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Determination of seleno-amino acids bound to proteins in extra virgin olive oils

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ABSTRACT

An analytical method has been developed to determine seleno-amino acids in proteins extracted from extra virgin olive oils (EVOOs). Different aqueous/organic solvents were tested to isolate proteins, an acetone:*n*-hexane combination being the best protein precipitant. In a first dimension chromatography, extracted proteins were analysed by size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) to identify S and Se associations as proteins marker. Two fractions of 66 kDa (A) and 443 kDa (B) were identified. These fractions were submitted to microwave-assisted acid hydrolysis (MAAH) to release seleno-amino acids. In a second dimension chromatography seleno-amino acids were determined by reversed-phase chromatography (RPC) coupled to ICP-MS. Selenomethylselenocysteine was determined with values ranging from 1.03–2.03 ± 0.2 μg kg^{-1} and selenocysteine at a concentration of 1.47 ± 0.1 μg kg^{-1}. Variations of protein and seleno-amino acid concentrations were observed between EVOO varieties, contributing to EVOO cultivar differentiation.

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1. Introduction

Olive trees are among the oldest cultivated crops. This type of tree has great longevity and is very adaptable to soil and weather conditions (Giannoulia, Banilas, & Hatzopoulos, 2007; Muzzalupo, Stefanizzi, Salimonti, Falabella, & Perri, 2009). Olive cultivars have achieved great economic importance, since high quality oil for human consumption is obtained. Extra virgin olive oil (EVOO) consumption has been associated with a decrease of the incidence of cardiovascular diseases, cancer, and Alzheimer's disease in the Mediterranean area (López-Miranda et al., 2010). There are now numerous studies that maintain the premise that sustained consumption of EVOO can have a protective effect against different types of cancer (Sánchez-Quesada et al., 2013). For this reason it is very important to identify the bioactive components responsible for its anticancer effects and the elucidation of the mechanisms by which bioactive components of food can prevent cancer. Currently multiple bioactive components that reduce the risk of contracting cancer derived from fruits and vegetables oils are being elucidated (Stan, Kar, Stoner, & Singh, 2008).

It has been proven in the last two decades that Se may be directly or indirectly linked to a large variety of human health disorders (Davis, Tsuji, & Milner, 2012). However, definitive knowledge concerning the mechanisms underlying the action of Se-proteins related to human diseases is still far from being reached (Roman, Jitaru, & Barbante, 2014).

In humans selenium absorption from products of plant origin is much easier than the absorption from products of animal origin. Therefore, scientists are interested in analysing selenium speciation in plant materials (Saygi, Melek, Tuzen, & Soylak, 2007; Tuzen & Pekiner, 2015; Tuzen, Saygi, & Soylak, 2007). Earlier work showed the presence of free selenomethylselenocysteine (SeMetSeCys) in virgin olive oils (Torres, Cerutti, Raba, Pacheco, & Silva, 2014). The presence of peptides and proteins has been reported in various types of olive oil (Crevel, Kerkhoff, & Koning, 2000; Hidalgo & Zamora, 2006). It is possible that seleno-amino acids are integrated in these molecules. During the extraction of olive oils a small amount of protein remains (Zamora, Alaiz, & Hidalgo, 2001).

In comparison to other samples, olive samples and plants in general are more problematic for protein extraction because plant tissues are rich in proteases and interfering compounds. Low protein concentration in oil and the high number of interferences, makes the number of methods for protein determination quite limited. Pigments, such as chlorophyll, phytochemicals, and lipid-based components, can also cause severe disturbances in protein extraction (Mitra, Walters, Clouse, & Goshe, 2009). Extraction of

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proteins from those matrices has usually been carried out using a first extraction with aqueous/organic solvents, followed by a protein isolation step (De Ceglie, Calvano, & Zambonin, 2014). More recently, extractions of proteins from olive oils have been based on their precipitation with solvents, such as acetone and acetone/*n*-hexane.

Separation and isolation of proteins has been performed using 1D- and 2D-PAGE (Martín-Hernández, Bénet, & Obert, 2008). Other techniques have been also employed for protein detection or characterisation, like size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), fast protein liquid chromatography (FPLC), liquid chromatography coupled to mass spectrometry (LC–MS) (Bodzon-Kulakowska et al., 2007; da Silva, Mataveli, & Arruda, 2011). Verola Mataveli, Fioramonte, Gozzo, and Zezzi Arruda (2012) used capillary electrophoresis (CE) to separate proteins in raw and table olive samples.

Protein hydrolysis for amino acids release can be achieved employing microwave radiation and microwave-assisted acid hydrolysis (MAAH). This method is fast and detergent-free, also offers good sequence coverage and no background peptides, such as those from protease autolysis in enzyme digestion (Zhong, Marcus, & Li, 2005). Seleno-amino acids separation can be achieved by coupling liquid chromatography to inductively coupled plasma mass spectrometry (LC–ICP-MS) (Uden, 2002), by liquid chromatography with electrospray ionisation tandem mass spectrometry (LC–ESI-MS) or isotachophoresis (ITP) (Gosetti et al., 2007; Infante, Hearn, & Catterick, 2005; Zembrzuska & Matusiewicz, 2010; Zembrzuska, Matusiewicz, Polkowska-Motrenko, & Chajduk, 2014). This technique has been recently applied to the analysis of free seleno-amino acids in EVOO (Torres et al., 2014).

This work describes a method to determine seleno-amino acids bound to proteins/peptides in a complex matrix such as EVOO. To this end, the aqueous fraction containing proteins was extracted. Proteins were isolated by SEC–ICP-MS. After these fractions were collected, seleno-amino acids were released by MAAH of proteins. Free seleno-amino acids were determined by reversed-phase chromatography coupled with ICP-MS (RPC–ICP MS). This method was applied to the analysis of EVOOs from different regions and varieties. This work represents an advance in oils analysis, since it overcomes some characteristics of EVOOs such as low protein concentration and oil matrix, identifying seleno-amino acids bound to proteins for the first time.

2. Experimental

2.1. Reagents

Se-Met, Se-MetSeCys, and Se-Cys standards were purchased from Sigma–Aldrich (St. Louis, MO). Water, methanol (MeOH), *n*-hexane, acetone and acetonitrile Optima LC–MS grade were purchased from Fisher (Fair Lawn, NJ). Formic acid, 98% and acetic acid (glacial, Trace Metal grade) were also obtained from Fisher. Ultrapure water (18 MΩ cm) was obtained from EASYpure® (Barnstead, Dubuque, IA). Sodium hydroxide was provided by Biopack (Buenos Aires, Argentina); 2-mercaptoethanol and hydrochloric acid, 65%, were provided by Sigma–Aldrich.

2.2. Preparation of standard solutions

Amino acid stock solutions were prepared by dissolving respective substances in 0.1 M hydrochloric acid, except for Se-Met, which was prepared in 0.5% 2-mercaptoethanol (0.3 mg g^{-1}). Stock solutions were prepared once and stored at −20 °C. Dilutions were made with 0.004% (*w*/*v*) aqueous solution of 2-mercaptoethanol to avoid oxidation of Se-Met. Working standard solutions were prepared by appropriate dilution with ultrapure water, adjusted to pH with hydrochloric acid or sodium hydroxide. Quantification was achieved by preparing spiked samples with proper amounts of the analytes.

2.3. Sample collection and extraction

A total of 10 samples of EVOO were obtained from olives processed within 24 h of harvest, and the process was carried out with the same equipment for all oils. In this way, environmental and experimental parameters that could affect the content of the analytes under study were reduced. Olive oils correspond to *Olea europaea* L., subspecies Arauco, Arbequina, Cornicabra and Empeltre.

The significant low concentration of proteins in EVOOs (between 0.2 and 0.6 mg kg^{-1}) (Martín-Hernández et al., 2008) together with the high amount of lipids and interfering compounds make the extraction of proteins very difficult.

2.3.1. Protein isolation methods from olive oils

Experimental protocols available in the literature were tested with modifications.

Hexane/acetone precipitation: The method described by Martín-Hernández et al. (2008) was employed with modifications. To 5 g of olive oil, 10 mL of cold *n*-hexane/acetone (1:1, v/v) (2 °C) were added, The mixture was shaken vigorously, kept for 1 h at 2 °C, and shaken every 10 min. The mixture was then centrifuged, and the supernatant was discarded. The precipitate was washed twice with 1 mL of cold *n*-hexane/acetone solution (1:1). After each washing, the mixture was centrifuged, and the supernatant was discarded. In both steps centrifugation lasted 10 min at 7000 rpm (6.026*g*) at 2 °C in a refrigerated centrifuge (Boeco U-320 R; Boeckel + Co (GmbH + Co), Hamburg, Germany).

After the centrifugation stage, the supernatant was discarded and the pellet obtained was redissolved. Four solutions were evaluated for redissolution: tris(hydroxymethyl)aminomethane hydrochloric acid (Tris–HCl) buffer at pH 7.5, water:methanol (80:20), acetonitrile:formic acid:water (10:10:80) and urea in NH_4HCO_3. Best results were obtained with water:methanol (80:20), since this solution could be frozen allowing an easier elimination of remaining oil. This solution was centrifuged 5 min at 3500 rpm (3.013*g*), followed by freezing at −18 °C for 1 h. The remaining oil froze on the tube walls, and a clear solution was obtained for size-exclusion chromatography (SEC).

Four more protein extractants/precipitants were tested: *n*-hexane:acetone (1:1), acetone:methanol (1:1), *n*-hexane/methanol (1: 1) and acetone. The volume used was 10 mL in each case.

2.4. Size-exclusion chromatography analysis

SEC was performed coupling the chromatograph (Series 200; Perkin-Elmer. Thornhill, Canada) to an ICP-MS (ELAN DRC-e; Perkin-Elmer SCIEX, Thornhill, Canada). Argon gas with a minimum purity of 99.996% was supplied by Praxair (Córdoba, Argentina). Buffer ammonium acetate (50 mM) was employed being adequate for coupling with ICP-MS, since its volatility prevented deposits on ICP cones. Bovine serum albumin (66 kDa) Alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa) and equine myoglobin (17 kDa) were employed for calibration. SEC analysis was applied to determine Se distribution within this fraction. S was determined simultaneously in order to investigate the presence of peptides and proteins (Spisso, Pacheco, Gómez, Silva, & Martinez, 2013). The employed SEC column separates over a wide range from 10 to 700 kDa. Since the extraction was performed with a Tris–water–MeOH solution, water-soluble selenium compounds, such

as proteins, polysaccharides, amino acids, polypeptides, and Se-protein complexes were extracted. This first dimension procedure, allowed the identification of Se–S fractions with molecular weight correspondent to proteins and peptides.

2.5. Fraction collection

Once separation was achieved by SEC, the different sulphur/selenium fractions were collected off-line and preconcentrated with 5 kDa MWCO filters (Amicon® Ultra-4 Millipore; Billerica, MA) prior to reverse-phase analysis. The total volume of the protein extract (1 mL) was injected in SEC for fraction collection; 5 injections of 200 μL were performed to reach a quantitative recovery.

2.6. Protein hydrolysis

To achieve seleno-amino acids determinations, fractions from SEC were treated for protein hydrolysis assisted by microwave, as reported by Zhong et al. (2005); 0.05% (*v*/*v*) phenol was added, to avoid any amino acid oxidation by acids used during digestion. The hydrolysis was carried out in a Milestone Start D microwave system (Sorisole, Italy) under mild conditions; 15% HCl (*v*/*v*) was added for a period of 5.5 min and a power of 900 watts. Afterwards, this solution was nitrogen evaporated at room temperature to avoid any volatilisation of seleno-amino acids. The residue was then dissolved in 1 mL of 0.02 M hydrochloric acid and filtered through a membrane filter before injection (200 μL) on LC–ICP-MS.

2.7. Determination of seleno-amino acids by reverse-phase chromatography

Hydrolysed fractions were analysed for seleno-amino acids using RPC with ICP-MS. The selected isotope for mass monitoring by ICP-MS was ^{82}Se, in order to avoid interference by polyatomic argon (Date, Cheung, & Stuart, 1987). In Table 1, RPC–ICP MS conditions for separation of selenospecies by reverse phase chromatography are summed up.

Table 1
Conditions for HPLC–ICP-MS of selenium species.

Chromatographic conditions	
Reversed-phase chromatography	
Stationary phase	Zorbax SB C18 (150 mm 4.6 mm × 5 μm)
Mobile phase	98:2 water:methanol, 10 mM TFA pH 3.0
Flow rate	1.0 mL min^{-1}
Injection volume	200 μL
Size exclusion chromatography	
Mobile phase	Ammonium acetate 50 mM, 5% methanol (*v*/*v*)
Elution mode	Isocratic
Flow rate	0.9 mL min^{-1}
Column	TSK gel G3000SW (300 mm × 7.5 mm × 10 μm)
Sample loop	200 μL
ICP-MS conditions	
RF forward power	1050 W
Gas flow rates	
Plasma	13 min^{-1}
Auxiliary	1.35 min^{-1}
Nebulizer	0.75 min^{-1}
Resolution	Normal
Scanning mode	Peak hop
Dwell time	500 ms
Isotope monitored	Se^{82} (with krypton correction)

3. Results and discussion

3.1. Protein extraction optimisation

Since protein concentrations levels in EVOO are low and its determination suffers from interferences, it is essential to find precipitating and extracting agents able to generate a higher protein concentration. Different methods have been tested based on organic solvents precipitation or isolation (Hidalgo, Alaiz, & Zambra, 2002; Hidalgo, Alaiz, & Zamora, 2001; Lerma-García, Ramis-Ramos, Herrero-Martínez, & Simó-Alfonso, 2007).

Mixtures of acetone:*n*-hexane (1:1), acetone:methanol (1:1) and hexane:methanol (1:1) and acetone were tested. In Fig. 1 the extraction efficiency, based on sulphur signal as protein marker (Campanella & Bramanti, 2014) is depicted for four oil samples by complete digestion of the obtained pellets. Sulphur was determined by ICP MS. Preliminary results showed that the best performance was accomplished with *n*-hexane:acetone (1:1), so this was chosen for subsequent extractions. After protein isolation; the mixture was centrifuged at 7000 rpm (6.026*g*). The supernatant was removed and the pellet was redissolved with water:methanol (80:20). Redissolution was completed by heating at 37 °C for 1 h. Se recovery percentage reached 48.6 ± 6.3%, compared to total selenium content in pellet samples reported in previous studies (Torres et al., 2014).

3.2. Size exclusion chromatography

After removing the protein fraction from EVOOs, the pellet was resuspended and injected for SEC–ICP MS analysis. SEC analysis allowed the identification of protein fractions and the determination of their molecular weight. Selenium and sulphur signals were monitored on-line to determine seleno-amino acids associations to proteins. Sulphur was monitored as protein and peptide marker (Spisso, Cerutti, Silva, Pacheco, & Martinez, 2014). Fig. 2 shows the chromatogram correspondent to ARB-4 olive oil sample. Two fractions were eluted, at a time of 7.83 min (Fraction A) corresponding to a molecular weight of ~443 kDa; and at a time of 15.03 min (Fraction B) corresponding to a molecular weight of ~66 kDa. These molecular weights suggest protein presence rather than peptide. These fractions were not detected in all the different

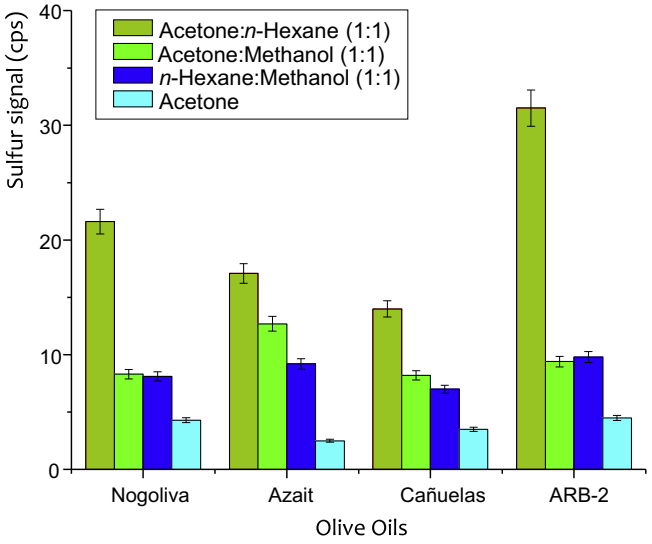

Fig. 1. Optimisation of protein extraction under different precipitant mixtures for different EVOO samples. Sulphur is employed as protein marker. Sample weight, 5 g; extractant volume, 10 mL.

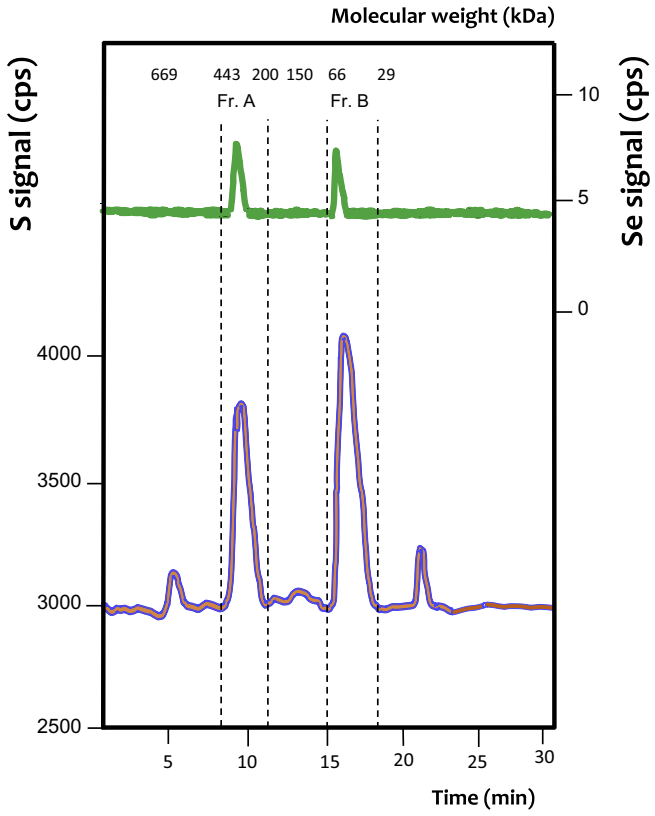

Fig. 2. Chromatogram from SEC–ICP MS corresponding to ARA-2 olive oil sample. Injected volume, 200 μL.

olive oil varieties analysed as observed in Table 2. Similar molecular weight of proteins in olives and olive oils of 63 kDa have been reported in the literature (Martín-Hernández et al., 2008; Montealegre, Esteve, García, García-Ruiz, & Marina, 2014). An overlapping of Se and S signals was observed in these two peaks, suggesting selenoproteins presence. For further analysis, these Se–S fractions were collected.

Table 2
Analysis of the fractions A and B, identification of selenoamino acids by reversed phase coupled to ICP-MS.

Olive oil sample	Olive tree subspecie	Fraction	Se-Cys (μg kg^{-1})	Se-MetSeCys (μg kg^{-1})
ARA-2	Arauco	A	ND*	ND*
		B	ND*	2.03 ± 0.2
ARB-4	Arbequina	A	ND*	ND*
		B	1.47 ± 0.1	1.65 ± 0.1
Blend Zucardi	–	A	ND*	1.90 ± 0.2
		B	ND*	1.85 ± 0.2
ARB-2	Arbequina	A	ND*	ND*
		B	ND*	1.33 ± 0.1
ARA-1	Arauco	A	ND*	1.40 ± 0.1
		B	ND*	1.13 ± 0.1
ARA-4	Arauco	A	ND*	ND*
		B	ND*	1.46 ± 0.1
Empeltre	Empeltre	A	ND*	1.79 ± 0.2
		B	ND*	1.93 ± 0.2
COR-1	Cornicabra	A	ND*	ND*
		B	ND*	1.22 ± 0.1
Blend Azait	–	A	ND*	ND*
		B	ND*	1.03 ± 0.1
Blend Nogoliva	–	A	ND*	ND*
		B	ND*	1.73 ± 0.1

* ND, not detected (detection limit, calculated on 3σ basis, of 0.17, 0.20 and 0.09 μg kg^{-1} for Se-Cys, Se-MetSeCys and Se-Met in proteins respectively, employing 5 g of EVOO for the optimised extraction procedure).

Seleno-amino acids detection possibilities were increased in a first step by means of protein extraction, since from a mass of 5 g of olive oil these were extracted into 1 mL of extraction solution. In addition, 5 injections of 200 μL each were made for fraction collection. Finally, the collected fractions were concentrated in 5-kDa molecular weight exclusion filters.

3.3. Reverse-phase separation

After collection, SEC fractions were submitted to protein hydrolysis assisted by microwave radiation. Further seleno-amino acids separation studies were conducted with a C18 column as reported by Bird et al. (1997). Separation conditions are in Table 1. An optimisation of variables was performed to improve resolution. The pH was modified from 2.0 to 6.0, the best resolution being for pH 3.0. In addition, the flow rate was evaluated from 0.5 to 1.5 mL min^{-1}. Best results were obtained for a flow rate of 0.9 mL min^{-1}, this flow rate being chosen for subsequent analyses.

In Fig. 3, the upper chromatogram shows a standard run of Se-Cys, Se-MetSeCys and Se-Met. The use of trifluoroacetic acid (TFA) in the mobile phase allowed a substantial improvement in peak shape, reducing noise (Bird et al., 1997). The lower chromatogram corresponds to a sample analysis of EVOO corresponding to Arauco (ARA-2) variety and the presence of Se-MetSeCys can be observed. The detection limit (*LOD*), calculated on 3σ basis after analysis of spiked olive oil samples with seleno-amino acid standards was

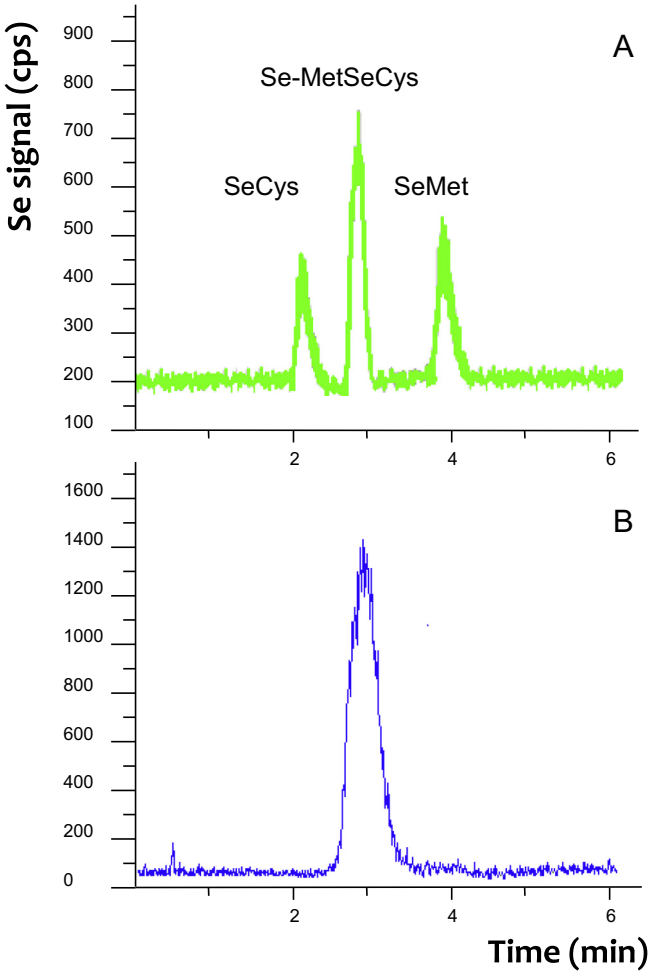

Fig. 3. Chromatogram from RPC–ICP MS corresponding to ARA-2 olive oil sample. Injected volume, 200 μL. (A) Selemoaminoacid standard of 5 μg L^{-1}. (B) Ara-2, Fr. B, sample.

0.17, 0.20 and 0.09 μg kg^{-1} and the quantification limit (*LOQ*) calculated according to 10σ definitions was 0.56, 0.66 and 0.3 μg kg^{-1} for Se-Cys, Se-MetSeCys and Se-Met respectively; employing 5 g of EVOO. *LOD* and *LOQ* are comparable with another research of our group where free selenium species were determined in EVOOs, reaching a *LOD* of 0.01 μg/kg and a *LOQ* of 0.09 μg/kg (Torres et al., 2014).

3.4. Distribution of seleno-amino acids in protein fractions of olive oils

By performing seleno-amino acids determination by RPC–ICP MS in hydrolysed fractions collected from SEC–ICP MS analysis, different seleno-amino acids bound to proteins were determined and results are shown in Table 2. In Fraction B corresponding to molecules with a molecular weight of 66 kDa, a higher prevalence of Se-MetSeCys was observed in most samples of EVOOs. Se-MetSeCys was determined in fraction B of the following extra virgin olive oils: Ara-2, Arb-2, Ara-4, Fra-2 Cor-1-Azait Blend and Blend Nogoliva. The analysis of the following olive oils: Blend (Zuccardi), ARA-1, Empeltre, ARA-3 and FRA-3, showed Se-MetSeCys presence in both fractions A and B. The FRA-4 oil was the only sample in which it was not possible to determine the proposed seleno-amino acids. The ARB-4 olive oil sample showed the presence of Se-Cys and Se-MetSeCys in fraction B, although in fraction A no selenoaminoacid was detected. This was the only case where Se-Cys was detected. SeMet was not detected in any of the analysed olive oils.

Fig. 4 shows S and Se distribution in different varieties of EVOO. Sulphur represents the extracted proteins employing the optimised method described in Section 3.1, distributed in fractions A and B from SEC. As described previously, according to the determined molecular weight, the presence of protein was inferred. Se corresponds to selenoaminoacid concentration within these fractions according to RPC. Sulphur concentration ranged from 149 to 283 μg kg^{-1} of EVOO, while seleno-amino acids concentrations range from 1.33 to 3.72 μg kg^{-1} of EVOO. Statistical results obtained from ANOVA (Table 3) show significant variation (CI = 95%) of Se and S concentration in protein fractions of EVOO from different olive tree varieties (n = 10). This fact reinforces the idea that proteins vary according to olive provenance. These observations are in good agreement with Montealegre et al. (2014). They separated proteins from olive oils and stated that protein profiles could be employed as a tool for differentiation of monovarietal olive oils.

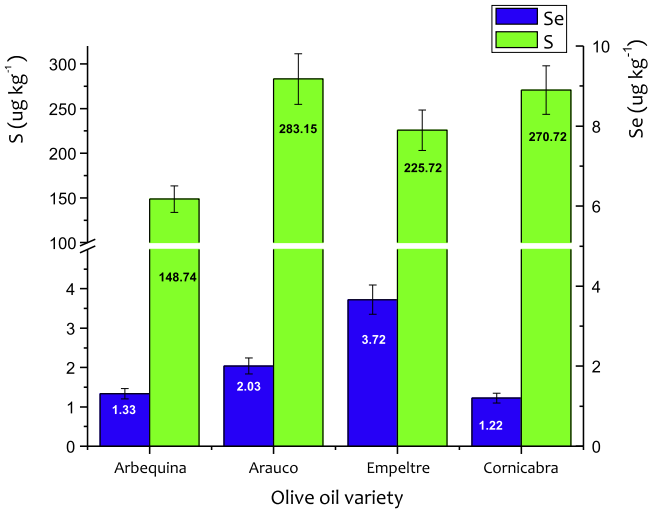

Fig. 4. Seleno-amino acids (as Se) and proteins (as S) distribution in olive oil samples according to olive tree species.

Table 3
One-way ANOVA for the effect of olive oil variety on proteic S and Se concentrations.[a]

Effect	Se			S		
	DF	*F*	*p*	DF	*F*	*p*
Olive oil variety	8	75.30	0.000003	8	19.57	0.0004

[a] F_{critic} = 4.066 (p = 0.05).

Previous research determined free seleno-amino acids in olive oils. Free SeMetSeCys was determined in olive oils using the method described by Torres et al. (2014). Differentiation between free seleno-amino acids and proteins bound to seleno-amino acids in EVOO becomes nutritionally important, considering that free seleno-amino acids are more bioavailable than seleno-amino acids integrated to proteins (Stipanuk & Caudill, 2013). From Table 2, seleno-amino acids bound to extracted proteins concentration ranges from 1.03 ± 0.1 to 2.03 ± 0.2 μg kg^{-1} of EVOO. Concentrations of free seleno-amino acids reported previously (Torres et al., 2014) ranged from 2.0 to 8.3 μg kg^{-1}. However, the only seleno-amino acid determined was Se-MeSeCys. It is worth mentioning that determinations of free seleno-amino acids are accurate since the proposed method was validated by a recovery test (Torres et al., 2014). Concentrations of seleno-amino acids bound to protein reported in this manuscript refer to extracted proteins, due to difficulties related to method validation.

4. Conclusions

The present work accomplished the determination of seleno-amino acids bound to proteins in olive oils. The organic solvent mixture, acetone:*n*-hexane, at low temperature, showed the best performance for protein extraction from EVOOs, in comparison with other extraction methods tested.

SEC–ICP-MS exhibited enough separation capacity and sensitivity to identify Se–S fractions correspondent with proteins' molecular weight. Collection of these fractions, along with protein concentration, encompass SEC–ICP MS analysis, through MAAH, with RPC–ICP MS determination. In this way, a sequence of extraction and separation steps reached enough selectivity and sensitivity for determination of seleno-amino acids integrated to proteins, in a complex matrix.

Determination of free and protein-bound seleno-amino acids in olive oils is relevant from a nutritional point of view considering their different bioavailability, and the role of selenium in the reduction of oxidative stress. This research validates the knowledge that olive oil consumption is associated with a decrease of the incidence of important human illnesses.

This paper provides useful data for further research on the use of protein fractions as a tool for cultivar origin differentiation of EVOOs.

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