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Effects of common cooking heat treatments on selenium content and speciation in garlic

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ABSTRACT

The effect of traditional thermal treatments, normally applied during cooking practices, on the stability of Se-amino acids and Se-proteins present in Se-enriched garlic was studied. Five heat treatments including, convection oven (180 °C, 10 min), boiling (100 °C, 10 min), microwave oven (800 W, 3 min), steaming (10 min) and stir-frying (180 °C, 3 min) were assayed. Modifications on the molecular weight fractions profiles of protein extracts were evaluated by size exclusion chromatography with UV–visible and inductively coupled plasma mass spectrometry (ICP-MS) detection using a 0.05 M Tris(hidroxymethyl)aminomethane solution at pH 7.3 as the mobile phase. Possible modifications of Se-amino acids were evaluated by anion exchange chromatography (AEC), with a 30 mM Na_2HPO_4 solution (pH 6) as the mobile phase, coupled to ICP-MS after enzymatic hydrolysis. SEC chromatograms showed the incorporation of Se to high (7 kDa) and low molecular weight fractions (2–4 kDa) while AEC chromatograms showed mostly the presence of Se-methylselenocysteine. However, it could be observed that heat treatments significantly affect this important Se-amino acid.

1. Introduction

The information regarding the chemical reactions occurring in food and the lost of nutritional value as a result of thermal treatments normally applied for household cooking or during industrial processing are topics of remarkable interest for consumers nowadays. Garlic (*Allium sativum L.*) is recognized as an important source of Se and it is widely used for cooking by several cultures (Cornelis et al., 2005). Therefore, an evaluation to determine if Se speciation in cooked garlic is correlated with that found in the raw food is really necessary.

Selenium is an essential micronutrient for humans and animals, and it naturally occurs ubiquitously in the environment (Winkel et al., 2015). Physiological Se concentration must be kept constant through an optimal diet and it is a prerequisite in reproduction, normal function of thyroid gland, immunity, general health preservation and it has been demonstrated to have an important role in cancer prevention (Duntas and Benvenga, 2014). However, biological activity of Se is dependent on its metabolic disposition, chemical species and concentration in food (Longchamp et al., 2015). This essential element occurs in food mainly as organic chemical species such as Se-cysteine (SeCys) and Se-methionine (Se-Met), while inorganic forms [selenite (Se(IV) or selenate (Se(VI))] are present at very low levels (Thiry et al., 2013). Absorption in the organism varies depending on the type of species as different Se species follow dissimilar transport routs through the intestinal barrier (e.g. Se(IV) is less bioavailable that Se-Met) (Thiry et al., 2013).

Garlic (*Allium sativum L.*), a widely used household kitchen condiment, can synthesize several sulfur compounds, but also it is capable of synthesizing Se-amino acids (El-Bayoumy et al., 2006). Studies have shown that garlic extracts can cause direct stimulation of immune cells, ameliorate the endogenous antioxidant status, modulate liver and kidney function parameters, prevent cardiovascular and muscle disorders and maintains neurological functions (Arreola et al., 2015; Suru and Ugwu, 2015). Moreover, Se-amino acids such as methyl-selenocysteine (methyl-SeCys), SeCys and Se-Met have much more antioxidant activity than their sulfur analogs (Battin et al., 2011). As a consequence, enriched garlic combines the benefits of Se and garlic, e.g. inhibiting the post-initiation phase of mammary carcinogenesis when it was given continuously and it is considered a nutritive food (Ip and

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Lisk, 1993; Ip et al., 1996). In fact, there are reports showing the high bioavailability of Se in enriched garlic (Ip and Lisk, 1993; Thiry et al., 2012). This high bioavailability is due to the presence of Se-Met and other species that are also precursors of methylselenol, an important anticancer in animals and humans (Thiry et al., 2012). However, when garlic is cooked before consumption, thermal treatment might modify chemical components of interest. Different studies have revealed that these treatments could lead to various changes in foods that involve the modification of their physical and chemical characteristics (e.g. protein changes, color and flavor) (Shabbir et al., 2015). It has also been shown that some cooking methods are able to preserve the antioxidant activity of some vegetables (e.g. artichoke) and even increase their activity after being cooked (e.g. green beam, celery and carrots), while other vegetables lose their properties (Jiménez-Monreal et al., 2009). Likewise, the concentrations of essential amino acids, except methionine and histidine, decrease in the soluble fraction with an increase in thermal exposure time after meat boiling (Deb-Choudhury et al., 2014). Other authors reported the effect of three cooking methods on amino acids content in veal meat, determining that grilling seems to be the treatment that changed the composition of most amino acids (Lopes et al., 2014). Due to the high accumulating capacity of garlic towards Se, this plant has been proposed as a natural source of Se for the human diet and a potential functional food (Arnault and Auger, 2006). However, despite the importance of garlic as well as Se content, no research has been focused on the transformation of Se speciation after household thermal treatments applied during cooking (Pyrzynska, 2009).

The aim of the present study was to assess the effect of five cooking heat treatments (heating in a convection oven, boiling, heating in a microwave oven, steaming and frying) on the stability of non-volatile Se-species and its association to proteins in Se-enriched garlic. For this purpose, anion exchange chromatography (AEC) and size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) were used in order to evaluate the association of Se to proteins and Se-amino acids naturally occurring in garlic.

2. Materials and methods

2.1. Instrumentation

Total Se determination was performed by hydride generation atomic fluorescence spectrometry (HG-AFS) using an instrument model AF-640A from Rayleigh (Rayleigh, Beijing, China) under the conditions indicated in Table 1. A high intensity Se hollow cathode lamp (HCL) (Rayleigh) was used as a light source. For lyophilization and homogenization, a freeze dryer (Virtis, New York, USA) Model 6 Lyophilizer 12L and a grinder (Ultracomb, Buenos Aires, Argentina) model MO-8100A were used, respectively. Molecular weight fractionation was performed by SEC with a BioSep-SEC-S-2000 column purchased to Phenomenex (Phenomenex, Torrance, USA). Samples were injected into an HPLC 200LC system from Perkin Elmer (Perkin Elmer, Norwalk, CT, USA), composed of a quaternary pump coupled to a UV–vis detector. Separation of Se-amino acids was performed by AEC using a PRP-X100 column from Hamilton (Hamilton, Nevada, USA). Measurements were performed by inductively coupled plasma mass spectrometry (ICP-MS) with an instrument model 350Q from Perkin Elmer. Instrumental and other analytical conditions were as mentioned in Table 1.

2.2. Reagents

All reagents were of analytical grade. Stock standard solutions of inorganic Se(IV) and Se(VI) species (100 $\mu g\,L^{-1}$) as sodium selenite (Na_2SeO_3) (99%) (Anedra, Buenos Aires, Argentina) and anhydrous sodium selenate (Na_2SeO_4) (98%) (Sigma-Aldrich, St. Louis, MO, USA), respectively, were prepared in ultrapure water (18 MΩ cm) obtained from a Milli-Q system OSMOION (Apema S.R.L., Buenos Aires, Argentina). Seleno-DL-methionine (Sigma-Aldrich) and Se-methylselenocysteine (Se-methylSeCys) (Sigma-Aldrich) stock standard solutions (100 $\mu g\,L^{-1}$) were prepared with ultrapure water. Rhodium (^{103}Rh) mono-elemental standard solution from Perkin Elmer Pure Plus Atomic Spectroscopy Standards was used as an internal standard.

Table 1
Instrumental and experimental conditions for Se determination and speciation analysis.

ICP-MS	
Forward power	1250 W
Plasma gas flow rate	16 L min^{-1}
Auxiliary gas flow rate	1.20 L min^{-1}
Carrier gas flow rate	0.87 L min^{-1}
Dwell time	35 ns
Isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se, ^{82}Se, ^{103}Rh (as internal standard)
AFS	
PMT voltage	270 V
Wavelength	196 nm
Primary current	40 mA
Argon gas flow rate	800 mL min^{-1}
Atomization temperature	300 °C
Atomatization mode	Flame
Reading time	20 s
Sampling time	8 s
Conc. $NaBH_4$	0.7% (w/v) in 0.05% (w/v) NaOH
Conc. HCl in carrier	5% (v/v)
SEC	
Column	BIOSEP-SEC-S-2000
Mobile phase	0.05 M Tris buffer, pH 7.3
Flow rate	1 mL min^{-1}
Injection volume	100 µL
UV detection	280 nm
AEC	
Column	Hamilton PRP X100
Mobile phase	30 mM Na_2HPO_4 buffer, pH 6
Flow rate	1 mL min^{-1}
Injection volume	100 µL

The mobile phase solution used for AEC was dibasic sodium phosphate (Na_2HPO_4) (99%) (JT.Baker, Phillipsburg, N.J., USA) which was prepared daily with ultrapure water and adjusted to pH 6 with acetic acid (99%) (JT. Baker). The mobile phase solution used for SEC was prepared with tris(hidroxymethyl)aminomethane ($C_4H_{11}NO_3$) (98.8%) purchased from J.T. Baker. This solution was prepared daily with ultrapure water and adjusted to pH 7.3 with acetic acid (99%) (JT.Baker).

Protease XIV enzyme (Sigma-Aldrich) was used for enzymatic hydrolysis. Hydrochloric acid (37%) and nitric acid (65%) were purchased to Merck (Merck, Darmstadt, Germany) and used for acid digestion. Sodium hydroxide (98%) (Sigma-Aldrich) was employed for extraction of Se-proteins and Se determination. Sodium borohydride (Sigma-Aldrich) was used for total Se determination by HG-AFS.

2.3. Cultivation of Se-enriched garlic

Selenium enrichment experiments of garlic were conducted at the San Carlos experimental station (Mendoza, Argentina) of the Instituto Nacional de Tecnología Agropecuaria (INTA) during the cultivation season ranges April 2014 to December 2014. The garlic clone “Fuego INTA” was used in this work. For plantation, 10 L pots filled with peat and 10% soil were used. Plants not fortified with Se were used as control. Selenium was not detectable in the control after HG-AFS determination. The application of Se to garlic plants (15 kg Se ha^{-1}) was made in August because garlic plant is found in the vegetative growth stage at this month and hence, it is able to assimilate and metabolize nutrients more efficiently (Burba, 2013). The plants were watered daily and fertilized with commercially available 15N:30P:15 K when needed. They were harvested on December. The bulbs were dried to commercial moisture and isolated from the leaves and roots. After that, they were stored at −18 °C until thermal treatment.

2.4. Heat treatments

Garlic samples were peeled and chopped, and five traditional household heat treatments used for cooking were applied as follows:

2.4.1. Convection oven

A convection oven (Philco HEE-PH65, Argentina) was pre-heated to 180 °C. An amount of 3 g of fresh garlic was placed on an individual dish and heated for 10 min in the convection oven. After baking, the sample was cooled to room temperature.

2.4.2. Boiling

An amount of 3 g of fresh garlic were immersed in 500 mL of boiling tap water for 10 min. Then, the sample was drained and cooled to room temperature. Selenium was not detected in tap water.

2.4.3. Microwave

The fresh garlic sample (3 g) was treated by MW-assisted digestion in a Whirlpool JQ278BL (Buenos Aires, Argentina) MW oven for 3 min at 800 W. After, sample was cooled to room temperature.

2.4.4. Steaming

Approximately 600 mL of tap water were boiled in a steamer from Smart Tek and model SD2071 (Smart Tek, Buenos Aires, Argentina) and 3 g of fresh garlic were steamed for 10 min. Then, the sample was cooled to room temperature. Selenium was not detected in tap water.

2.4.5. Stir-frying

Sunflower oil (50 g) was added to a pan and fire-heated until the oil temperature reached 180 °C. A small cloth bag was filled with 3 g of fresh garlic and immersed in the hot sunflower oil for 3 min. After cooking, the garlic was drained and cooled.

After each heat treatment, samples were lyophilized, milled and stored at −18 °C for subsequent analysis.

2.5. Total Se determination

Total Se determination was performed based on the conditions recommended by the AFS manufacturer manual. Basically, an amount of 1.0 g of dried (lyophilized) sample was digested with 10 mL HNO_3 (65%) and 2 mL HCl (37%). The mixture was left to stand for 12 h and then subjected to sequential heating steps (1 h at 50 °C, 1.5 h at 100 °C, 1.5 h at 150 °C). Prior to total Se determination, Se(VI) present in the sample was reduced to Se(IV) by adding 3 mL of 6 M HCl and boiling the mixture for 7 min. Finally, the sample was filtered and diluted to 50 mL in a volumetric flask and Se determined by HG-AFS under the instrumental parameters listed in Table 1.

2.6. SEC-ICP-MS analysis

For molecular weight distribution studies, the SEC column was calibrated, using a standard mixture of vitamin B_{12}: (1.3 kDa), cytochrome C: (12 kDa), carbonic anhydrase: (29 kDa), and albumin: (66 kDa) (Sigma-Aldrich St Louis, USA). A linear response was obtained for the calibration curve of log10 molecular weight vs. retention time ($r^2 = 0.9668$). Detection of calibration standards was performed with UV at 280 nm. A solution of 50 mM Tris buffer at pH 7.3 was used as mobile phase, which led to obtain good resolution with short retention times. This buffer solution avoided protein precipitation inside the column and reduced hydrophobic interactions of these compounds with the stationary phase, which otherwise would have affected SEC separations.

Before SEC separation, 0.2 g of each treated sample was extracted with 3 mL of 0.05 M NaOH for 1 h with constant magnetic stirring. Subsequently samples were centrifuged (3500 rpm) for 10 min. The supernatant was filtered through a poly ether sulfone membrane filter (0.22 µm) and 100 µL of the filtrate were injected into SEC-ICP-MS for Se fractionation under the conditions shown in Table 1. Additionally, the sample extracts were injected into SEC-UV for molecular weight fractionation.

2.7. AEC-ICP-MS analysis

For Se(IV), Se(VI) and Se-amino acids determination, the Se species were extracted from 0.2 g of raw or heat-treated garlic samples adding 5 mL of Tris buffer solution (0.05 M, pH 7.5) and 0.02 g of Protease XIV for enzymatic hydrolysis. The solution was kept at a constant temperature of 50 °C and it was constantly stirred for 24 h. The final mixture was filtered with 0.22 µm regenerated cellulose filter. A volume of 100 µL of the resulting solution was injected immediately into AEC-ICP-MS for Se speciation analysis under the conditions given in Table 1.

3. Results and discussion

3.1. Effect of traditional heat treatment on total Se content in garlic

Total Se was determined by HG-AFS technique after acid digestion of garlic samples. A recovery study was performed by spiking the samples before the digestion procedure. The recovery values were calculated using the following equation: $100 \times [(C_{Se\_final} - C_{Se\_initial})/C_{Se\_spiked}]$; where C_{Se_final} was the Se concentration found in the samples after addition at 50 µg L^{-1} Se, $C_{Se\_initial}$ was the concentration found in the samples without addition of Se and C_{Se_spiked} corresponds to the spiked concentration level (50 µg L^{-1}). Determinations were performed in triplicate in each case. The obtained analytical recoveries were in the range of 97.2 to 102%. Furthermore, the accuracy of the method was assayed by analyzing a certified reference material (CRM), BCR 402 white clover from Sigma-Aldrich, with a certified value of 6.70 ± 0.25 mg Se kg^{-1}. Using this methodology, Se concentration found in the CRM was 6.58 ± 0.010 mg Se kg^{-1} (n = 6 and 95% confidence interval) and no significant differences were observed with the certified value, thus indicating an acceptable accuracy of the method ($p < 0.01$).

Total content of Se found in raw garlic is summarized in Table 2. The results confirmed the high efficiency that garlic plants have to accumulate Se after they were treated with Se(VI). The highest Se content found in garlic was 39 µg Se g^{-1}. The results also showed that Se did not inhibit the growth and maturity of garlic plants significantly at the concentration range of Se added to the growth mixture. Therefore, the treatment with Se(VI) is a good option to increase the Se content in garlic for two reasons, i.e., the better bioavailability of inorganic forms for plants and the lower price of Na_2SeO_4 as compared to organic Se compounds, which might favor the application of this element to large-scale cultures.

The studies performed in this work were focused on how the total Se content could be modified when garlic was cooked under different thermal treatments (Table 2). Total Se concentration was decreased by 6 to 11% after garlic was boiled, steamed or microwaved. On the other hand, Se losses were more significant in baked and fried samples (16

Table 2
Total Se concentration in garlic samples submitted to different heat treatments.

Garlic sample	Se concentration (µg Se g dry garlic^{-1})[a]	Se loss (%)
Raw	39.3 ± 0.05	–
Baked	32.9 ± 0.08	16
Boiled	36.7 ± 0.01	7
Microwaved	35.1 ± 0.02	11
Steamed	37.0 ± 0.06	6
Fried	14.1 ± 0.03	64

[a] Concentration values are the result of 3 determinations on 3 replicates treated samples.

and 64%, respectively), where the highest temperatures were reached (180 °C). Boiling and steaming were found to have little effect on Se content in enriched garlic. Similar results were reported when a similar heat process was applied to other vegetables (mushrooms and asparagus), showing losses of Se of 44 and 29%, respectively (Higgs, 1972). However, other authors have found that Se is minimally lost when broccoli is boiled (Pedrero et al., 2007). Thus, the analysis of the aqueous boiling extracts in our work showed a Se distribution profile that is very similar to fresh broccoli. By analogy, the lixiviation of soluble Se species to boiling water and steam is the cause of these small Se losses. Moreover, since Se is mainly associated to proteins, extraction of Se species in boiling water or steam is expected to be minimal.

The thermal treatment of garlic by microwave oven caused no consistent losses of Se, however baked samples treated in a conventional oven showed a more significant effect on Se content. This may be attributed to the different conditions used in both cooking systems, having different heating modes that led to higher temperature in a conventional oven than in a microwave one. The combination of voltage/time used in the microwave oven represents a less intense heat treatment than the conditions used in the convection oven, and hence, Se losses were minor (Bratakos et al., 1988). Other authors have obtained similar results for sulfur during the thermal treatment of onion samples using convection and microwave ovens (Cavagnaro and Galmarini, 2012).

Finally, the highest losses of Se were reached when Se-enriched garlic was fried. This fact may be attributed to the lixiviation of Se from garlic to the heated oil following the decomposition of Se-aminoacids into volatile Se-compounds like dimethylselenide or dimethyldiselenide due to the high temperatures reached during the frying process (Bratakos et al., 1988; Khanam and Platel, 2016). Furthermore, it has to be mentioned that free-aminoacids are more reactive than proteins under these conditions, so the proteins losses are lower because they are denatured only (Boskou and Elmadfa, 2010). Similar results have been obtained for other plants, such as wheat and rice, and other foods like eggs and chicken (Higgs, 1972). It has also been reported that Se content and bioavailability in plants decreased when they were subjected to cooking processes (Khanam and Platel, 2016). This is an important aspect to consider because the main sources of Se in the human diet are vegetables and they are usually cooked before consumption.

3.2. Fractionation profiles of Se in garlic and changes observed after heat treatments

Chemical association of Se to different molecular weight fractions in raw and cooked garlic samples was monitored with HPLC-UV and HPLC-ICP-MS techniques (Fig. 1a and b). Before SEC fractionation, the raw and cooked garlic samples were treated with a NaOH solution for Se-proteins extraction and the obtained extracts were analyzed. It was observed that Se was associated to high (7 kDa) and low molecular weight (2–4 kDa) fractions in raw garlic (Fig. 1a). The fractions corresponded to the presence of low molecular weight proteins, free Se-amino acids and probably inorganic Se that are usually extracted under alkaline conditions. Similar results have been observed for onion where Se was associated to both, high and low molecular weight fractions (Shah et al., 2004; Wróbel et al., 2004). The *Allium* gender plants, such as garlic and onion, have the ability to accumulate and metabolize Se following the same path than sulfur (Pilon-Smits and Quinn, 2010). Therefore, Se can be mostly metabolized into various non-protein Se-amino acids (e.g. Se-methylSeCys and γ-glutamyl-Se-methylSeCys) and non-essential proteins, which can be understood as the fundamental mechanism of plants to overcome Se toxicity (Pilon-Smits and Quinn, 2010; Pyrzynska, 2009). On the other hand, in non-accumulating plants, Se can be metabolized as Se-Met and SeCys species, which upon replacement of methionine and cysteine in proteins that are essential to plant, might cause higher toxicity (Rayman et al., 2008). In our work, the main association of Se to low molecular weight protein fractions can be explained by these differences in Se metabolization, but also primarily to the way garlic plants were supplemented with Se during their cultivation. Thus, it has been observed that when Se is supplemented as Se(VI) species, low molecular weight fractions accounts for most of the Se occurring in plants. On the other hand, Se can be found equally distributed among low and high molecular fractions when it is supplemented as Se(IV) species (Kápolna et al., 2007; Wróbel et al., 2004). This effect could be attributed to the ability of Se(VI) for activating specific enzymes that hydrolyze proteins presents in the high molecular weight fractions (Wróbel et al., 2004).

The changes in the association of Se to proteins caused by the different heat treatments were studied in this work by SEC-ICP-MS. The fractionation profiles of Se evidenced the association of this element to high (7 kDa) and low molecular weight fractions (2–4 kDa) (Fig. 2(a)). In fact, for some heat treatments such as steaming, boiling, baking and microwaving, similar fractionation profiles were observed with most of the Se associated to low molecular weight fraction of 2.2 kDa and other minor fractions found in the range of 4 to 7 kDa. Interestingly, it was observed that the fraction about 2.2 kDa decreased with respect to that found in raw garlic and this effect was more intense when the heating temperature was increased. Ultimately, fractionation profile was completely modified and Se was almost exclusively associated to a single molecular weight fraction of 4.4 kDa (Fig. 2(b)) in the fried garlic sample, i.e. at the highest cooking temperature.

Some possible explanations for these changes in Se fractionation profiles upon the different heat treatments can be proposed based on chemical modifications of foods. Generally, heat treatments produce conformational changes in proteins structures and cause denaturalization (Shabbir et al., 2015). Likewise, temperature, time of cooking and type of heat treatment could have an important effect on the denaturalization process (Shabbir et al., 2015). In fact, during heat treatments some biochemical reactions like hydrolysis and aggregation lead to modification of protein structures (Deb-Choudhury et al., 2014). Finally, changes in Se fractionation profiles observed in our work can be explained considering two combined effects: (i) the hydrolysis of proteins by heating to produce a mixture of peptides with lower molecular weight, and (ii) the formation of heat-induced insoluble protein aggregates that decreases the size of high molecular weight fractions.

3.3. Study of Se-amino acids and inorganic Se species by AEC-ICP-MS

Since Se-amino acids and inorganic Se species are ionic over a wide pH range, AEC coupled to ICP-MS detection was chosen for Se speciation studies in garlic submitted to the different heat treatments. A mobile phase composed of Na_2HPO_4 buffer at pH 6 was initially selected for Se species separation. Also, a flow rate of 1 mL min^{-1} was fixed for all separation studies as this value was highly compatible with optimal sample introduction into the ICP-MS instrument. The effect of Na_2HPO_4 concentration on the separation of Se species was assayed in the range of 20 to 40 mM, obtaining the best results at 30 mM. Concentrations below this range led to very high retention times, while higher concentrations caused peaks overlapping. A typical chromatogram obtained after injection of a Se standards mixture under the conditions mentioned in Table 1 is shown in Fig. 3(a).

It is also noteworthy that AEC was developed with no addition of organic solvents in the mobile phase to avoid plasma instability and carbon deposition on sampling cone of ICP-MS instrument, thus yielding optimal analytical figures of merit. The limits of detection (LOD) for each Se species were calculated based on the signal at the intercept and three times the standard deviation about regression of the calibration curve. The LODs obtained in this work were 32, 43, 58 and 15 ng Se g^{-1} for Se(IV), Se(VI), Se-Met and Se-MeSeCys, respectively. Also, reagent blanks were analyzed and the presence of Se species was not observed. The calibration curves showed an acceptable linearity ($R > 0.998$) for each Se species. The accuracy of the speciation analysis was evaluated by means of a recovery study by spiking the samples

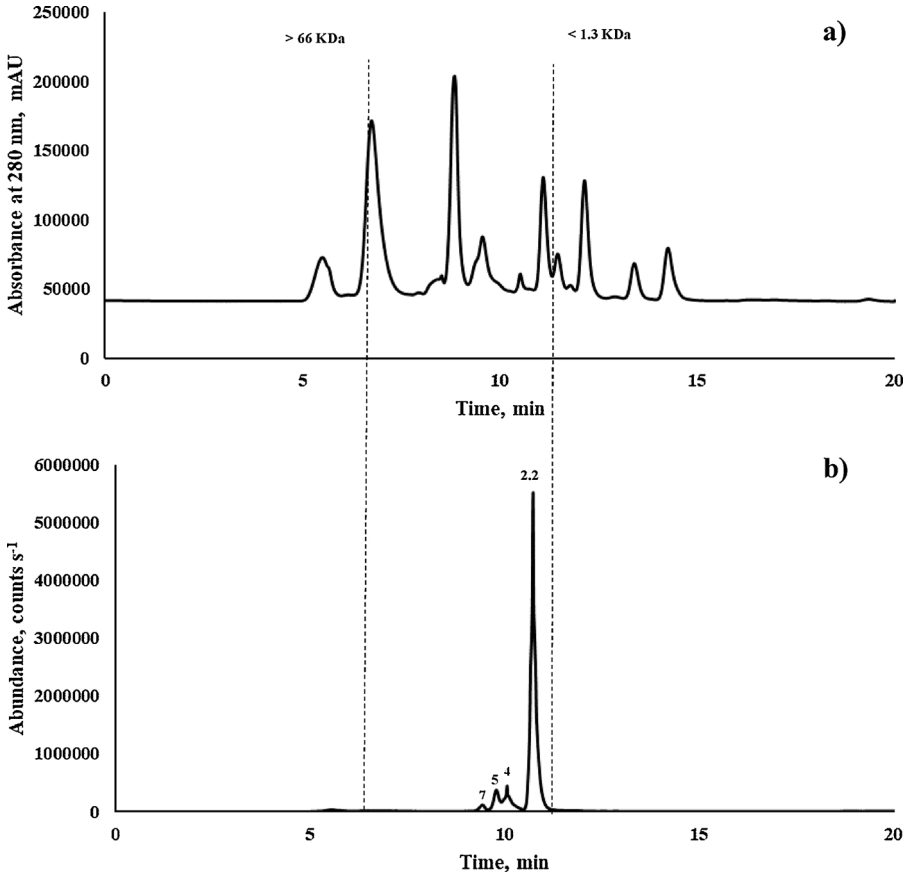

Fig. 1. Size exclusion chromatograms showing the association of Se to different molecular weight fractions in raw garlic. a) SEC-UV and b) SEC-ICP-MS (^{82}Se).

at 10 and 50 µg Se L^{-1} with Se species standards. The recovery percentages of Se species varied in the range of 92 to 103%, thus confirming the accuracy of the method for Se speciation analysis.

In order to release Se naturally associated to proteins occurring in garlic, enzymatic hydrolysis was performed with Protease XIV on both raw and cooked samples before AEC-ICP-MS. The extraction efficiency of total Se by enzymatic hydrolysis was 85%. Different Se species including, Se-methylSeCys, Se(VI), Se-Met and unknown species were observed in raw garlic as shown in Fig. 3(b). This chromatogram reveals that Se was metabolized mainly as Se-methylSeCys. In fact, about 80% of total Se found in the enzymatic extract was under this important Se-amino acid that has anticancer activity. Similar results have been found in other *Allium* and *Brassica* gender plants (Kápolna et al., 2007; Lavu et al., 2012; Michalska-Kacymirow et al., 2014; Zhong et al., 2014). On the other hand, γ-glutamyl-Se-methylSeCys was not detected in our work. This result is in agreement with what has been reported in a previous work published by Larsen et al. after the analysis of garlic samples (Larsen et al., 2006). However, in order to confirm that all Se species were being eluted from the column and no permanent retention on the stationary phase might be responsible for some Se species missing, a Se recovery study of the chromatographic process was performed in this work. Total Se determination performed in the enzymatic extract injected into HPLC and the eluent collected during the AEC separation of Se species, showed that 98% of the Se occurring in the extracts was eluted from the HPLC column. Therefore, all Se species present in the enzymatic extracts were detected and no Se species was permanently retained inside the column during the analysis.

Modifications of Se speciation in garlic after heat treatments were also evaluated by AEC-ICP-MS. Several Se species including, Se(VI), Se-methylSeCys, Se-Met and unknown Se species were identified in raw garlic, but also in baked, boiled, microwaved and steamed garlic with similar chromatographic patterns. Only the chromatogram obtained for microwaved garlic is shown in this work as an example (Fig. 3(c)). However, in fried garlic the presence of Se(IV), Se(VI) and three unknown Se species was confirmed (Fig. 3(d)), indicating that Se-methylSeCys and Se-Met could have been partially decomposed into other unidentified Se species due to the high temperatures achieved by this cooking procedure. Future studies will be developed to identify the unknown species by MS/MS. Furthermore, it was studied the effect of the different heat treatments applied for cooking the garlic samples over each of the Se-species detected in this work. The results in Fig. 4 shows how the area of peaks was modified with the cooking procedures. It has to be observed that Se-methylSeCys concentration decreased when the temperature of the cooking procedure was increased, while the other Se species remained practically unchanged. These changes were more evident when fried garlic samples were analyzed, most probably due to the high temperatures reached with the boiling oil. Certainly, these effects could be attributed to the high instability and volatility of some Se-aminoacids (Higgs, 1972). Since Se and S share chemical similarities and S content in garlic is very significant, it is valid to compare some of their properties to clarify the results of the present work. In fact, it has been reported that Se-compounds tend to be more thermally instable than S analogous compounds as electronegativity of S (2.58) is higher than that of Se so the molecular radio increase from 1.27 to 1.40 Å and the electron density decreases (Jiménez et al., 2015). For this reason, the electrostatic interactions established by these atoms weaken, resulting in a decrease of intra and intermolecular stability. The results obtained in our work also show that Se-methylSeCys is less stable than Se-Met despite the fact that their structures are similar. Since Se-methylSeCys is not associated to proteins, as Se-Met does, it could be more easily lost under heating or cooking treatment (Higgs, 1972).

Finally, it has to be mentioned that the modifications observed in this research on the Se speciation patterns are in agreement with those

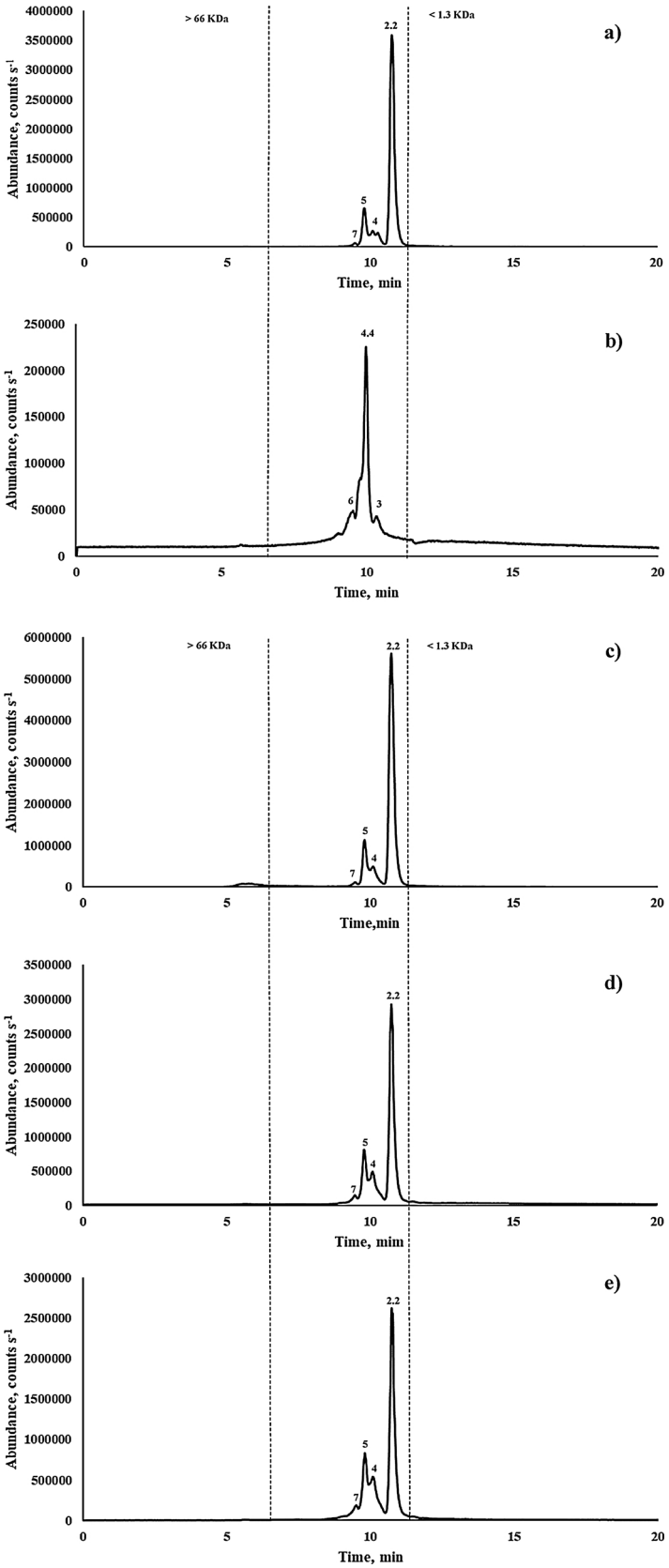

Fig. 2. SEC-ICP-MS (^{82}Se) chromatograms of cooked garlic. a) Boiled, b) Fried, c) Steamed, d) Microwaved and e) Baked.

reported in other works about Se speciation analysis in vegetables like boiled broccoli, where it has been demonstrated that Se-methylSeCys can be decomposed into other compounds (Pedrero et al., 2007). Similarly, it has been reported that the speciation profile for organic Se species can be modified in boiled cabbage (Funes-Collado et al., 2015; Pedrero et al., 2007).

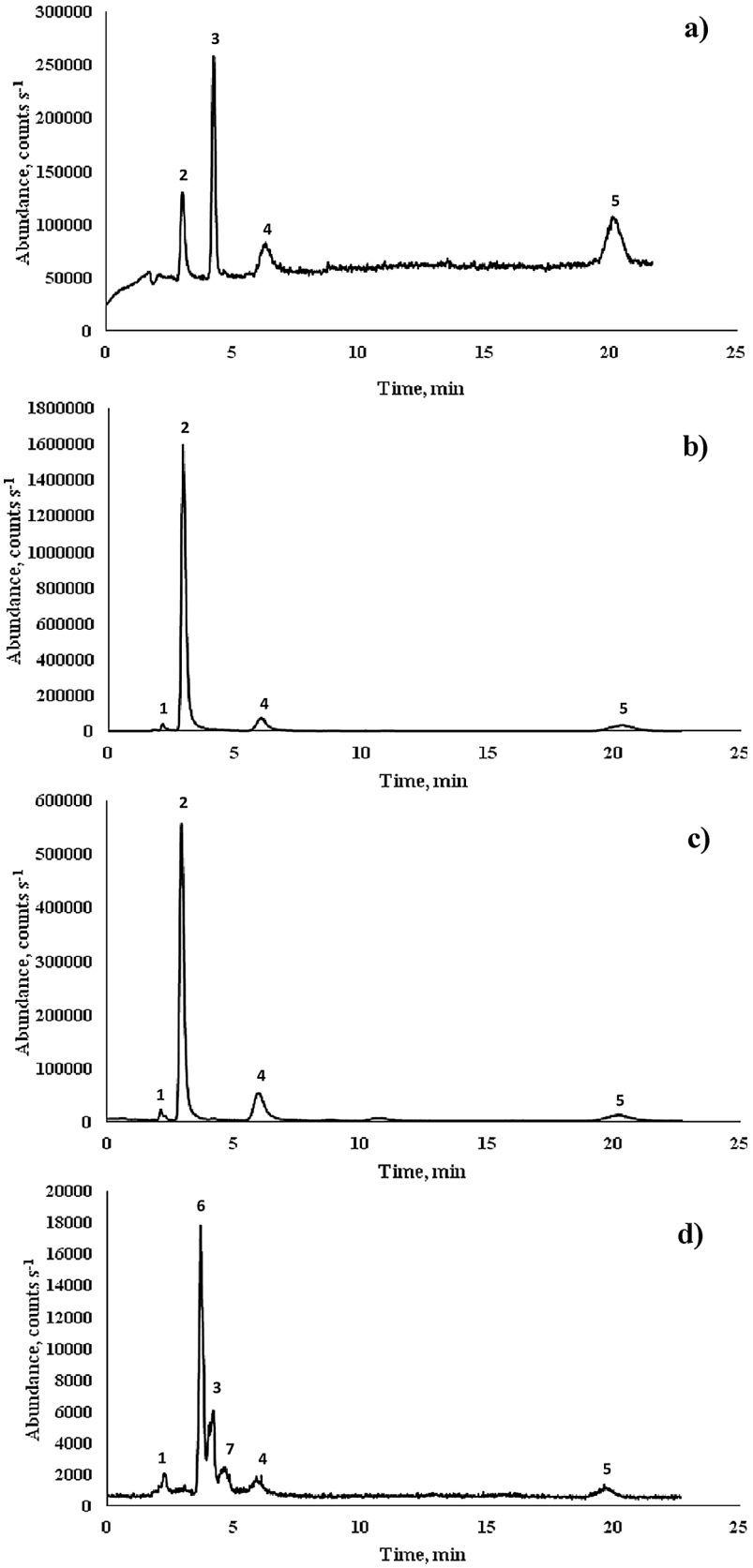

Fig. 3. AEC-ICP-MS (^{82}Se) chromatograms: a) 100 μg L^{-1} Se species standards mixture, b) Raw, c) Microwaved and d) Fried garlic. (1) Unknown 1, (2) Se-Methylselenocysteine, (3) Se (IV), (4) Se-Methionine, (5) Se(VI), (6) Unknown 2 and (7) Unknown 3.

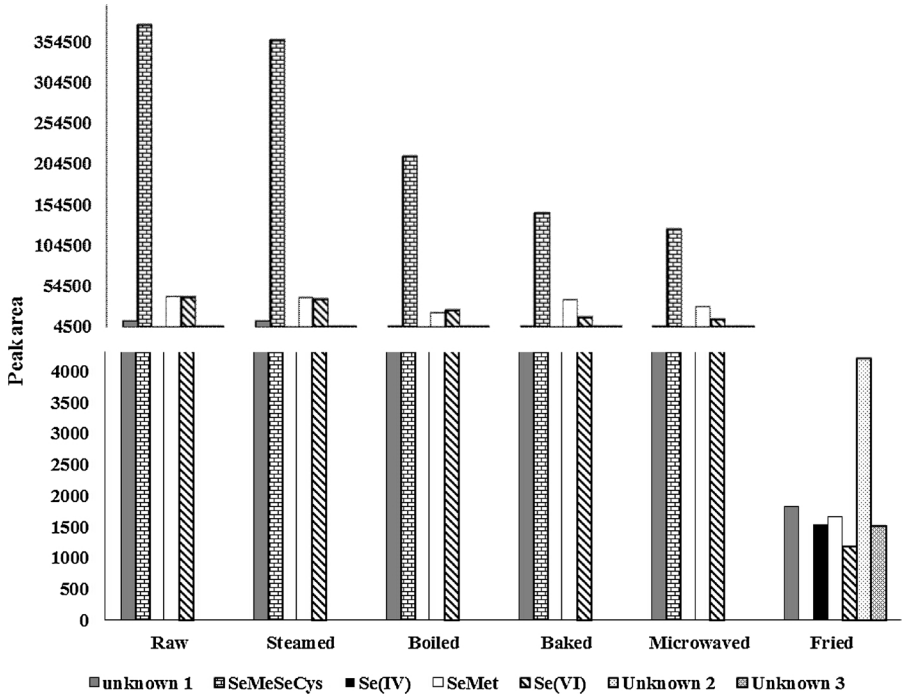

Fig. 4. Effect of traditional cooking heat treatments on Se species distribution and concentration.

4. Conclusions

The results obtained in this work have shown that Se is metabolized by garlic plants as Se-methylSeCys, an important Se-amino acid with anticancer activity, and hence Se-enriched garlic may be useful as a source of dietary Se. This study has demonstrated that traditional heat treatments involved in typical cooking processes cause changes on Se fractionation profiles, which is most probably due to protein denaturalization and hydrolysis by activation of natural enzymes occurring in garlic. Therefore, the way garlic is cooked does not only change Se speciation in this food, but also could have a remarkable impact on Se properties, such as bioavailability, pointing out the need for further studies to fully evaluate the real benefits of consuming Se-enriched foods after they have been submitted to different cooking procedures.

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