume of 10 mL.

Atomic fluorescence spectroscopy (AFS) was used for the determination of the selenium total content. The signal was obtained by a continuous selenium hydride generation system connected to AFS equipment, under the operating conditions given in **Table 2**. The total selenium concentration was determined by the standard addition method.

Proteolytic Digestion of Yogurt for Selenium Speciation. *Protocol B*: Enzymatic hydrolysis was performed in polypropylene bottles, adding 20 mg of protease XIV and 1 mL of Milli-Q water to 1–3 g of yogurt samples. The mixture was sonicated with an ultrasound probe during 50 s using a power of 20 W (*32*). After digestion, extracts were filtered through 10 kDa cutoff centrifuge filters. Reversed-phase and/or anion-exchange HPLC columns coupled to an ICP-MS detector were used for the Se speciation study of the filtrates.

Preparation of Water-Soluble Proteins. *Protocol C1*: Yogurt samples (3 g) were extracted with 3 mL of Milli-Q water in an ultrasonic bath at room temperature for 30 min. Extracts were then centrifuged at 14000*g* for 30 min at 4 °C, and supernatants were injected into the SEC column, previously coupled to an ICP-MS.

Protocol C2: Yogurt samples (3 g) were homogenized with 3 mL of Milli-Q water for 2 min using a potter. Extracts were then centrifuged as in protocol C1.

Protocol C3: Yogurt samples (3 g) with 3 mL of Milli-Q water were treated using a combined extraction including a potter for 2 min and an ultrasonic bath for 30 min. The resulting extracts were then centrifuged as in protocol C1.

The SEC column was calibrated with the following molecular mass standards: Blue dextran (2000 kDa), alcohol deydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), aprotinin (6.5 kDa), coenzyme B12 (1.58 kDa), and selenourea (0.123 kDa). The logarithm of molecular mass versus the ratio elution volume/void volume followed a linear response and was obtained using a UV detector at 280 nm and by ICP-MS while monitoring ^{59}Co, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{77}Se, ^{78}Se, and ^{82}Se isotopes, elements bonded to protein standards.

Stock protein standard solutions were prepared at a concentration of approximately 1000 mg L^{-1} in 50 mmol L^{-1} Tris-HCl/100 mmol L^{-1} KCl at pH 7.5. Diluted solutions at protein concentrations of 20–100 mg L^{-1} were prepared daily from the stock solutions with deionized water.

Table 3. Operating Conditions for Se Determination by Anion-Exchange, Reversed-Phase, and Size-Exclusion Chromatography Coupled to ICP-MS

ICP-MS Operating Conditions	
forward power	1250 W
plasma gas (Ar) flow rate	15 L min^{-1}
auxiliary gas (Ar) flow rate	0.8 L min^{-1}
carrier gas (Ar) flow rate	0.72 L min^{-1}
nebulizer type	Meinhard
spray chamber type	impact bead quartz
data acquisition mode	time-resolved analysis (TRA)
Chromatographic Conditions IPRP-HPLC	
analytical column	C18 Gemini
mobile phase	0.05% TFA, 0.1% HFBA solution (pH 2.0), 2% methanol
flow rate	0.2 mL min^{-1}
elution program	isocratic
injection volume	20 μL
Chromatographic Conditions Anion-Exchange HPLC	
analytical column	Hamilton PRP-X100
mobile phase	10 mM ammonium citrate (pH 5.0), 2% methanol
flow rate	1.0 mL min^{-1}
elution program	isocratic
injection volume	100 μL
Chromatographic Conditions SEC-HPLC	
analytical column	BioSep SEC-2000
mobile phase	5 mM phosphate buffer (pH 7.5)
flow rate	1.0 mL min^{-1}
elution program	isocratic
injection volume	100 μL

Determination of Selenium Species by HPLC-ICP-MS. Extracts obtained by enzymatic hydrolysis were injected in anion-exchange HPLC or IP-RP-ICP-MS systems. Operating conditions are summarized in **Table 3**. The identification and quantification of selenium species were done comparing the retention times of standards and using the standard addition method.

RESULTS AND DISCUSSION

Yogurt Fermentation under Increasing Concentrations of Inorganic Selenium. A wide range of concentrations of sodium selenite or selenate salts, spanning from 0.1 to 5000 μg g^{-1}, were added to the growing media, to determine the maximum amount of selenium compatible with the lactic fermentation. The maximum tolerated concentration for Se(IV) was 500 μg g^{-1}; above this limit, the formation of yogurt was impelled. The presence of red Se^0 was observed at concentrations of Se(IV) of 50 μg g^{-1} and higher. Interestingly, it has been reported that *Lactobacillus* uses the reduction of Se(IV) to Se^0 as a detoxification mechanism to survive in the presence of high Se concentrations (*30*). For further experiments Se(IV) concentration was kept between 0.1 and 20 μg g^{-1}. Se(VI) is compatible with the lactic fermentation when used at concentrations up to 5000 μg g^{-1}. However, unlike Se(IV), the percentage of biotransformation observed for Se(VI) is very low (see following sections).

Quantification of Total Selenium in Yogurt Samples and Extracts. Total selenium content in yogurt samples, aqueous yogurt extracts, and yogurt supernatants obtained after enzymatic proteolysis was analyzed. The quantification was performed using hydride generation atomic fluorescence spectroscopy (HG-AFS). Method validation was performed by recovery studies of spiked samples and using the certified reference material nonfat milk powder NIST 1549 (0.11 ± 0.01 μg g^{-1}). Recoveries were in the range of 99−104%. No significant differences were obtained between experimental and certified

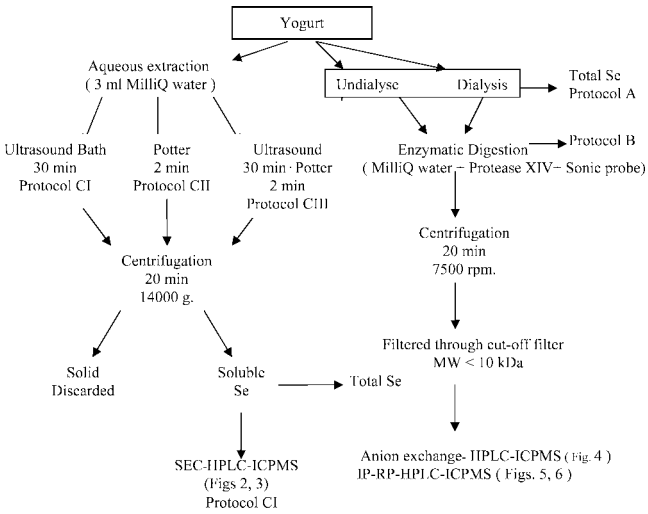

Figure 1. Flow chart for the different treatments used for total Se determination and speciation in yogurt samples.

Table 4. Percentage of Total Se Found in Yogurt after Dialysis[a]

	selenium (%)	
addition (μg g^{-1})	fermentation time 12 h	fermentation time 24 h
Se(IV)		
0.100	98 ± 2	
0.500	98 ± 2	99 ± 2
1.00	98 ± 1	
2.00	89 ± 1	94 ± 1
5.00	58 ± 1	69 ± 1
20.0	13 ± 0.5	27 ± 0.5
Se(VI)		
1.00	2 ± 0.2	12 ± 1
2.00	6 ± 0.2	8 ± 0.2

[a] MW > 3.5 kDa. Three independent determinations were averaged.

values at 95% probability. The use of ICP-MS was not ideal because of the presence of ^{78}Se and ^{82}Se isobaric interferences, especially at low selenium concentrations (*33*). A flow chart of the different treatments used for Se determination and speciation is shown in **Figure 1**.

Total content of Se in undialyzed and dialyzed yogurt samples was determined to evaluate the possible biotransformation of Se(IV) and Se(VI) during the fermentation process. Dialysis was performed using 3.5 kDa membranes following the protocol described under the Materials and Methods section. Compounds with molecular masses lower than 3.5 kDa are able to diffuse out of the membrane, while large organic compounds with MW > 3.5 kDa remain inside the membrane. As shown in **Table 4**, when Se(IV), in the 0.1–1.0 μg g^{-1} range, was added, the percentage of selenium bound to macromolecules is around 98%. This indicates that inorganic selenium is biotransformed efficiently. Longer fermentation times (from 12 to 24 h) lead to slightly higher recoveries, reaching 99%. However, when Se(IV) was added at higher concentrations, such as 5 and 20 μg g^{-1}, the percentage of selenium bound to macromolecules decreases to 58% and 13%, respectively, after 12 h of yogurt incubation at 37 °C.

On the other hand, when selenium was added to the growing media as Se(VI), less than 12% of it was converted to compunds with a MW higher than 3.5 kDa, even at the lowest concentrations of Se tested with fermentation times (from 12 to 24 h) (see **Table 4**. This indicates that, unlike Se(IV), Se(VI) was, basically, not biotransformed. Low biotransformation rates for Se(VI) have also been reported by other authors (*29*, *30*, *34*, *35*).

Table 5. Total Selenium in the Whole Sample and in Aqueous and Enzymatic Hydrolysis Extracts[a]

Se(IV) added (μg g^{-1})	Se in whole sample (μg g^{-1} ± SD)	Se in aqueous extract (μg g^{-1} ± SD)	% Se	Se in enzymatic hydrolysis extract (μg g^{-1} ± SD)	% Se
unenriched	0.058 ± 0.02	0.014 ± 0.002	24	0.07 ± 0.03	121
0.500	0.57 ± 0.02	0.058 ± 0.01	10	0.61 ± 0.02	106
1.00	1.08 ± 0.03	0.098 ± 0.01	9.1	0.99 ± 0.02	91.5
2.00	2.14 ± 0.05	0.20 ± 0.02	9.2	1.97 ± 0.04	92.0
5.00	5.08 ± 0.06	0.70 ± 0.03	14		

[a] Three independent determinations were averaged; fermentation time 24 h. Following protocol C1 (ultrasonic bath).

Table 6. Selenium Extracted from Yogurt Samples (Fermentation Time 12 h) under Different Extraction Protocols[a]

	total Se (μg g^{-1})		
Se(IV) added (μg g^{-1})	ultrasonic bath, protocol C1	potter, protocol C2	ultrasonic + potter, protocol C3
unenriched	0.014 ± 0.002	0.007 ± 0.001	0.018 ± 0.004
2.00	0.21 ± 0.02	0.09 ± 0.04	0.25 ± 0.02
5.00	0.70 ± 0.03	0.41 ± 0.03	0.71 ± 0.02

[a] Three independent determinations were averaged.

After these initial findings, experimentation was focused in the study of the fermentation process using Se(IV) at concentrations within the 0.1−5.0 μg g^{-1} range.

Total Se contents in the whole yogurt and in the aqueous and enzymatic extracts are shown in **Table 5**. Selenium recoveries in the aqueous extracts of enriched yogurts were about 10% of the total selenium present in the growing media and were not dependent on the absolute amount of the selenite added up to 2 μg g^{-1}. These results are in good agreement with those obtained using dialysis (see **Table 4**). Furthermore, selenium recoveries were higher than 92% for enzymatic extracts after filtering them through 10 kDa cutoff membranes (see **Table 5**).

As can be observed, a high percentage of Se(IV) is biotransformed into organic selenium during the process of lactic fermentation, and the selenium bioavailability is consequently increased in supplemented yogurts, thus enabling a feasible way for food supplementation.

Study of the Incorporation of Se to High Molecular Mass Biomolecules Using SEC-HPLC-ICP-MS. The incorporation of Se(IV) into high molecular mass biomolecules was evaluated using size-exclusion chromatography coupled to Se-specific detection by ICP-MS. Sample treatment for SEC-ICP-MS analysis was an extraction with ultrapure water (Milli-Q). Several methods were assayed to maximize the extraction efficiency and to evaluate the possible procedure-dependent changes on the integrity of the different species. Either an ultrasonic bath, a potter homogenizer, or the combination of both, was used to help in the sample extraction procedure. The specific protocols are described in the Materials and Methods section as C1, C2, and C3, respectively. Total Se contents in the different extracts were quantified (see **Table 6**). When the ultrasonic bath was used, the amount of selenium extracted was double than when using the potter homogenizer. The combination of both techniques did not improve the percentage of extraction. The chromatographic profiles obtained with and without applying the ultrasonic bath were very similar (data not shown). Therefore, the protocol C1 was selected for further experiments.

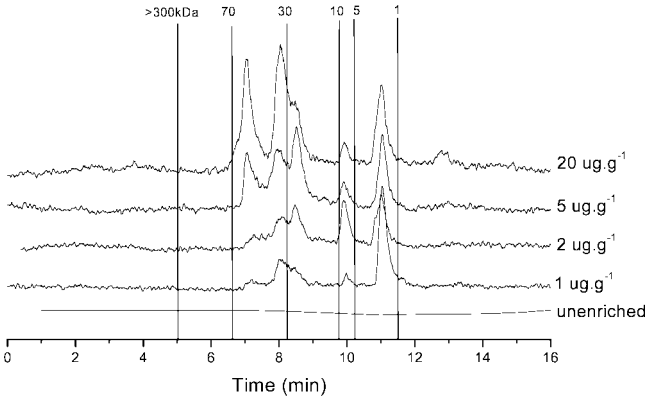

Figure 2. Size-exclusion chromatograms showing the separation of the aqueous extracts from the Se-enriched (1, 2, 5, 20 μg g^{-1}) and plain yogurt samples. Elution times for the molecular mass markers at 30, 70, and 300 kDa are highlighted.

No signal for Se was found in the chromatograms of unenriched yogurt samples (see **Figure 2**). Five new chromatographic peaks were found when the fermentation was produced in the presence of Se(IV), indicating that Se was associated with high molecular mass species. Two of these peaks correspond to molecular masses lower than 30 kDa, and the other three peaks to molecular masses between 30 and 70 kDa. The intensity of the peaks between 30 and 70 kDa increases with the amount of Se added (up to 20 μg g^{-1}). The effect of the fermentation time in the incorporation process was studied as well. Panels **a** and **b** of **Figure 3** show the differences between the chromatograms of the samples fermented for 12 and 24 h. The intensity of the peaks corresponding to low molecular mass species diminishes with longer fermentation times while the intensity of peaks corresponding to high molecular mass species increases.

Speciation Studies of the Enzymatic Extracts by HPLC-ICP-MS. Dialyzed and undialyzed yogurt samples were incubated with protease XIV using an ultrasonic probe, following the procedure described above. After enzymatic hydrolysis, samples were centrifuged and the supernatants filtered through 10 kDa cutoff membranes. The selenium species released by the enzymatic hydrolysis of the enriched yogurt at different levels (1, 2, 5, 20 μg g^{-1}) were analyzed using two chromatographic methods.

Anion-Exchange Chromatography. Separation of selenium species was performed in a Hamilton PRPX100 column (*36*) using 10 mM ammonium citrate, pH 5.0, in 2% methanol as the mobile phase (**Table 3**). Under these conditions, a complete separation of selenium standards was possible within 12 min (**Figure 4a**). The chromatographic profiles of the enzymatically hydrolyzed samples are shown in panels **b** and **c** of **Figure 4**, respectively. Initially, the selenium species $SeCys_2$, MeSeCys, and Se(IV) were identified in the yogurt samples by their retention time and by standard additions as peaks 1 (2.2 min), 2 (2.8 min), and 3 (3.2 min), respectively (see **Figure 4b**). Additinally, two unidentified peaks, named (U), were detected. $SeCys_2$ was the only Se amino acid unambiguously identified by this experiment because MeSeCys coelutes with selenomethionine oxide (SeMetO) in this chromayographic system. The ability of *Lactobacillus* to transform Se(IV) to $SeCys_2$ was previously reported by Calomme et al. (*29*), which suggests that the incorporation of Se to selenoproteins is a specific process encoded by the UGA codon.

In the hydrolyzed samples from plain, unenriched yogurts, two species were identified (see protocol B), $SeCys_2$ and SeMet

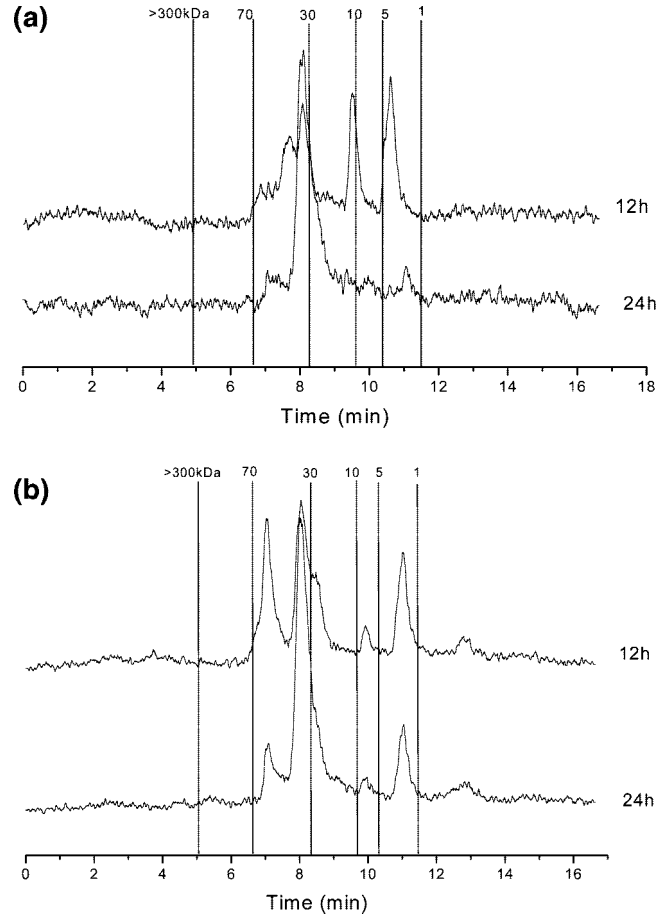

Figure 3. Size-exclusion chromatograms showing the separation of aqueous extracts from yogurts enriched with (**a**) 2 μg g^{-1} and (**b**) 5 μg g^{-1} Se at 12 and 24 h fermentation time.

(peaks 1 and 4 in **Figure 4c**). Although traces of SeMet were detected in these samples, increases in the signal for this seleno amino acid were not observed when the amount of Se added to the culture was increased. Consequently, the unspecific incorporation of selenium into proteins as SeMet, reported for other microorganizms such as *S. cerevisiae* in selenized yeast (*12*), was not observed in the present experiments.

The selenium species present in the undialyzed enriched yogurt after enzymatic hydrolysis were quantified using the standard addition method. Results obtained for yogurt samples enriched with 0.50, 1.00, and 2.00 μg g^{-1} Se(IV) during 12, 24, or 36 h of fermentation are shown in **Table 7**. The amounts for $SeCys_2$ and MeSeCys, the two major species in which Se is transformed, increased proportionally with the concentration of inorganic selenium present in the growing media. Signals for these two species also increased with culture time up to 24 h while Se(IV) concentration decreased in these samples. Quantification of Se(IV) was made in samples from undialyzed yogurts (chromatograms not shown). The low content of Se(IV) in these samples indicates that the biotransformation was higher than 90% after 24 h of fermentation for yogurts produced in the presence of up to 2 μg g^{-1} Se(IV).

Reversed-Phase HPLC-ICP-MS. To confirm the identity of the selenium species found by anion exchange, a separation by RP-HPLC, widely used for Se species analysis (*18*, *23*), was performed. A mixture formed by 0.1% heptafluorobutyric acid (HFBA) and 0.05% trifluoroacetic acid (TFA) was used for effective ion pairing. Under optimized conditions (see **Table 3**), the separation of Se compounds was achieved within 20 min. **Figure 5** shows the chromatogram for selenium standards

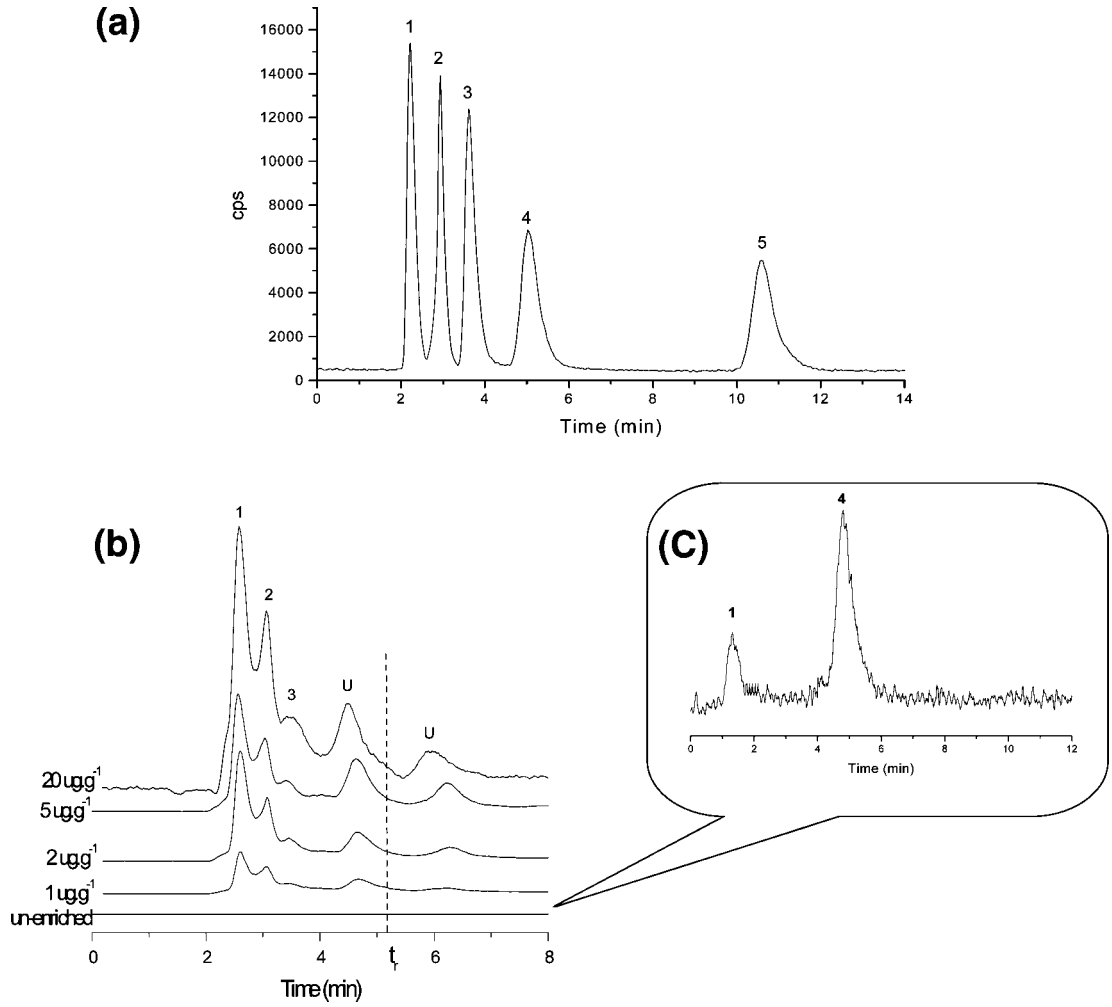

Figure 4. Anion-exchange chromatograms showing separations. (**a**) Mixture of Se standards containing 20 μg L^{-1} of each Se species: (1) $SeCys_2$; (2) MeSeCys; (3) Se(IV); (4) SeMet; (5) Se(VI). (**b**) Enzymatic extraction of Se-enriched (1, 2, 5, 20 μg g^{-1}) yogurt samples at 24 h fermentation time: (1) $SeCys_2$; (2) MeSeCys; (3) Se(IV); (U) unknown. The dashed line is the t_r for SeMet. (**c**) Enzymatic extraction of unenriched yogurt at 24 h fermentation time: (1) $SeCys_2$; (4) SeMet.

Table 7. Selenium Species Quantification[a] in the Hydrolyzed Extract of Unenriched and Enriched Yogurt Supplied with 0.50, 1.00, and 2.00 μg g^{-1} Selenium at 12, 24, and 36 h Fermentation Time

	unenriched	0.50 μg g^{-1}			1.00 μg g^{-1}			2.00 μg g^{-1}		
species	sample	12 h	24 h	36 h	12 h	24 h	36 h	12 h	24 h	36 h
Se(IV)		31	5.7	1.3	202	110	92	200	162	136
$SeCys_2$	3.3	69	102	179	53	107	383	800	581	542
MeSeCys					90	145	167	409	312	272
SeMet	24	14	24	12	39	33	44	14	10	5.7
Se(VI)		15	15	2.9	14	13	16	7.6	6.7	6.5

[a] ng g^{-1}; average of three replicates; RSD range 10–15%.

using this method. The flow rate was optimized between 0.1 and 0.4 mL min^{-1} using a micronebulizer for the ICP-MS instrument, and a flow rate of 0.2 mL min^{-1} was chosen. The methanol concentration in the chromatographic mobile phase was maintained below 10% (v/v). These conditions allowed the use of ICP-MS without adding oxygen in the plasma gas, thus avoiding instability.

Using this RP column, two peaks corresponding to MeSeCys and $SeCys_2$ were detected. In this system, SeMeO does not coelute; therefore, the presence of MeSeCys can be confirmed (**Figure 6**). The intensity of both peaks (2 and 3) increases when samples are spiked with $SeCys_2$ and MeSeCys (panels **b** and **c** of **Figure 6**, respectively).

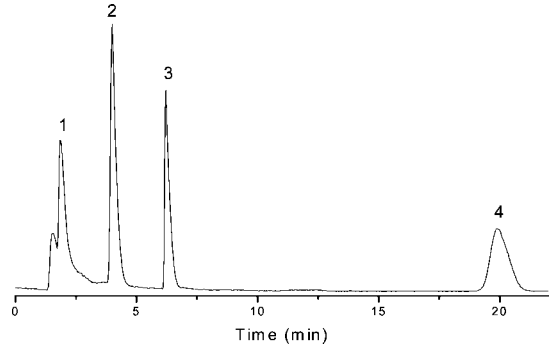

Figure 5. Reversed-phase chromatogram showing the separation of Se standards containing 50 μg L^{-1} each of Se species: (1) Se(IV) and Se(VI); (2) $SeCys_2$; (3) MeSeCys; (4) SeMet.

These results corroborate the previous findings obtained by anion-exchange chromatography. Although MeSeCys in protein hydrolysates is not easy to explain, the structure of this particular compound awaits further investigations using molecular mass spectrometry.

CONCLUSIONS

Tolerance of yogurt to inorganic selenium depends on the nature of the species added to the growing media. Fermentation

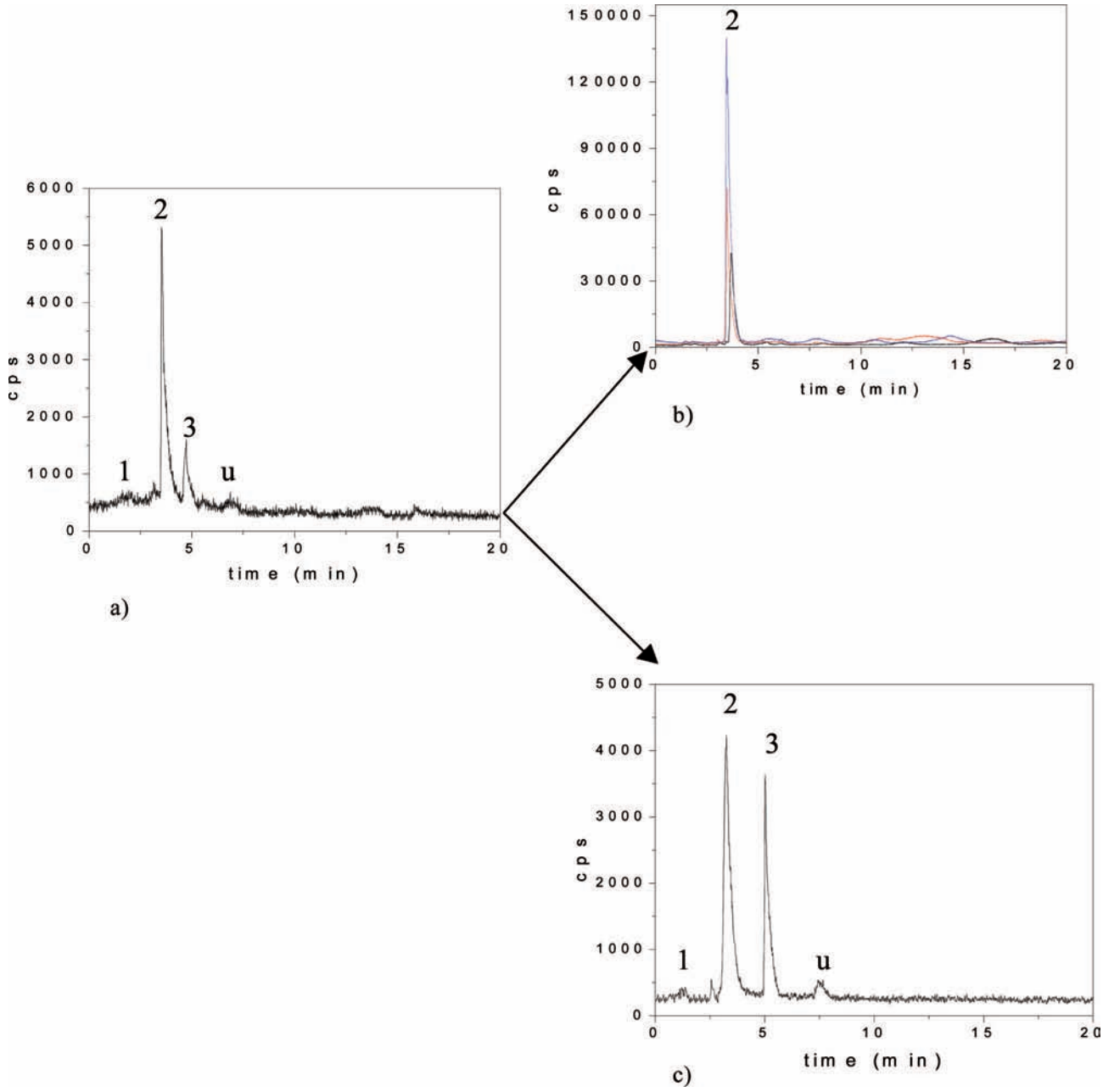

Figure 6. Reversed-phase chromatograms showing the separation of Se species after enzymatic digestion of dialyzed yogurt samples enriched with 2 μg g^{-1} of Se(IV). (**a**) (1) Se(IV) + Se(VI); (2) $SeCys_2$; (3) MeSeCys; (U) unknown. (**b**) Sample spiked with $SeCys_2$, 10−50 μg L^{-1}. (**c**) Sample spiked with MeSeCys, 10 μg L^{-1}.

of yogurt can take place in the presence of up to 500 and 5000 μg g^{-1} selenium Se(IV) and Se(VI), respectively. While a high percentage of the Se(IV) added (up to 2 μg g^{-1}) is incorporated into proteins after 24 h, Se(VI) is not biotransformed, remaining unchanged during fermentation times up to 36 h. Water-extractable proteins containing selenium have molecular masses between 30 and 70 kDa.

The metabolic transformation of selenite increases with time, reaching a plateau after 24 h of fermentation. Speciation studies of the enriched yogurt after enzymatic hydrolysis have demonstrated the presence of $SeCys_2$ and MeSeCys with their concentrations increasing with the amount of Se(IV) added. SeMet was found at same levels in both the plain and the enriched yogurt, indicating that *Lactobacillus* does not metabolize Se(IV) to SeMet. The capability (higher than 90%) of *Lactobacillus* to biotransform Se(IV) has been demonstrated by three independent assays: speciation of selenium in enzymatic hydrolysates and determination of the total selenium in dialyzed yogurt samples and in aqueous extracts.

Future activity will be focused on a bioavailability study of the Se-enriched supplemented yogurt by gastric and intestinal in vitro digestion.

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