



SPECIATION OF SELENIUM IN *Pleurotus ostreatus* AND *Lentinula edodes* MUSHROOMS

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Abstract- *Pleurotus ostreatus* and *Lentinula edodes* are two of the most commercialized mushrooms species in the world and exhibit the potential to accumulate selenium (Se). The form of Se in the protein extracts of *P. ostreatus* and *L. edodes* mushrooms enriched with Se has been investigated with the aim of obtaining a protein extract with high concentrations of selenomethionine (SeMet). For an approach to the speciation of Se incorporated in *P. ostreatus* proteins, size exclusion high performance liquid chromatography was used in combination with inductively coupled plasma mass spectrometry (SEC-HPLC-ICP-MS) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Se-enriched mushrooms of *P. ostreatus* and *L. edodes* were used. The proteins were extracted with phosphate buffer, borate buffer and solubilization buffer (2DE) and were purified by acetone precipitation. The extracts were analyzed by reversed phase high performance liquid chromatography in combination with inductively coupled plasma mass spectrometry (RP-HPLC-ICP-MS). The efficiency of the extraction of Se compounds varied according to the buffer type and the species of fungus. The 2DE buffer was the most efficient, reaching a recovery of 60% and 30% of all Se in the protein extracts of *P. ostreatus* and *L. edodes*, respectively. The main form of Se in the protein extracts was SeMet. The Se concentration was higher in *P. ostreatus* than in *L. edodes*. The *P. ostreatus* protein extracts with 2DE buffer represent a great potential for obtaining biomass proteins with high SeMet concentrations on an industrial scale. The 2D-PAGE analyses revealed the presence of eight Se-containing proteins. The nutritional value of protein biomass containing SeMet presently has a high potential to be explored by pharmaceutical industries.

Keywords- Protein extract, selenomethionine, solubilization buffer, HPLC, ICP-MS, selenium-containing proteins

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Introduction

Selenium (Se) is recognized as an essential micronutrient for humans and animals and has an important role in biological functions that is mediated primarily by selenoproteins. These proteins are vital for the antioxidant activities of cells and the metabolism of thyroid hormones [1,2]. Se has also demonstrated properties in protecting against cancer when ingested in concentrations that exceed the nutritional guidelines [3].

The chemical forms in which Se can be ingested are important factors that determine not only its bioavailability but also its metabolic pathway, distribution and nutritional function [4]. There is a consensus that Se should be consumed in an organic form because it has a higher bioavailability than in an inorganic form. Consequently, the preferred foods are those rich in organic Se, such as some plants and microorganisms that have been enriched during their growth [5].

Se enters the body primarily through food. Fish, seafood and some

vegetables have been the main sources of Se [1]. Foods such as mushrooms, garlic, and Brazilian nuts contain high concentrations of Se [1].

In nature, high Se levels between 10 and 30 $\mu\text{g g}^{-1}$ of dry weight have been observed in widely consumed species of mushrooms including *Boletus edulis*, *B. Aestivalis* and *Xerocomus badius* [6,7]. When grown in substrates with high concentrations of Se, some mushrooms are able to accumulate this element. *Pleurotus ostreatus* and *Lentinula edodes* are species-accumulative and may accumulate up to 858 and 170 μg of Se per g of dry weight, respectively [8,9]. Depending on the species and the mode of cultivation, mushrooms are able to absorb inorganic selenium from the substrates and transform it into organic forms, incorporating it as protein components or by synthesizing it as free selenoamino acids [8-10]. These aspects are an important point for using the mushrooms as biotechnological tools for the production of protein biomass rich in selenoamino acids.

The investigation of the presence of proteins containing selenoamino acids in mushrooms is of high interest [11-13]. Because *P. ostreatus* and *L. edodes* mushrooms contain relatively high protein levels and can accumulate large amounts of Se, it is reasonable to expect that Se could be incorporated into their proteins [8,9].

Improvements in the methodologies for the speciation of Se have made it possible to show more details of the species of Se present in mushrooms [1]. There are studies evaluating the concentration of Se-species in Se-enriched mushrooms [10,14-17], but this is the first study to propose a methodology for the extraction and identification of SeMet in two mushroom species with distinct enrichment processes and to characterize proteins containing Se in Se-enriched *P. ostreatus* mushrooms.

Materials and Methods

Instrumentation

The chromatographic separations were made with an Agilent 1100 series high performance liquid chromatography system (HPLC). The column used was a Phenomenex Luna C18, 250 mm x 4.60 mm, 5 μ m, and with a pore size of 100 Å. The volume of injection was 50 μ l. The elemental detection was performed using an inductively coupled plasma mass spectrometer (ICP-MS), Agilent 7500 ce (USA). The ICP-MS conditions are presented in [Table-1].

Table 1- Experimental conditions for ICP-MS

Instrumental parameters	
Forward power	1500 W
Sampling depth	7-8 mm
Plasma gas flow rate	15 mL min ⁻¹
Nebulizer gas flow rate	0.9 mL min ⁻¹
Auxiliary gas flow rate	1 mL min ⁻¹
Extract 1	0-3 V
Extract 2	-137.5 V
Omega Bias-ce	-20 V
Omega Lens-ce	-1.6 V
Cell entrance	-40 V
QP Focus	-15 V
Cell Exit	-44 V
OctP RF	190 V
OctP Bias	-18 V
H2 gas flow rate	4 mL min ⁻¹
QP Bias	-16 V
Discriminator	8 mV
Analog HV	1840 V
Replicates	3
Analysis time per isotope	0.3 s

Size-exclusion chromatography used for the fractionation of the mushroom extract and was carried out with a Superdex™ 75 10/300 GL GE Healthcare column and a preparative Hiload™ 26/60 column.

A lyophilizer (Enterprise II, Terroni) was used for drying the mushrooms. An ultrasonic probe (HD2200, Bandelin electronic, GmbH and Co. Kg, Berlin, Germany) was used for the extraction of the Se compounds. A centrifuge (Sigma Laborzentrifugen 4-10, Osterode, Germany) was used for separating the extracts.

The digestion of the samples for the determination of total Se content was conducted in a microwave oven with an acceleration system of reaction (MARS®, CEM Corporation, Matthews, North Carolina, USA) using vessels MARSX.

The first dimension separation was carried out using an IPGphor isoelectric focusing (IEF) Ettan™ dedicated for an immobilized pH gradient IEF with a pH gradient of 3-10. These IPG strips featured a non-linear pH gradient. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a Mini Protean 3 Electrophoresis System (Bio-Rad Laboratories) with 5% polyacrylamide for the stacking gels and 12% polyacrylamide for the resolving gels (8 cm x 7 cm x 0.075 cm).

Standards and Reagents

All the reagents used were of a high purity grade. Deionized water (18 M Ω cm⁻¹) was obtained from a system of Milli-Q purification (Millipore, UK). Proteases of *Streptomyces griseus* type XIV, selenocystine (SeCys₂), selenomethylselenocysteine (SeMetSeCys), D,L-selenomethionine (D-LSeMet) and sodium selenite (Na₂SeO₃) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium selenate (Na₂SeO₄) and tetraethylammonium chloride (TEAC) were obtained from Fluka (Buchs, Switzerland). Ammonium acetate, sodium phosphate (Na₂HPO₄), boric acid (H₃BO₃), sodium hydroxide, nitric acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany).

Stock solutions of 100 μ g mL⁻¹ were prepared from L-SeMet in deionized water, and SeMetSeCys, SeCys₂, Na₂SeO₃ and Na₂SeO₄ in HCl solution (3%, v/v). All these solutions were stored at 4°C in the dark until use. The working solutions were prepared daily by diluting the stock solutions with deionized water.

A solution of tetraethylammonium chloride (0.075%, w/v), pH 4.5, adjusted with HCl (10%, v/v), was used as a mobile phase and was prepared daily.

Mushroom Enrichment with Selenium

The isolates of *P. ostreatus* (Plo 02) and *L. edodes* (UFV 73) belonged to the collection of fungi from the Laboratory of Mycorrhizal Associations, Department of Microbiology/BIOAGRO, in the Federal University of Viçosa, Brazil. The *P. ostreatus* isolates were inoculated on a coffee husk based substrate [8], and eucalypt sawdust was used as a substrate for *L. edodes* [9]. Briefly, one kilogram of coffee husk, previously autoclaved at 121°C for 70 min, was added to 25 mL of a solution of Na₂SeO₃ (1 g mL⁻¹) to produce *P. ostreatus* enriched with Se. For the *L. edodes* enrichment, the eucalypt sawdust substrate was previously autoclaved at 121°C for 70 min, and the primordia induction was made by the submersion of the substrate in a solution of Na₂SeO₃ (50 mg L⁻¹) for 24 hrs. *P. ostreatus* and *L. edodes* mushrooms without Se enrichment were also used for comparative analyses. The collected mushrooms were lyophilized for 48 h, weighed, ground in a mill of knives (General Electric, MOD.SXBGOOG), packed in sealed plastic bags and stored at a room temperature of 25°C until analysis.

Protein Extractions

Three different protein extracts were investigated to obtain information about the recovery of the species of Se: phosphate buffer, borate buffer and 2DE buffer; these solutions were used for further identification of the selenoamino acids present in the samples of enriched mushrooms.

Two grams of lyophilized mushrooms were added to 40 mL of phosphate buffer (50 mM, pH 7.2), borate buffer (50 mM, pH 8.5), or 2DE buffer (8 M urea, 4% CHAPS (p/v) and 30 mM Tris-Cl, pH 8.5)

in 50 mL centrifuge tubes; the tubes were kept under agitation for 48 hrs. After this period, the tubes were centrifuged at 10000 *g* for 20 min, and the supernatant was collected and stored in the refrigerator at 10°C for the precipitation of proteins in acidified acetone (80%, v/v, pH 1.5). After the protein precipitation, the samples were centrifuged at 10000 *g* for 20 min, and the precipitate was collected and lyophilized for 24 hrs.

Selenium Content in the Mushrooms and Protein Extracts

Approximately 40 mg of mushrooms and dried protein extracts were digested with 1 mL of HNO₃ (65%, v/v) and 0.25 mL of H₂O₂ (30%, v/v). This mixture was placed into the closed tubes and transferred to a microwave oven with a programmed power of 400 W, at temperature of 160°C, and a ramp of 15 min; this was maintained for 30 min. Three replicates of each sample were prepared and analyzed. A control without an added sample was digested in the same way. After digestion, the samples were cooled, diluted in 10 mL of deionized water and filtered with a PTFE filter of 0.45 µm. The reference material was utilized to evaluate the accuracy of the readings and consisted of extractions of selenium enriched yeast at a concentration of 2059 mg kg⁻¹. The total concentration of Se was determined by ICP-MS by monitoring the isotopes ⁷⁴Se, ⁷⁶Se, ⁷⁷Se, ⁷⁸Se, ⁸⁰Se and ⁸²Se.

Selenium Speciation by RP-HPLC-ICP-MS

Samples of 20 mg of mushrooms and 20 mg of protein extracts were mixed with 20 mg of protease XIV and 5 mL of deionized water at 5°C using an ultrasonic probe at 25% power for 2 min. These mixtures were centrifuged at 10000 *g* for 20 min, and the supernatants were collected. The precipitates were submitted again to the same procedure. The supernatants of both extractions were mixed, filtered with a PTFE filter of 0.45 µm and stored at -80°C until analysis.

To identify the forms of Se, the supernatants, were injected into the RP-HPLC-ICP-MS system, which utilized Phenomenex Luna C18 columns (250 mm x 4.60 mm, 5 µm, with a pore size of 100 Å). The injection volume was 50 µL with a flow rate of 1 mL min⁻¹, and the mobile phase was composed of TEAC (0.075%, pH 4.5).

For determining and quantifying the forms of Se, a calibration curve was composed of the following standards: DLSeMet, SeMetSeCys, SeCys₂, Na₂SeO₃ (Se IV) and Na₂SeO₄ (Se VI), at concentrations of 1, 2, 5, 10, 25, 50, 100, 150 and 200 µg L⁻¹ of each type; these substances were injected into the RP-HPLC-ICP-MS system. The retention time and peak area of each standard substance were analyzed.

Extractions and Mapping of Se-Containing Proteins from Se-enriched Mushrooms

Protein Extraction

Proteins from the mushrooms were extracted by combining to 500 mg of powdered mushrooms samples, 7 mL of 20 mM ammonium acetate (NH₄Ac) at a pH of 7.4 that contained phenylmethanesulfonyl fluoride (PMSF, protease inhibitor activity) and 1 mM Tris(2-carboxyethyl) phosphine hydrochloride (TECP, antioxidant) on ice for 10 min. The extracts were centrifuged at 10000 *g* at 4°C for 30 min and decanted in a 500 mL flask. The supernatants were concentrated and underwent dialysis with Amicon Ultra 3K-3000 NMWL membranes; the supernatants were then stored at -80°C until use. To check for the presence of proteins containing Se using SEC-

HPLC-ICP-MS, an aliquot of 50 µL of the supernatant was injected into a Superdex™ 75 10/300 GL GE Healthcare column. The isocratic elution was from 0 to 40 min and was carried out in 20 mM ammonium acetate (pH 7.4, adjusted with ammonium (1:100, v/v) at a flow rate of 0.7 mL min⁻¹). The standards used for calibration of the masses were: ferritin (440 kDa), glutathione peroxidase (84.5 kDa), superoxide dismutase (32.5 kDa), myoglobin (19 kDa), metallothionein (7 kDa), cobalamin (1.3 kDa) and selenomethionine (0.196 kDa).

Additionally aliquots of 2 mL (5 replicates) of the supernatant were injected into a preparative Hiload™ 26/60 column. The elution was performed in 20 mM ammonium acetate (pH 7.4) at a flow rate of 2 mL min⁻¹. The eluate was collected every 15 min over 3 h, and the Se was selectively determined in each fraction by ICP-MS. Three fractions of proteins containing Se were grouped, concentrated and underwent dialysis with Amicon Ultra 3K-3000 NMWL membranes; the fractions were then stored in a freezer at -80°C until use.

Protein Determination

Protein quantitative determinations were performed using a Bio-Rad® Bradford Protein Assay kit. Bovine serum albumin was used as a protein standard.

SDS-PAGE

An aliquot of 60 µL containing 50 µg of protein of the elution of the mushroom enriched extracts was fractionated by molecular exclusion chromatography in accordance with the protocol described above; these aliquots were concentrated to a volume of 10 µL and dissolved in 0.02 M Tris-HCl buffer, pH 6.8. Samples of 10 µL were loaded onto an SDS-PAGE gel, and electrophoresis was performed in 25 mM Tris-HCl, pH 8.4 to 25 mA, for 50 min. The molecular weight of the proteins were determined by the electrophoresis of 8 proteins with known molecular weights (aprotinin 6.5 kDa; lysozyme 14.4 kDa; trypsin inhibitor 21.5 kDa; carbonic anhydrase 31 kDa; ovalbumin 45 kDa; albumin 66.2 kDa; β-galactosidase 116.25 kDa and myosin 200 kDa).

2D Gel

The first dimension separation was carried out using an IPGphor Isoelectric Focusing (IEF) System (Amersham Biosciences) that was dedicated for an immobilized pH gradient IEF with a gradient of pH 3-10. These IPG strips featured a non-linear pH gradient. Isoelectric and SDS-PAGE were performed with a volume of approximately 200 µL of the eluate from the SEC after the eluate was cleaned with a 2-D Clean-Up Kit (Amersham Biosciences). After cleaning, the sample containing 50 µg of protein was dissolved in a solution containing 30 mM Tris-HCl, 2 M thiourea, 7 M urea, and 4% CHAPS (w/v), pH 8.5. An aliquot of 100 µL of the dissolved sample was placed into the loading trench and strip (pH 3-10). The SDS-PAGE was performed in 25 mM Tris-HCl, pH 8.4 to 25 mA for 50 min.

Results and Discussion

Selenium Concentration and Protein Content

Selenium accumulated in both *P. ostreatus* and *L. edodes*. The total Se concentration increased 152- and 330-fold in the *P. ostreatus* and *L. edodes* Se-enriched mushrooms, respectively [Table-2]. This shows the high capability of these mushrooms to absorb inorganic Se and accumulate it into the fruitbody of the mushroom. Edible

mushrooms display variable concentrations of Se depending on the species, compost and growth substrate conditions, such as the nutrient content and availability [10]. In commercial mushrooms, such as *Agaricus bisporus*, *P. ostreatus* and *L. edodes* low levels of

Se have been found [8,9,18], but when grown on Se-enriched substrates, these levels can reach 347, 857.8 and 355.7 $\mu\text{g g}^{-1}$ of dry weight, respectively [8,10,14].

Table 2- Selenium content in mushrooms dried and proteins of *Pleurotus ostreatus* and *Lentinula edodes*.

Sample	Se content in mushrooms						Se content in protein					
	Phosphate buffer			Borate buffer			2DE buffer					
	$\mu\text{g g}^{-1}$	SD	RSD %	$\mu\text{g g}^{-1}$	SD	RSD %	$\mu\text{g g}^{-1}$	SD	RSD %	$\mu\text{g g}^{-1}$	SD	RSD %
PoNE	1.76	0.02	1.25	1.52	0.17	11.21	1.66	0.09	5.56	2.69	0.05	1.92
PoSe	267.55	0.29	0.11	136.77	8.08	5.91	178.94	0.83	0.46	369.17	4.9	1.33
LeNE	0.53	0.04	3.8	0.33	0.09	29.64	0.38	0.01	3.43	0.57	0.04	6.18
LeSe	174.46	4.07	2.33	92.27	1.33	1.44	162.96	4.79	2.94	285.56	0.16	0.05

PoNE: Non-enriched *P. ostreatus*, PoSe: Se-enriched *P. ostreatus*, LeNE: non-enriched *L. edodes*, LeSe: Se-enriched *L. edodes*, SD: Standard Deviation, RSD: Relative Standard Deviation of the mean (n=3).

The protein content of the *P. ostreatus* mushrooms was higher than that of the *L. edodes* mushrooms for all buffers tested [Fig-1A]. The 2DE buffer was the best buffer for protein extraction, and this finding can be attributed to the different ion concentrations in the buffers that affect important properties such as solubility and the dissociation of substances. Thus the higher ionic strength in the 2DE buffer, which is a function of the concentration of all ions present in a solution, promoted the greater solubility of the compounds contained in the mushrooms and thereby provided an increased solubilization of the proteins.

The 2DE buffer promoted the best recovery percentage of total Se for both species of mushrooms [Fig-1B], which is an important characteristic that enables its use as efficient extractant for the production of protein biomass rich in Se. In addition, *P. ostreatus* had a higher recovery percentage of Se in the dry weight of proteins than *L. edodes* [Fig-1B], which can be explained by the higher concentration of protein found in *P. ostreatus* in relation to *L. edodes* [Fig-1A]. These results show that the effect of the extractant depends on the fungal species. For example, when pronase was used as an extractant of Se-enriched *A. bisporus* mushrooms, the efficiency of the extraction (78%) was increased two-fold compared to when water was used (42%) [19]. However, for Se-enriched *L. edodes*, the pronase treatment had little effect compared with water extraction (77.5 vs 68%) [14]. The values observed in our experiment indicate that the level of recovery of Se bound proteins is higher than the recovery of Se non-bound proteins.

Selenium Speciation

The accumulation of five known forms and two unknown forms of Se was observed in the Se-enriched mushrooms of *P. ostreatus* and *L. edodes* [Fig-2]. In non-enriched *P. ostreatus*, only SeMet was observed [Fig-2A], whereas for non-enriched *L. edodes*, it was not possible to detect any form of Se [Fig-2B]. Additionally, in the Se-enriched *P. ostreatus*, the main form identified was SeMet, whereas in the Se-enriched *L. edodes* the main form identified was Se IV [Fig-2C], [Fig-2D].

All the buffers tested increased the protein extract, in the following order: 2DE buffer > phosphate buffer > borate buffer [Fig-1], [Fig-3], demonstrating the efficiency of the 2DE buffer in recovering SeMet, which is a less toxic form of Se that is more bioavailable to organisms. Using the 2DE buffer, we obtained protein biomasses containing up to 88.42% and 92% for Se-enriched *P. ostreatus* and *L. edodes* mushrooms, respectively [Fig-3B].

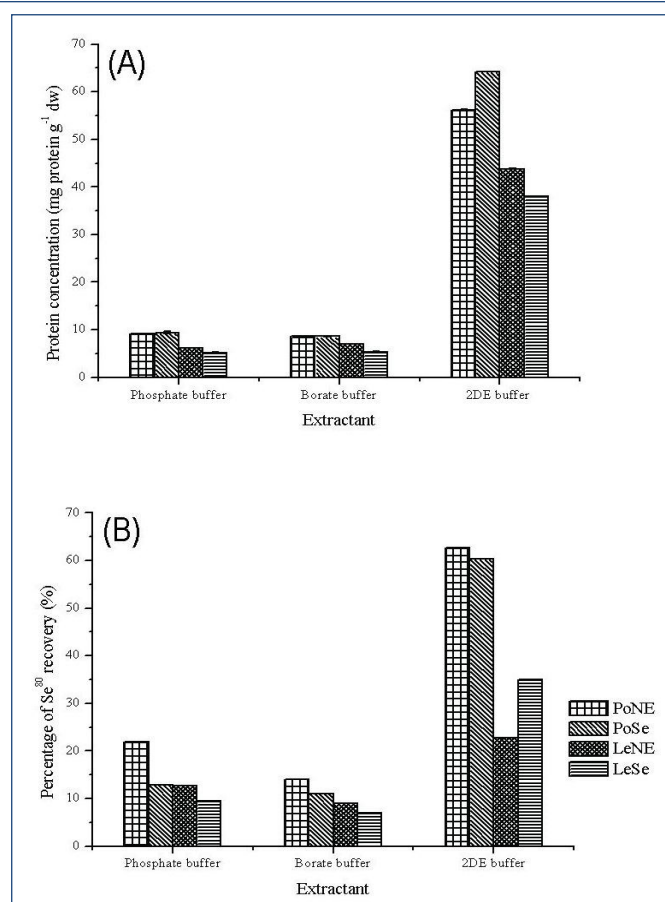


Fig. 1- Concentration of buffer soluble proteins in non-enriched *P. ostreatus* (PoNE), Se-enriched *P. ostreatus* (PoSe), non-enriched *L. edodes* (LeNE) and Se-enriched *L. edodes* (LeSe) mushrooms (A), and the percentage of Se recovery in these proteins using different buffers extractants (B). The error bars indicate the standard deviation of the mean (n=3).

In non-enriched mushrooms of both fungi species, SeMet was the only form of Se identified, corresponding to 63.46% of the total Se [Fig-3A]. In non-enriched *L. edodes*, SeMet was detected only when the protein was extracted with 2DE buffer [Fig-3A]; this may be due to the low concentration of SeMet, because for the equipment used, the minimum concentration threshold for detection was 2.6 ng g^{-1} Se. This result confirmed the low efficiency of the phosphate and borate buffers as extractants of Se compounds.

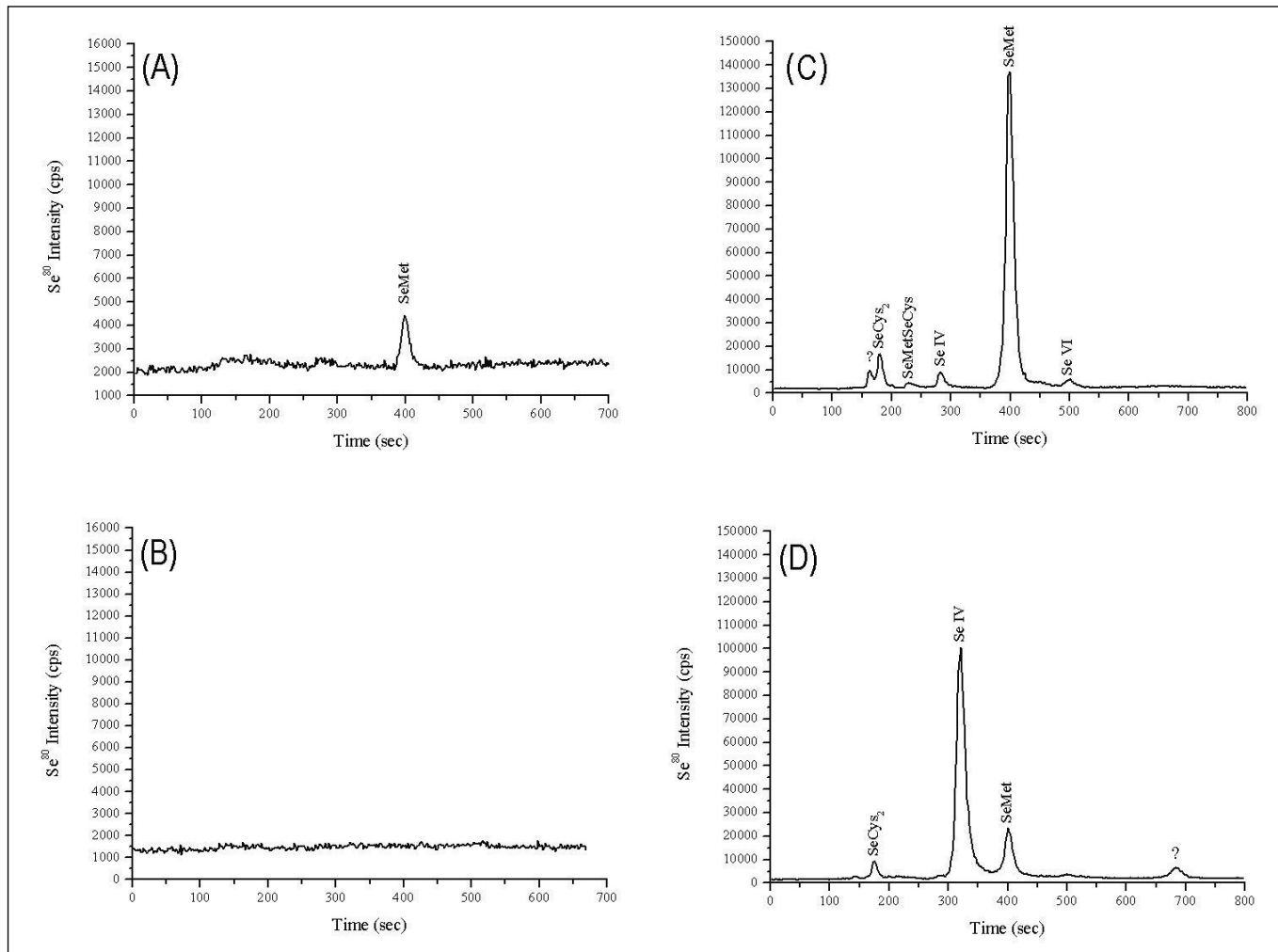


Fig. 2- RP-HPLC-ICP-MS chromatograms of selenium compounds: selenocystine (SeCys₂); selenomethylselenocystein (SeMetSeCys); selenomethionine (SeMet); sodium selenite (Se IV); sodium selenate (Se VI) and unknown forms of selenium (?) in the following mushrooms: non-enriched *Pleurotus ostreatus* (A); non-enriched *Lentinula edodes* (B); Se-enriched *P. ostreatus* (C); Se-enriched *L. edodes* (D).

Se-enriched mushrooms of *P. ostreatus* exhibited a significant increase in SeMet levels in relation to other species of Se, such as the SeMetSeCys, SeCys₂, Se IV and Se VI [Fig-3B]; they also had a higher content of organic-Se than inorganic-Se, with SeMet being the main type. In contrast, in *L. edodes*, the main type of Se was inorganic Se (Se IV) although the protein extraction buffers were able to concentrate the organic form of Se [Fig-3B].

Selenium speciation studies have identified but not quantified SeMet, SeCys₂ and SeMetSeCys in mushrooms [14-16]. Some analyses have reported SeMet to be the main selenoamino acid in *A. bisporus* and *L. edodes* mushrooms grown on compost enriched with inorganic Se [20,21]. However, three selenoamino acid types, SeMet, SeCys₂ and SeMetSeCys were accumulated by *A. bisporus*, with SeCys being the main selenoamino acid [10,17].

The differences in these results may in part be explained by different methods used in the extraction and quantification of the selenoamino acids and also by the oxidative degradation of the selenium compounds [22,23]. The cultivation process of the fungus, the concentration and form of Se added during enrichment and the physiology of the fungus all influence the forms of Se absorption and also the capability for biotransformation [8-10].

There is evidence that the increased Se status attained after

supplementation with organic forms of Se is retained for a longer period after supplementation has ceased than is the case with Se IV or Se VI [24]. The whole body half-lives of SeMet and Se IV or Se VI are reported to be 252 and 102 days, respectively, implying that Se administered as SeMet is retained 2.5 times longer in the body than is Se IV [25]. Accordingly, foods or supplements containing SeMet can maintain the activities of selenoenzymes during depletion for longer periods of time than those containing inorganic Se due to the recycling of SeMet that is catabolized from stored protein [25]. Thus, the high level of SeMet in protein extracts from the 2DE buffer of Se-enriched mushrooms causes increased biological benefits compared to the Se-enriched mushrooms with high concentrations of inorganic form, such as Se (IV) and Se (VI).

The content of 326.4 or 262.7 µg of SeMet per g detected in the dried protein of *P. ostreatus* and *L. edodes* mushrooms, respectively, were approximately five-fold greater than the Recommended Dietary Allowances (RDA) provided in the Dietary Reference Intakes developed by the Institute of Medicine of the USA for Se. This level is currently 55 µg for adults of both sexes [26]. The consumption of only 0.17 or 0.21 g of dried protein of Se-enriched *P. ostreatus* and *L. edodes* mushrooms, respectively, meets the daily needs for Se in the form of SeMet, indicating that the Se-enriched

P. ostreatus and *L. edodes* mushrooms can be considered to be an excellent source of this element.

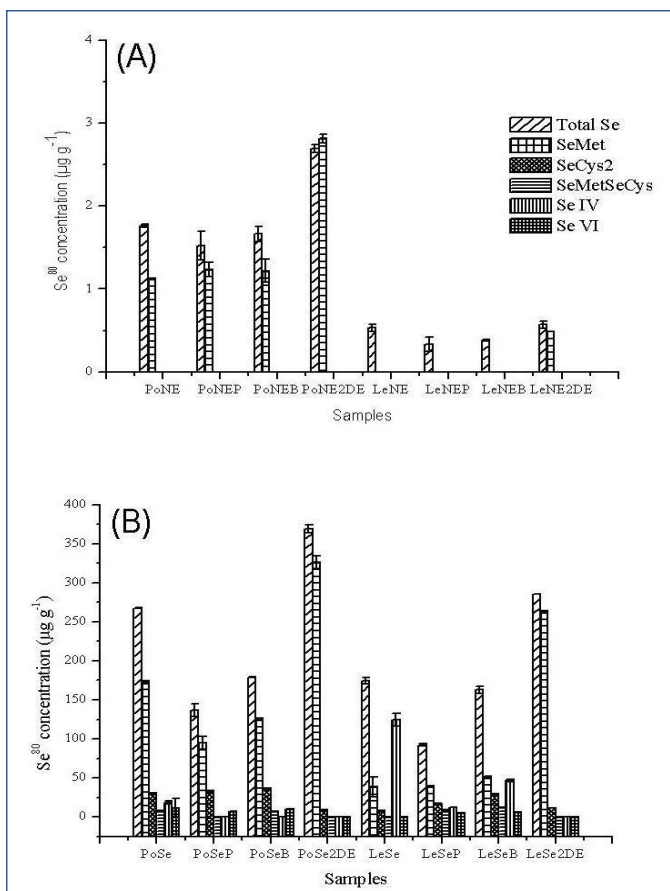


Fig. 3- Total concentration of the Se forms in the mushrooms and in protein extract of non-enriched (A) and Se-enriched (B) mushrooms. Mushrooms of non-enriched *Pleurotus ostreatus* (PoNE); protein extract of non-enriched *P. ostreatus* extracted with phosphate buffer (PoNEP), borate buffer (PoNEB) or solubilization buffer (PoNE2DE); mushrooms of non-enriched *L. edodes* (LeNE); protein extract of non-enriched *L. edodes* extracted with phosphate buffer (LeNEP), borate buffer (LeNEB) or solubilization buffer (LeNE2DE). Se-enriched mushrooms of *P. ostreatus* (PoSe); protein extract of Se-enriched *P. ostreatus* extracted with phosphate buffer (PoSeP), borate buffer (PoSeB) or solubilization buffer (PoSe2DE); Se-enriched mushrooms of *L. edodes* (LeSe), protein extract of Se-enriched *L. edodes* extracted with phosphate buffer (LeSeP), borate buffer (LeSeB), or solubilization buffer (LeSe2DE). The error bars indicate the standard deviation of the mean ($n=3$).

Fractions Purifications of Proteins Containing Se by SEC-HPLC-ICP-MS in *P. ostreatus* Mushrooms

The SeMet content in the Se-enriched *P. ostreatus* mushrooms was four-fold higher ($172.21 \mu\text{g g}^{-1}$) compared to the Se-enriched *L. edodes* mushrooms ($38.45 \mu\text{g g}^{-1}$). In the non-enriched mushrooms, it appeared that a large portion of the Se accumulated in the protein biomass in the fungus [Fig-3]. Thus, size exclusion chromatography was applied in this study to isolate fractions of proteins that were Se-linked in Se-enriched *P. ostreatus*, revealing the intensity of the signal traced by Se in the Se-enriched mushrooms [Fig-4], which contrasts with the lack of a signal in the non-enriched mushrooms (data not presented).

The Se-enriched mushrooms showed a peak that correlated to the selenoamino acid SeMet ($M_r = 196 \text{ Da}$) in a free form. In addition to this peak, three peaks were found, corresponding to compounds with molecular weights greater than 19, 32.5 and 84.5 kDa [Fig-4]. Several extraction procedures were performed previously to resolve these three peaks; however, these attempts were not successful. The chromatogram of Se-enriched *P. ostreatus* showed peaks that corresponded to proteins containing Se with molecular weights between 19 and 440 kDa [Fig-4].

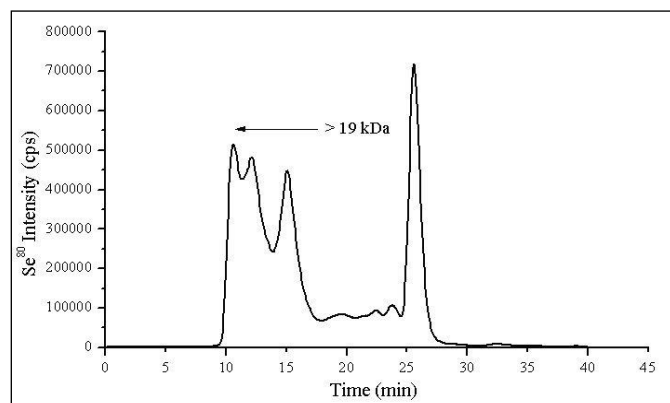


Fig. 4- Se-linked to biomolecules in Se-enriched *Pleurotus ostreatus* mushrooms by SEC-ICP-MS. Chromatographic conditions: column - Superdex™ 75 ($10 \times 300 \times 13 \mu\text{m}$); mobile phase - 20 mM ammonium acetate (pH 7.4); flow rate - 0.7 mL min^{-1} ; injected volume - $50 \mu\text{L}$.

Thus, we conclude that each peak corresponds to proteins containing Se; however, this analysis is based on retention times and the signal intensity of Se. Therefore, it does not provide enough information to predict the exact structure of these compounds [12]. An approach using mass spectrometry and protein gels is required to identify the proteins and confirm the Se content of the same.

Mapping of the Selenopeptides

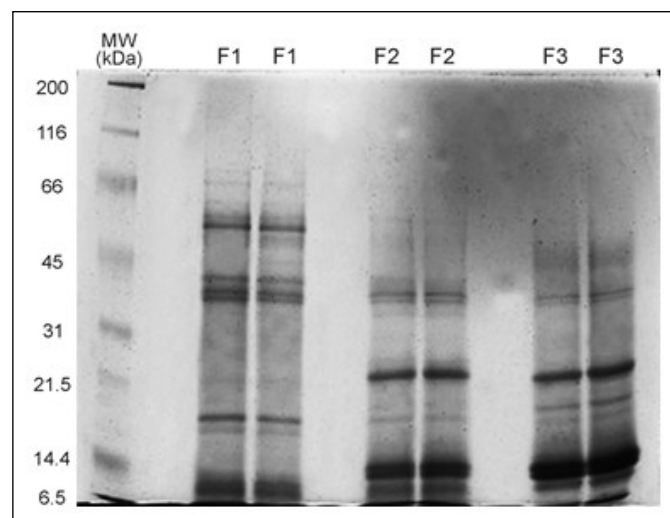


Fig. 5- Gel electrophoresis SDS-PAGE of the fraction one (F1), fraction two (F2) and fraction three (F3) purified in duplicates of Se-enriched *Pleurotus ostreatus* mushrooms. Markers of known molecular weight (MW): aprotinin 6.5 kDa; lysozyme 14.4 kDa; trypsin inhibitor 21.5 kDa; carbonic anhydrase 31 kDa; ovalbumin 45 kDa; albumin 66 kDa; β -galactosidase 116 and myosin 200 kDa.

The profile of the proteins and the presence of Se in the fractions of Se-enriched mushrooms, retrieved by SEC-HPLC-ICP-MS, showed that the fraction one (F1) exhibited a distinct profile from the fractions two (F2) and three (F3), with the presence of proteins with molecular weights ranging from 6.5 to 66 kDa [Fig-5]. After nitric digestion, of all the bands of each fraction, the total content of Se total was 40.47, 24.24, 34.22 $\mu\text{g}\cdot\text{g}^{-1}$, for F1, F2 and F3, respectively, confirming that all the collected fractions contained Se.

The 2D gel electrophoresis of the three fractions collected from *P. ostreatus* enriched with Se presented 12 well defined spots [Fig-6]. Spots 1, 2 and 3 corresponded to proteins with isoelectric points of 2.3, 2.5 and 5, respectively. Spot 4 had an isoelectric point of 6 and a molecular weight of approximately 14.4 kDa, which corresponded to the gel SDS-PAGE [Fig-5], [Fig-6A]. Additionally, the gels corresponding to the second and third fractions seemed to present the same profile of proteins with the spots 5, 6, 7 and 8, showing isoelectric points of 2, 2.4, 3.1 and 5, respectively [Fig-6B], [Fig-6C].

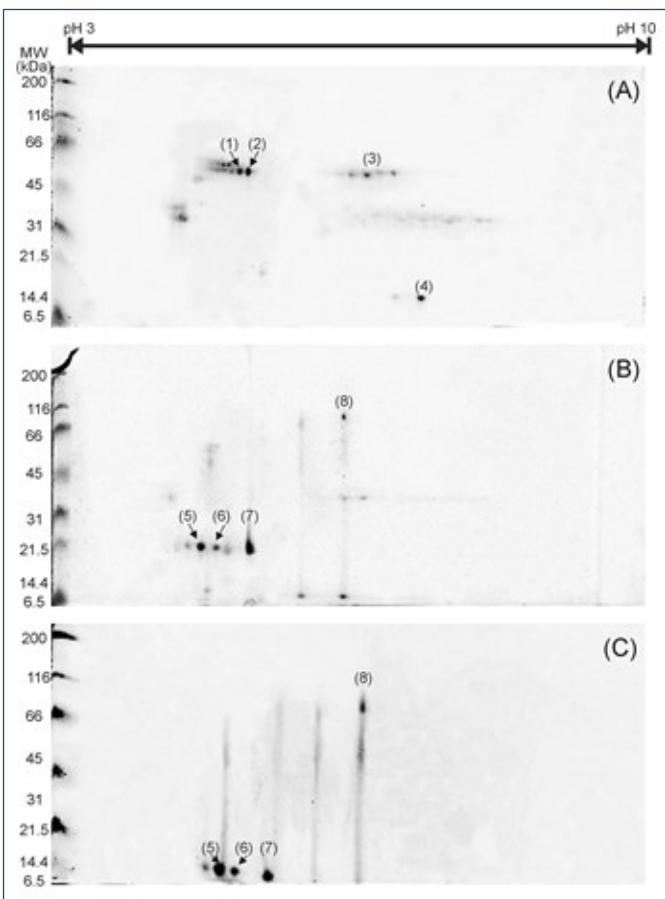


Fig. 6- Bidimensional electrophoresis gels of fraction one containing Se proteins 1, 2, 3 and 4 (A), fraction two (B) and fraction three (C) containing Se proteins 5, 6, 7 and 8, obtained from extracts of *Pleurotus ostreatus* mushrooms enriched with Se purified by SEC-HPLC-ICP-MS. Isoelectric focusing (IEF) with a pH gradient of 3-10. Markers of known molecular weight (MW): aprotinin 6.5 kDa; lysozyme 14.4 kDa; trypsin inhibitor 21.5 kDa; carbonic anhydrase 31 kDa; ovalbumin 45 kDa; albumin 66 kDa; β -galactosidase 116 and myosin 200 kDa.

The aqueous extract analysis of the Se-enriched yeast that underwent 2D gel electrophoresis, nanoHPLC-ICP MS and nanoHPLC-ESI MS/MS revealed the presence of two selenium-containing pro-

teins: the HSP12 protein (11693 Da) corresponding to a spot with an apparent mass of 14.4 kDa and the SIP18 protein (17748 Da) corresponding to a spot with an apparent mass of 20.1 kDa [11]. This was in agreement with the spots 4, 5, 6 and 7 observed in this study, which shows that a similar Se-protein is present, although it belongs to a different species of the fungi [Fig-6].

The results showed that *P. ostreatus* and *L. edodes* edible mushrooms, if enriched with Se, present a high potential for use as functional foods. Additionally, *P. ostreatus* Se-enriched mushrooms could provide better biological benefits than *L. edodes* Se-enriched mushrooms, which contains mainly inorganic Se.

Conclusions

Fungi species and enrichment methods affect the form of Se that bioaccumulates in mushrooms. The element is distributed in five known types including SeCys₂, SeMet, SeMetSeCys, Se IV and Se VI. Se-enriched *P. ostreatus* mushrooms, in which Se is added to the substrate, bioaccumulate Se mainly as SeMet, while *L. edodes* mushrooms, in which Se is added only in the shock water, bioaccumulate this element mainly as Se IV.

Solubilization buffer was a better extractant for protein biomass rich in SeMet. Thus, this technique has a high potential for obtaining protein biomass containing high concentrations of selenoamino acid on an industrial scale.

There are potentially eight Se-containing protein in Se-enriched mushrooms of *P. ostreatus*, that may be of interest for the pharmaceutical industries.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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