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An attempt to differentiate HPLC-ICP-MS selenium speciation in natural and selenised *Agaricus* mushrooms using different species extraction procedures

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Abstract Total determination and speciation analysis of Se in commercial and selenised *Agaricus* mushrooms have been performed to investigate the Se species naturally occurring in non-enriched mushrooms as well as those present in specimens grown in a Se-enriched medium. Mushroom aqueous and enzymatic extracts have been analysed by three complementary chromatographic separation mechanisms (size-exclusion, anion-exchange and reversed-phase) coupled to an inductively coupled plasma mass spectrometer with an octopole reaction system. Post-column isotope dilution analysis has been used on-line with the separations for quantification of the Se species eluted. The ^{78}Se -to- ^{77}Se isotope ratio was monitored after adequate corrections for both total determinations and Se species quantitative speciation. The results showed marked differences not only in total Se contents but also in Se species found in the two types of *Agaricus* mushrooms investigated. Selenomethionine was detected in both of them (free in commercial mushrooms and incorporated into proteins in selenised ones) together with a number of unknown selenocompounds.

Keywords *Agaricus* · Mushroom · Se speciation · Hybrid high-performance liquid chromatography inductively coupled plasma mass spectrometry · Isotope dilution analysis

Introduction

Despite the relatively high levels of Se found in mushrooms, one of the most important vegetable Se sources,

some studies have reported relatively low Se bioavailability in these foods [1, 2]. As bioavailability and absorption strongly depend on the chemical form in which the element is present, rapid, accurate and precise analytical methodologies for the qualitative and quantitative speciation analysis of Se in foodstuffs are now mandatory.

On the other hand, the accurate quantification of the separated Se species is not always possible by the “traditional” techniques (such as external or standard addition calibration methods), owing to the fact that most selenocompounds detected are unknown. In this sense, an attractive approach to get reliable species determinations in biological materials consists of the application of isotope dilution (ID) techniques [3], with the species-unspecific spiking mode proposed by Heumann et al. [4] being the approach that allows accurate quantification of the heteroatom measured in a compound, even in unknown species [5]. For such an approach species separation must be performed first (e.g. by chromatography) and the enriched isotope is added after the column for inductively coupled plasma (ICP) mass spectrometry (MS) specific detection [3]. This methodology was recently applied in our group to Se speciation in human serum [6] and in yeast, wheat flour and cod muscle extracts [7, 8]. As ID is only useful for elements having at least two isotopes free of interferences, the application of this technique to the analysis of Se cannot be performed in conventional quadrupolar ICP-MS and reaction cells or high-resolution instruments have to be used.

Very few papers have been published so far on total determination and speciation analysis of Se in mushrooms and most of the work reported has been performed in Se-enriched specimens [9–11]. In this sense, the most widely investigated mushroom is *Agaricus bisporus* [1, 12, 13], whose consumption has been shown to retard the growth of chemically induced tumours [14].

The aim of this work was a quantitative comparison of Se species occurring in *Agaricus* mushrooms, both natural and grown in a Se-enriched medium (selenised), using three different Se extraction procedures.

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Experimental

Instrumentation

A Milestone (Socisole, Italy) model 1,200 microwave oven with an EM-457(A) extractor module and an AC-100 open/close module and medium-pressure PTFE vessels were employed for digestion of samples.

All the extractions were carried out in a Digiterm 100 digitally controlled immersion thermostat (Selecta, Barcelona, Spain) and a Biofuge stratos centrifuge (Heraeus, Hanau, Germany) was employed for centrifugation of the extracts.

A Shimadzu LC-10 A high-performance liquid chromatography (HPLC) pump (Kyoto, Japan) was used for chromatographic analysis, and injections were carried out using a model 7,725 injection valve (Rheodyne, CA, USA) fitted with a 100- μ l loop. Reversed-phase (RP) HPLC was performed using a Waters Spherisorb ODS2 column (250 mm \times 4.6-mm inner diameter) (Milford, Massachusetts, USA) with particles of 5- μ m diameter. Anion-exchange (AE) HPLC was carried out using a Hamilton PRP-X100 column (250 mm \times 4.1 mm, 10 μ m) (Hamilton Company, Reno, Nevada, USA). Size-exclusion chromatography (SEC) separations were achieved using a Superdex Peptide column (300 mm \times 7.5-mm inner diameter) (Pharmacia, Uppsala, Sweden). A model HP4 peristaltic pump (Sharlau Sciences, Barcelona, Spain) was used for on-line introduction of the enriched isotope at the exit of the chromatographic column.

An Agilent 7,500c (Agilent Technologies, Tokyo, Japan) ICP octopole reaction system (ORS) mass spectrometer was used as a specific detector. A continuous flow of 4 ml min⁻¹ of hydrogen was introduced into the octopole cell as the reaction gas under mass flow control. Plasma conditions and acquisition parameters were those previously reported [7].

Reagents and materials

Suprapur hydrogen peroxide (Merck, Darmstadt, Germany) and nitric acid from Merck (after purification by subboiling distillation) were used for sample digestions.

Protease XIV for enzymatic hydrolyses was supplied by Sigma-Aldrich (Steinheim, Germany).

Enriched ⁷⁷Se (91.17% of abundance for ⁷⁷Se) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as an elemental powder and it was dissolved in a minimum volume of subboiled nitric acid and diluted then with ultrapure water, which was obtained from a Milli-Q system (Millipore Co., Bedford, MA, USA).

A standard solution of 1,000 mg l⁻¹ of Se was purchased from Merck.

Samples

Natural (non-Se-enriched) *Agaricus* mushrooms were purchased from a local fruit shop and freeze-dried, while the lyophilised Se-enriched sample was provided by Peter Fodor (Szent István University, Budapest, Hungary).

Procedures

The procedures for microwave digestion as well as for the chromatographic separation and the determination of Se in the digested samples and in the mushroom extracts are the same as already reported in a previous paper dealing with different wild edible mushrooms [15]. The extraction procedures used here were as follows:

1. Extraction with water (37 or 85°C): 5 ml of Milli-Q water was added to 0.2 g of freeze-dried samples. The mixture was kept in a digitally controlled immersion thermostat at the corresponding temperature (37 or 85°C) for 1 h.
2. Enzymatic hydrolysis with protease: 20 mg of protease and 5 g of Milli-Q water were added to 0.2 g of freeze-dried mushrooms and then incubated for 16 h at 37°C.

After the extraction, the samples were centrifuged (4000 g, 45 min, 15°C) and filtered through 0.45- μ m filters.

Results and discussion

Total Se determination and evaluation of extraction recoveries

Total Se was determined first in three aliquots of each mushroom (previously digested) by ID-ICP-MS using the measurement of the ⁷⁸Se-to-⁷⁷Se ratio. The same technique was used for determination of extracted Se in three replicates of each extract.

The observed total Se concentrations after both digestion and the three different extractions as well as the corresponding extraction efficiencies for natural and selenised *Agaricus* mushrooms and for each sample treatment investigated are shown in Table 1.

Total Se present in selenised *Agaricus* mushrooms was found to be up to 30 times higher than that found in natural (commercial) samples. Moreover, in commercial mushrooms the use of proteolytic enzymes did not improve the extraction yields reached using hot water (suggesting that in such samples no significant binding of Se to the primary chain of proteins occurs), while in selenised *Agaricus* the highest recovery of Se was obtained for enzymatic hydrolysis using protease, indicating that the exposure of the mushroom to high Se concentrations stimulates to some extent the incorporation of the element into its proteins.

Table 1 Total Se concentrations found (referred to a solid sample) after digestion and different extraction procedures of commercial and selenised *Agaricus* mushrooms and the corresponding extraction recoveries (values expressed as mean \pm standard deviation)

	Total Se in the solid ($\mu\text{g g}^{-1}$)	Extraction procedure	Total Se in the extract ($\mu\text{g g}^{-1}$)	Extraction yield (%)
Commercial <i>Agaricus</i>	1.6 \pm 0.1	Water 85°C	0.98 \pm 0.03	60 \pm 2
		Water 37°C	1.13 \pm 0.01	68.8 \pm 0.5
		Protease	0.99 \pm 0.06	61 \pm 3
Selenised <i>Agaricus</i>	51.4 \pm 0.8	Water 85°C	21.5 \pm 0.4	41.8 \pm 0.8
		Water 37°C	20.9 \pm 0.4	40.6 \pm 0.7
		Protease	29 \pm 4	56 \pm 7

Quantitative speciation of Se: species determination by LC-ID-ICP-MS

Mushroom extracts (three replicates of each) were then analysed by SEC, AE and RP chromatographies coupled to the ICP mass spectrometer to identify extracted compounds containing Se. Post-column ID was used for quantification

of Se in every detected peak following the protocol previously used [15].

Natural (commercial) *Agaricus* mushrooms

SEC-ICP-MS

The extracts injected in the SEC-ID-ICP-MS system provided the mass flow chromatograms shown in Fig. 1, chromatograms I, which pointed out that the speciation of Se in water extracts varied depending on the temperature. Thus, using warm water (at 37°C) a Se species was detected at 10.8 min (corresponding to around 7 kDa), which seems to be degraded when the extraction is performed in hot water (at 85°C). The degradation of some Se species present in mushrooms when the extraction is performed using water at high temperature was also recently reported by Ogra et al. [10].

AE-HPLC-ID-ICP-MS

The same extracts were also analysed by AE-HPLC-ICP-ORS-MS using post-column ID for species quantification. The mass flow chromatograms corresponding to the different

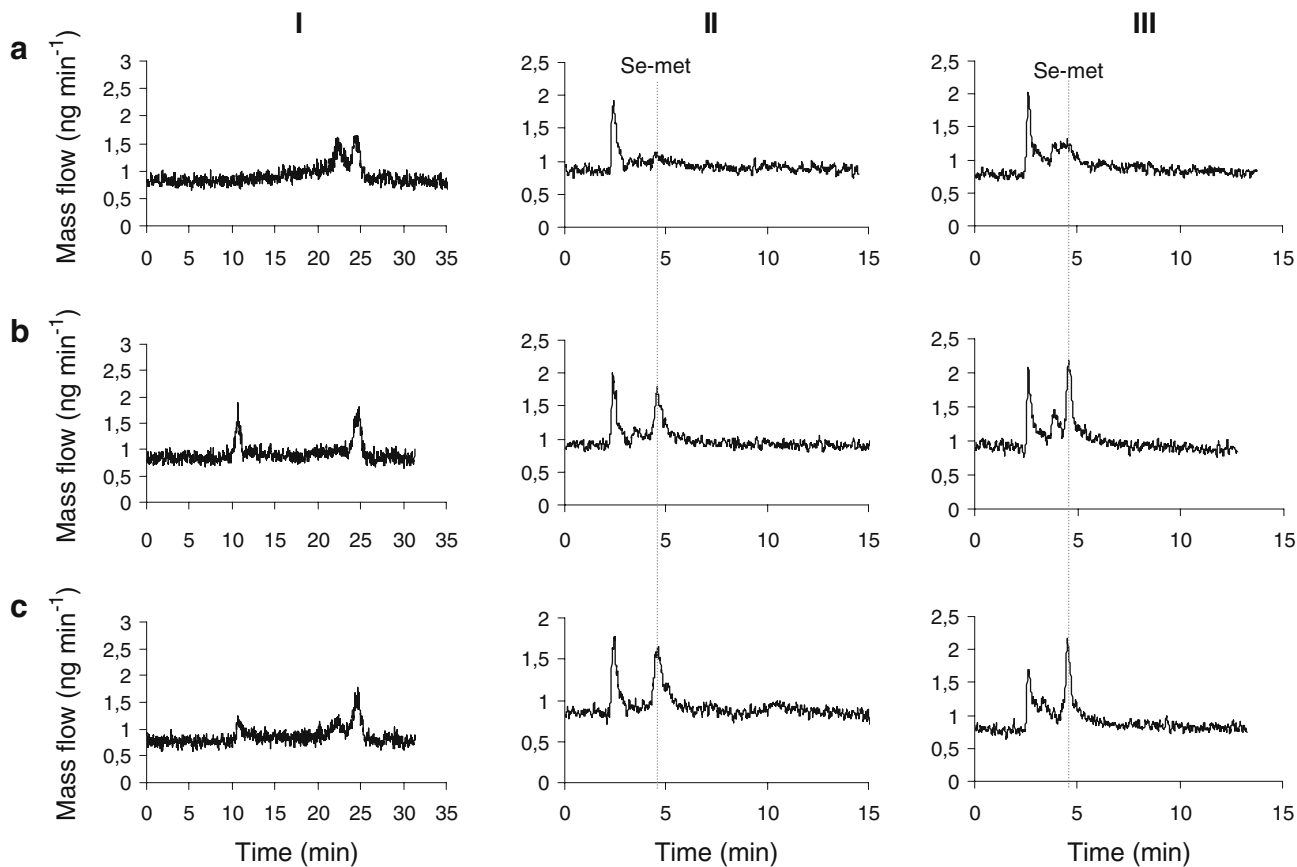


Fig. 1 Mass flow chromatograms obtained by size-exclusion (I), anion-exchange (II) and reversed-phase (III) liquid chromatography isotope dilution inductively coupled plasma mass spectrometry (LC-

ID-ICP-MS) for different extracts of commercial *Agaricus* mushrooms: **a** water at 85°C, **b** water at 37°C, **c** protease

extracts obtained from the natural (commercial) *Agaricus* mushrooms are shown in Fig. 1, chromatograms II.

As seen in the figure, the three extracts showed two major Se peaks on the AE column, one close to the void volume and the other matching the retention time of selenomethionine.

Table 2 contains the concentration of Se found in each of those two chromatographic peaks detected in commercial mushrooms, as calculated by post-column ID. As can be seen, the concentrations of Se in both peaks corresponding to warm water and enzymatic extractions were not significantly different (this indicates that the amount of Se bound to proteins seems very low).

RP-HPLC-ID-ICP-MS

RP chromatography was also used for separation of selenocompounds in the different extracts of commercial *Agaricus* to confirm the presence of selenomethionine (complementary separation mechanisms principle [16]). The corresponding chromatograms are presented in Fig. 1, chromatograms III, and the quantification of Se in the chromatographic peaks was again performed by post-column ID.

These new results confirmed the different Se speciation observed before in aqueous extracts of commercial *Agaricus* mushrooms depending on the temperature. In such chromatograms as shown in Fig. 1, chromatograms III, again one main species appeared close to the void volume (at 2.6 min) and the other one matched the retention time of selenomethionine. That is, two complementary separation mechanisms point to selenomethionine as one of the main Se species in natural (non-enriched) *Agaricus*. Moreover, Se does not seem to be incorporated into peptidic primary structures (very similar profiles were obtained for enzymatic hydrolysates and 37°C water extracts).

The concentrations of Se (referred to the freeze-dried sample) found in each Se RP peak in commercial *Agaricus* extracts are shown in Table 3 (where hot water extract peaks appearing at 3.9 and 4.6 min were quantified together as they appeared overlapped, Fig. 1a, chromatogram III).

It is remarkable that the concentrations of Se found as selenomethionine in 37°C water and in enzymatic extracts were not significantly different from those previously found by AE-HPLC, indicating the chromatographic purity of that particular selenocompound. Also, the similarity

Table 2 Concentrations of Se (as nanograms per gram, referred to a freeze-dried sample, $n=3$ independent extracts) found in the Se species detected in different extracts of commercial *Agaricus* mushrooms by anion-exchange (AE) high-performance liquid chromatography (HPLC)

t_R (min)	Commercial <i>Agaricus</i> AE-HPLC		
	Water 85°C	Water 37°C	Protease
2.4	100±20	107±3	97±14
4.6 ^a	20±2	134±28	157±7

^a t_R of selenomethionine

Table 3 Concentrations of Se (as nanograms per gram, referred to a freeze-dried sample, $n=3$ independent extracts) found in the Se species detected in different extracts of commercial *Agaricus* mushrooms by reversed-phase (RP) HPLC isotope dilution inductively coupled plasma mass spectrometry (ID-ICP-MS)

t_R (min)	Commercial <i>Agaricus</i> RP-HPLC		
	Water 85°C	Water 37°C	Protease
2.6	122±12	94±19	91±3
3.3	–	–	58±6
3.9	163±16	61±7	–
4.6 ^a	–	121±21	161±15

^a t_R of selenomethionine

found confirms that in natural *Agaricus* mushrooms selenomethionine is not incorporated in the primary structure of the proteins.

Selenised *Agaricus* mushrooms

SEC-ICP-MS

As in natural mushrooms, Se speciation was first investigated in selenised *Agaricus* extracts using SEC. However, the effect of water temperature on the species of Se extracted (previously pointed out in commercial specimens) was not observed in selenised mushrooms (Fig. 2a, chromatogram I, b chromatogram I).

The differential comparison of the SEC profiles obtained for the two groups of *Agaricus* investigated showed that Se speciation was different in natural (Fig. 1) and in selenised mushrooms (Fig. 2).

AE-HPLC-ID-ICP-MS

Again, conversely to the observed facts in commercial *Agaricus* (Fig. 1, chromatograms II), the AE chromatograms of selenised mushrooms (Fig. 2, chromatograms II) showed almost identical profiles for the two aqueous extracts under scrutiny. At least three peaks were apparent: a peak at 4.6 min that matched the retention time of selenomethionine and two peaks at 3.6 and 5.4 min, respectively (peaks B and C in Fig. 2, chromatograms II). In the enzymatic extracts of such selenised samples the Se peaks at 3.6 and 4.6 min (previously observed in aqueous extracts) were also detected, as well as an intense peak in the void volume (peak A) and a small peak at 6.1 min (peak D).

Table 4 shows the observed concentrations of Se (calculated by post-column ID) in each peak, as separated by AE in the selenised *Agaricus*.

RP-HPLC-ID-ICP-MS

RP-HPLC was again carried out as well as a complementary separation mechanism to confirm the results obtained by AE.

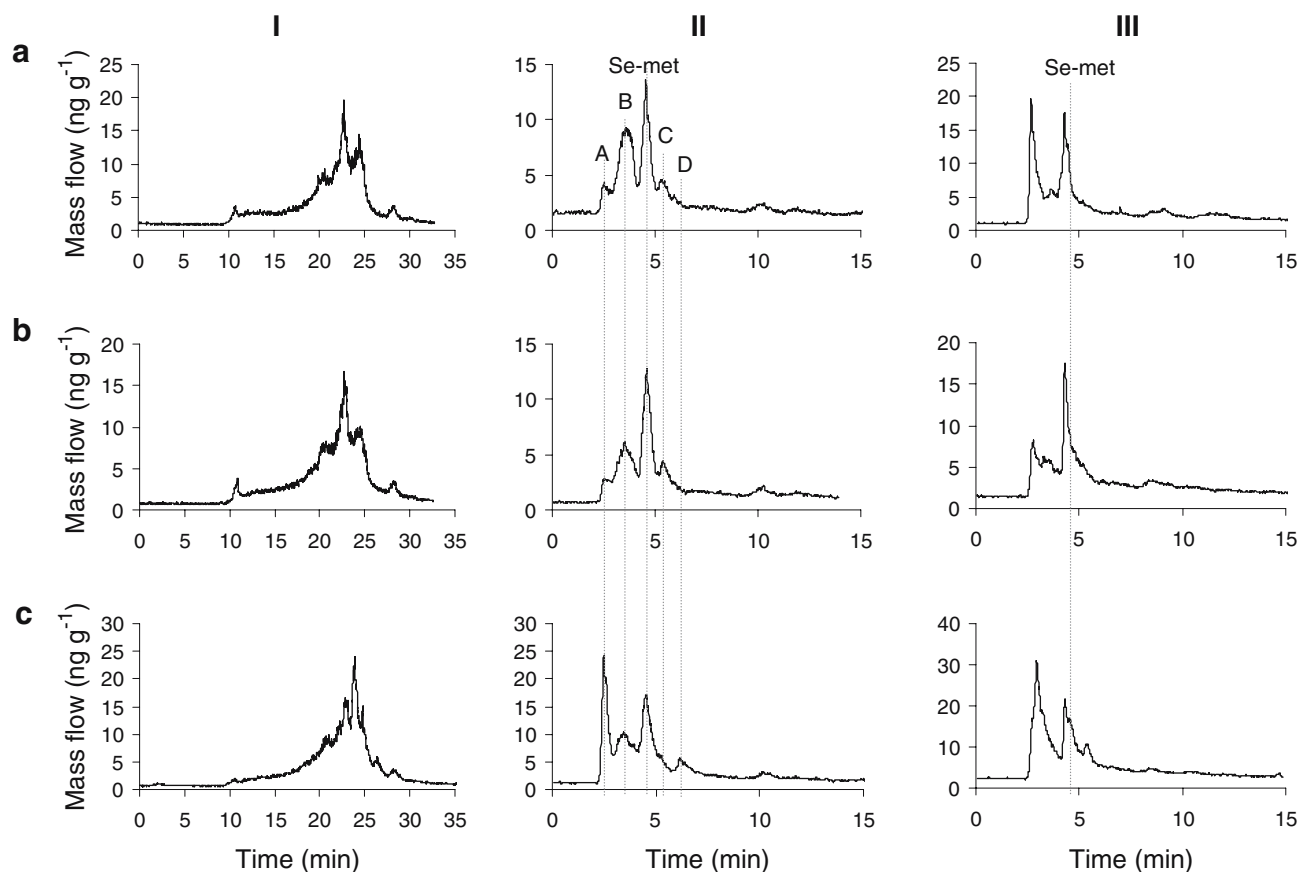


Fig. 2 Mass flow chromatograms obtained by size-exclusion (I), anion-exchange (II) and reversed-phase (III) LC-ID-ICP-MS for different extracts of selenised *Agaricus* mushrooms: **a** water at 85°C, **b** water at 37°C, **c** protease

The corresponding chromatograms obtained with this column can be seen in Fig. 2, chromatograms III. The profiles observed for the two aqueous extracts (85 and 37°C) were similar, presenting at least two overlapping species that are eluted close to the void volume (which were quantified together) and a Se peak at 4.3 min (slightly lower retention time than that of selenomethionine under the chromatographic conditions used). Perhaps most striking was the fact that selenomethionine does not appear at significant levels in the aqueous extracts of selenised samples (though a peak matching its retention time was detected by AE, see Fig. 2, chromatograms II), a fact that was verified by spiking the extract with selenomethionine. This fact shows once more the

limitation of using retention times for characterization, a task needing confirmation using more than one complementary chromatographic separation or, even better, using molecular identification techniques.

Concerning enzymatic hydrolysates of selenised mushrooms, a Se species matching the retention time of selenomethionine was found overlapped with another compound (which was eluted at 4.3 min and had been previously detected in aqueous extracts). In other words, in selenised *Agaricus* mushrooms, it appears that selenomethionine is present and should be mostly incorporated into proteins, as it is only detected after a proteolytic hydrolysis. Additionally, a peak close to the void volume (2.7 min) and another at 5.4 min were detected (Fig. 2c, chromatogram III).

Table 4 Concentrations of Se (as micrograms per gram, referred to a freeze-dried sample, $n=3$ independent extracts) found in the Se species detected in different extracts of selenised *Agaricus* mushrooms by AE-HPLC-ID-ICP-MS

t_R (min)	Selenised <i>Agaricus</i> AE-HPLC		
	Water 85°C	Water 37°C	Protease
2.5	–	–	2.2±0.3
3.6	1.60±0.02	1.6±0.5	2.2±0.1
4.6 ^a	1.5±0.2	1.53±0.09	2.32±0.04
5.4	0.5±0.1	0.48±0.07	–
6.1	–	–	0.8±0.1

^a t_R of selenomethionine

Table 5 Concentrations of Se (as micrograms per gram, referred to a freeze-dried sample, $n=3$ independent extracts) found in the Se species detected in different extracts of selenised *Agaricus* mushrooms by RP-HPLC-ID-ICP-MS

t_R (min)	Selenised <i>Agaricus</i> RP-HPLC		
	Water 85°C	Water 37°C	Protease
2.7	2.35±0.07	1.49±0.06	4.6±0.4
4.3	1.90±0.04	1.74±0.08	3.1±0.1
4.6 ^a	–	–	–
5.4	–	–	1.02±0.01

^a t_R of selenomethionine

Se concentrations observed in each RP chromatographic peak for selenised *Agaricus* extracts are shown in Table 5.

Conclusions

The possibility of using complementary mechanisms in chromatographic separations for speciation in order to confirm the presence of a given compound (even if exclusively retention time data are available) is highlighted here. This approach can be a useful alternative when molecular identification techniques such as electrospray ionization-(MS)ⁿ are not available or when the concentration of the Se species is not high enough to detect them by such molecular techniques.

Qualitative speciation results obtained from natural (commercial) *Agaricus* mushrooms indicate that they do not contain significant amounts of Se firmly bound to proteins. Also, the Se speciation in natural *Agaricus* was found to change with the temperature at which the aqueous extraction is performed, but this effect was not observed in selenised mushrooms.

In addition, the use of ID analysis on line with the chromatographic separation has enabled quantification of selenomethionine and a number of unknown Se species. These quantitative data allowed us relate some of the unknown Se peaks detected by AE to the corresponding species eluted from the RP column and also to confirm the chromatographic purity of some Se species detected.

Finally, the qualitative and quantitative results obtained indicate that selenomethionine was present free (or, at least, not incorporated in the primary structure of the proteins) in commercial mushrooms while it may appear as protein-bound in selenised ones. Of course, the ICP-MS results used here are a preliminary guide to further uses in

subsequent studies of molecular MS techniques in order to ascertain the nature of each Se species.

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