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Determination of arsenic species distribution in extra virgin olive oils from arsenic-endemic areas by dimensional chromatography and atomic spectroscopy

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ABSTRACT

Arsenic species were determined in extra virgin olive oils (EVOOs) by two dimension chromatography and atomic spectroscopy: inductively coupled plasma mass spectrometry (ICP MS) and electrothermal atomic absorption spectrometry (ETAAS). A first approach determined total As concentration in EVOOs samples by microwave assisted digestion and ICP MS from 2.01 to 152 μg kg^{-1}. EVOOs with elevated As concentration were selected for protein extraction. In a first dimension, by size exclusion chromatography (SEC) coupled to ICP MS, a fraction of 66 kDa was identified and collected for analysis. Free and proteic As were separated by molecular weight cut off filters. Proteic and free arsenic concentrations determined by ETAAS range from 0.56–4.44 and 0.67-3.89 μg kg^{-1} of As respectively. Finally in a second dimension, by anion exchange chromatography (AEC) coupled to ICP MS, dimethylarsenate and arsenite were determined in proteic fraction of EVOOs in concentrations of 1.68–2.86 and 0.53–0.55 μg kg^{-1} respectively.

1. Introduction

Arsenic is a metalloid present in environmental and biological systems (soil, water and foodstuffs) (EPA, 2012). Arsenic is present in environment mostly as arsenite, arsenate and methylated forms generated by microorganisms metabolism (Jia et al., 2013; Zangi and Filella, 2012). The trivalent compounds of arsenic are thiol-reactive, and thereby inhibit enzymes or alter proteins by reacting with proteinaceous thiol groups. Pentavalent arsenate is an uncoupler of mitochondrial oxidative phosphorylation, by a mechanism likely related to competitive substitution (mimicry) of arsenate for inorganic phosphate in the formation of adenosine triphosphate (Casarett et al., 2001). In certain parts of Taiwan and South America, among others worldwide, the water contains high levels of this metalloid, and the inhabitants often suffer from dermal hyperkeratosis and hyperpigmentation (Hodgson, 2011). Contamination of shallow groundwater aquifers with As has been reported in over 20 countries around the world (Nordstrom, 2002). Many regions of Latin America are widely reported for the occurrence of high As in groundwater and surfacewater due to a combination of geological processes and/or anthropogenic activities, such as mining and smelting (Bhattacharya et al., 2006; Bundschuh et al., 2004). There are indications that the use of As-contaminated water for irrigation has led to accumulations of As in surface soils which further lead to bio-accumulations of As in edible plants and crops (Rosas-Castor et al., 2014; Sadee et al., 2016). Livestock and humans may be exposed to As toxicity through plants and vegetables consumed (Khan et al., 2009).

The arsenic concentrations in the edible parts of crops depend on the availability of soil and the ability of a crop to take up As and translocate it to target organs (Zheng et al., 2011). In plants, the chelation phenomenon detoxifies arsenite through complexation with the thiol-rich peptide (Bluemlein et al., 2008; Castillo-Michel et al., 2011). Methylated forms of As have been found in plant tissues e.g. dimethylarsonate (DMA) and monomethylarsonate (MMA) (Huang et al., 2011; Ye et al., 2012). Generally, plants are less efficient at absorbing methylated species than inorganic As, but some plant species can accumulate higher concentrations of methylated As forms (Raab et al., 2007).

Determination of the chemical forms of arsenic in food (Corguinha et al., 2015; Jitaru et al., 2016) is critical because of the varying toxicity of different arsenospecies. Although the toxicity of the organoarsenicals is less than that of the inorganic As compounds (Liao et al., 2003; Nischwitz and Pergantis, 2006), the toxicity of organoarsenic species is of concern because of bioaccumulation in the organism (Kaise and

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Fukui, 1992). In this sense it is necessary to assess the levels of As in edible vegetable oils and to report possible contamination that would represent a health hazard (Zhuravlev et al., 2015).

A few researchers have made some progress on the determination of heavy metals in edible oils (Mendil et al., 2009; Sele et al., 2014). A novel approach, ultrasound-assisted dispersive liquid–liquid microextraction combined with liquid chromatography–mass spectrometry (UA-DLLME with LC–MS) has been reported (Wang et al., 2011). The results show that trace amounts of DMA were detected at concentration of 6 ng g^{-1} in frying oils. Chu et al. by means of inductively coupled plasma mass spectrometer (ICP MS) and ion chromatographic (IC) detector reported a method for speciation analysis of arsenic in edible oil (Chu and Jiang, 2011). However no arsenospecies were determined in unusued oils. Determination of arsenospecies in extra virgin olive oils (EVOOs) has not been reported before.

The present research describes a method for arsenic species determination in EVOOs from previously reported As endemic areas of Argentina (Bardach et al., 2015; Bhattacharya et al., 2006; Bundschuh et al., 2004). In a first approach a screening of As concentration in EVOOs was performed by microwave assissted digestion (MAD) followed by total As concentration determination by ICP MS. Since As shows high affinity for sulphidryl groups of proteins, this fraction was extracted, identified and isolated by size exclusion chromatography (SEC) in a first dimension. As distribution in proteic and non-proteic fractions of EVOOs was stablished by electrothermal atomic absorption spectrometry (ETAAS). Finally in a second dimension arsenospecies were determined in the proteic fraction, previous preconcentration with molecular weight cutoff filters (MWCO), by anion exchange chromatography (AEC). In both dimensions ICP MS was introduced for As monitoring. Arsenite, arsenate, MMA and DMA were determined for the first time in EVOOs.

2. Experimental

2.1. Reagents

Unless otherwise stated, the chemicals used were of analytical reagent grade and, therefore, no further purification was required. The standard As^{3+} solution (1000 μg mL^{-1}) was prepared by dissolving 0.3300 g of As_2O_3 (99.95% purity, Sigma, St. Louis, USA) in 10 mL of 1 M NaOH solution and diluting to a final volume of 250 mL with 2 M HCl. The standard As^{5+} solution (1000 μg mL^{-1}) was prepared by dissolving 0.4526 g of $As_2O_5{\cdot}2H_20$ (99% purity, Aldrich, St. Louis, USA) in 10 mL of 2 M NaOH solution diluted to a final volume of 250 mL with 2 M HCl. Standards of monomethylarsonate and dimethylarsonate solution (1000 μg mL^{-1}) containing 543.21 μg mL^{-1} of As were prepared from analytical grade $CH_3AsO(ONa)_2{\cdot}6H_2O$ (99% purity, Merck, Darmstadt, Germany) (Torralba et al., 1994). Water, methanol (MeOH), *n*-hexane and acetone Optima LC–MS grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA) as well as ammonium acetate. Ultrapure water (18 MΩ cm) was obtained from EASY pure (RF Barnstead, IA, USA). Sodium hydroxide was used provided by Biopack (Buenos Aires, Argentina) and nitric acid and hydrochloric acid, 65%, were provided by Sigma–Aldrich (St. Louis, USA).

2.2. Sample collection and treatment

Ten samples of EVOOs were obtained from olives processed before 24 h of harvest, and the process was carried out with the same equipment for all oils. Samples were collected from the most important EVOOs producing regions of Argentina. Details of procedence and origin are described in Table 1. The most important olive plants varieties grown corresponds to Arauco, Arbequina, Cornicabra and Frantoio. In this way, environmental and experimental parameters that could affect the content of the analytes under study are minimized. Olive oils correspond to *Olea europaea* L., subspecies Arauco, Arbequina, Cornicabra and Empeltre.

Table 1
Extra virgin olive oils characteristics and Arsenic concentrations determined.

	Procedence[b]	Variety	Total As (μg kg^{-1})[a]	As^{3+} (μg kg^{-1})	DMA[f](μg kg^{-1})
1	Mendoza	Arbequina	24.9 ± 1.4	ND[d]	ND[e]
2	Mendoza	Frantoio	ND[c]	ND[d]	ND[e]
3	Mendoza	Cornicabra	6.87 ± 0.2	ND[d]	ND[e]
4	Mendoza	Arauco	11.7 ± 0.9	ND[d]	ND[e]
5	Córdoba	Arbequina	ND[c]	ND[d]	ND[e]
6	Córdoba	Frantoio	16.5 ± 0.9	ND[d]	ND[e]
7	Mendoza	Blend	152 ± 7.4	0.55 ± 0.1	1.68 ± 0.4
8	San Luis	Arauco	13.2 ± 0.6	ND[d]	ND[e]
9	La Rioja	Frantoio	2.01 ± 0.2	ND[d]	ND[e]
10	La Rioja	Frantoio	66.5 ± 2.7	0.53 ± 0.1	2860 ± 0.5

[a] Mean of 5 replicates.
[b] Argentinian regions.
[c] ND: no detected (Limit of detection: 0.01 μg kg^{-1}.
[d] ND: no detected (Limit of detection: 0.00001 μg kg^{-1}.
[e] ND: no detected (Limit of detection: 0.00017 μg kg^{-1}.
[f] DMA: dimethylarsonate.

MAD for total As determination was carried out in a microwave digestor (Milestone, Sorisole, Italy). Digestion was performed according to the manufacturer indications: 0.5 g of olive oil samples were weighed and placed in individual microwave reactors. The aliquots were treated with 7 mL concentrated HNO_3 and 1 mL H_2O_2. Reactors were placed in the digestor at a ramp temperature of 10 min up to 200 °C and hold for 10 more minutes. The employed microwave power was up to 1000 W.

The method described by Martín-Hernández et al. (Martín-Hernández et al., 2008) was employed with modifications to extract proteins from EVOOs. 10 mL of cold *n*-hexane/acetone (1:1, v/v) (2 °C) were added to 5 g of olive oil. 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 with water:methanol (80:20). This solution was centrifuged for 5 min at 3500 rpm (3.013*g*), followed by freezing at −18 °C for 1 h. The remaining oil was kept frozen on the tube walls and a clear solution was obtained for analysis.

2.3. Size exclusion chromatography analysis

SEC was performed by coupling the chromatographer (Series 200, Perkin-Elmer (Thornhill, Canada) to ICP MS (Perkin-Elmer SCIEX, ELAN DRC-e; Thornhill, Canada). The 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 do not generate deposits on ICP cones. Bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amilase (200 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa) and equine myoglobin (17 kDa) were employed for calibration. Sulphur was monitored to investigate the presence of peptides and proteins, according to amino acids with sulphur residues like methionine and cysteine. The employed SEC column separates in a wide range from 10 to 700 kDa. This first dimension procedure, allowed the identification of As-S fractions correspondent with proteins and peptides molecular weight.

2.4. Fraction collection

Once separation was achieved by SEC, the different sulphur fractions were collected off-line and preconcentrated with 5 kDa MWCO filters (Amicon(R) Ultra-4 Millipore; Billerica, MA, USA) 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.

2.5. Arsenic determination in protein fractions

Arsenic was determined in the proteic fraction of EVOOs and in the filtrated fraction from MWCO filters, non proteic. To this end, a Shimadzu Model AA-7000 atomic absorption spectrometer (Tokyo, Japan) equipped with a background correction system employing a continuum source, a GFA-EX7 electrothermal atomizer, and an ASC-7000 auto sampler. L'vov graphite tubes (Shimadzu, Tokyo, Japan) were used in all experiments. An arsenic hollow-cathode lamp (Hamamatsu, Photonics K. K., Japan) was employed as radiation source operated at 10 mA, the analytical wavelength of 193.7 nm was employed for all measurements.

2.6. Determination of arsenospecies by anion exchange chromatography

Arsenic species were only determined in the proteic fractions of EVOOs by AEC with ICP MS determination. The method reported by Zheng et al. was selected since it does not required organic solvents and do not extinguish plasma (Zheng and Hintelmann, 2004). The selected isotope for mass monitoring by ICP-MS was ^{75}As to avoid interference by polyatomic of argon (Date et al., 1987). In Table 2, AEC-ICP MS conditions and for separation of arsenospecies by reverse phase are resume.

2.7. Statistical analysis

Five aliquots of each EVOO samples were analyzed in duplicate and the duplicate tests were statistically similar as paired-samples *t* test (p = 0.05). The average results were used to represent the data. Microsoft Excel was used to test one-way analysis of variance (ANOVA) at 95% confidence to investigate As variations in EVOOs.

Table 2
HPLC–ICP MS conditions.

Chromatographic conditions	
Anion Exchange Chromatography	
Stationary phase	Hamilton PRP X100 (4.6 mm x 25 cm x 10 µm)
Mobile phase	100% 20 mM ammonium dihydrogenphosphate, pH 5.6
Flow rate	1.0 mL min^{-1}
Injection volume	200 µL
Size Exclusion Chromatography	
Mobile phase	Ammonium acetate 50 mM, 5% methanol (v v^{-1})
Elution mode	Isocratic
Flow rate	0.9 mL min^{-1}
Column	TSK gel G3000SW (7.5 mm x 300 mm x 10 µm)
Sample Loop	200 µL
ICP MS conditions	
RF Forward power	1050 W
Gas flow rates:	
Plasma	13 min^{-1}
Auxiliar	1.35 min^{-1}
Nebulizer	0.75 min^{-1}
Resolution	Normal
Scaning mode	Peak hop
Dwell time	500 ms
Isotope monitored	As^{75}

3. Results and discussion

3.1. Total As determinations in EVOOs

Samples were digested by MAD and analyzed by ICP MS. MAD effectiveness assures total oil dissolution and ICP MS sensibility allows reaching As concentration ranges in EVOOs. Results are shown in Table 1. Arsenic concentrations values ranged from 2.01 to 152 µg kg^{-1}. Results are comparable with those reported by Beltran et al. (Beltrán et al., 2015) ranging from 53 to 77 µg kg^{-1}of As in virgin olive oils in Beas, Gibraleon, Niebla and Sanlúcar de Guadiana, southwestern regions from Spain. However they are higher compared to those reported by Llorent-Martínez et al. (Llorent-Martínez et al., 2011) where no As concetrations were detected (LoD < 3 µg kg^{-1}) for olive oils.

EVOOs with higher As concentration correspond to Argentinian regions of La Rioja (66.5 µg kg^{-1}) and Mendoza (152 µg kg^{-1}). In Argentina the highest As contents in groundwater were found in the Chaco–Pampean Plain (provinces of Buenos Aires and La Pampa), in the Northwest and Cuyo regions (provinces of Mendoza, San Juan and San Luis) (Bardach et al., 2015). In order to study differences in As concentration between samples, an ANOVA analysis was performed. Statistical results obtained show significant variation (CI = 95%), F = 910, $p = 1.36{\cdot}10^{-19}$ (F_{crit} = 2.66, p = 0.05). In coincidence, variations of As concentrations in EVOOs according to different regions of Argentina are correspondent with As variations reported in soil and groundwaters samples (Bhattacharya et al., 2006; Bundschuh et al., 2004). Regard safety, only EVOO sample #7, from Mendoza, blend (mixture of different olivés variety) is above the limit set by the Argentinian Alimentary Code (CAA, 1971) of 100 µg kg^{-1} for edible oils.

3.2. Protein distribution according to molecular weight in As contaminated olive oils

As species are transported and metabolized in plants by different proteins (Farooq et al., 2016). In addition arsenite has affinity for sulphydryl groups of proteins (Casarett et al., 2001). For these reasons As distribution in olive oils in proteic fraction was determined. To this end, proteins were extracted by the hexane-cold acetone method, since it has shown higher protein extraction yields (Torres et al., 2016). Despite the fact that SEC analysis provides lower separation resolution compared to other techniques that evaluates molecular weight, like gel electrophoresis, SEC prevents any metals separation from proteins and species interconversion. Results can be observed in Fig. 1. Fractions of ~66 kDa were determined for EVOO sample #7 and #10. This molecular weight value is correspondent with proteins related to As metabolization in plants reported by Farooq et al. and displayed in Table 3 (Farooq et al., 2016). The molecular weight of these proteins, according to UniProt protein sequence database, ranged from 17.705 to 63.519 kDa (Table 3). These MW range is correspondent with SEC analysis were a wide peak area can be observed around 66 kDa. Specifically in olives a 66 kDa protein fraction has been identified as a constituent protein of seeds, particularly in endosperm and embryo (Krebesová et al., 2015).

For proteic As studies, fractions from 12.5 to 17.5 min from SEC chromatograms of EVOOs sample #7 and #10 were collected and preconcentrated in 10 kDa MWCO filters. In this way, proteins diluted in a volume of 4.5 mL were concentrated in 200 µL, increasing the techniqués sensibility.

3.3. Arsenic determination in proteic fractions by ETAAS

As mentioned previously, arsenic has an elevated affinity for proteins, specifically As^{3+} species has been identified to bind sulfhydryl groups from proteins. In this sense, arsenic was analyzed in the proteic fractions collected from SEC analysis. ETAAS was chosen for As analysis

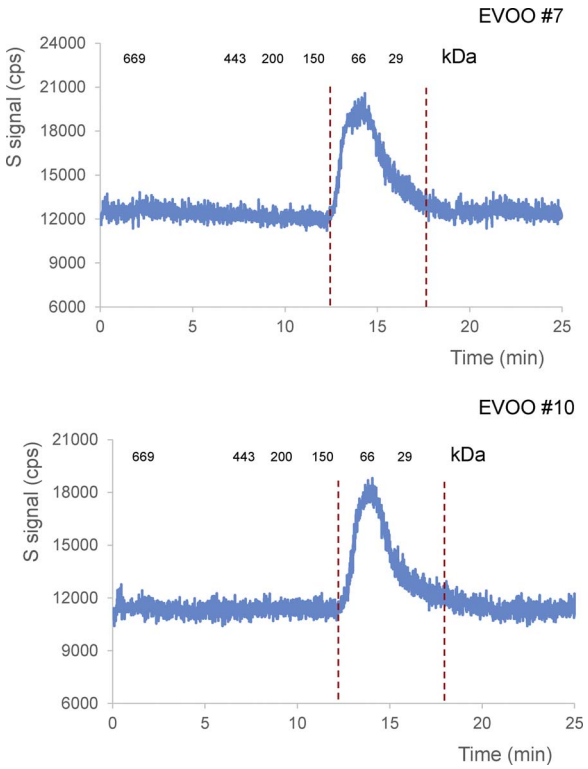

Fig. 1. Chromatogram from SEC–ICP MS corresponding to protein extract of EVOOs samples #7 and #10. Volume injected: 200 µL.

Table 3
Proteins function and molecular weight involved in Arsenic metabolism.

Entry[a]	Protein	Mass (kDa)	Function
Q8VYM2	Pi transporter Family members	57.616	Arsenate transporters
Q8VZW1	Aquaporin NIP-1	31.715	As species absorption
Q6Z2U2	As(III) methyltransferase	31.485	As methylation
Q336V5	Arsenate reductase 2.1	17.705	Reduction of arsenate to arsenite
D7KES1	Inositol transporter	63.519	Arsenic transporter
Q8VYM2	Inorganic phosphate transporter 1-1	57.616	Arsenic transporter
D2KZ47	Nodulin-26 like intrinsic protein	29.502	Arsenic transporter

[a] UniProt (http://www.uniprot.org/).

according to the collected volume of 200 µL and to avoid dilutions and the consequental sacrifice of sensibility.

According to the possible As-proteins associations, a pyrrolysis temperature optimization was performed previous to fractions analysis to avoid problems in As atomization in graphite furnace. There are several problems associated with As determination using ETAAS, including the low wavelength of the most intense line of absorption, the formation of volatile compounds, possible interactions of As or its compounds with the graphite and spectral interferences (Welz and Sperling, 2008). In this context, As pyrolysis was optimized in SEC collected fractions from 200 to 900 °C. A pyrolysis temperature of 600 °C was chose for further experiments corresponding to the maximum As signal reached.

Results of ETAAS analysis can be observed in Fig. 2. A higher concentration of proteic As was determined in both EVOOs samples #7 and #10, 4.44 and 3.89 µg kg^{-1} respectively. Non-proteic As concentrations were of 0.56 and 0.67 µg kg^{-1}, respectively. These non proteic As fraction can be formed by species with lower affinity for sulfhydryl groups of proteins, as As^{5+}. ANOVA analysis showed no significant

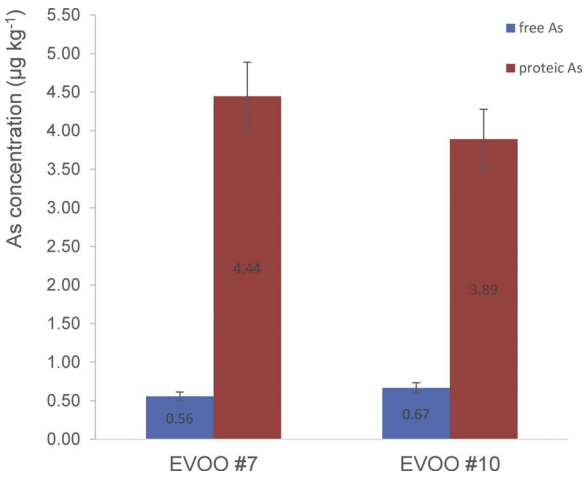

Fig. 2. Arsenic concentration in proteic and non-proteic fractions of EVOOs samples #7 and #10 determined by ETAAS. Volume injected: 50 uL.

variation (CI = 95%) in free As concentration between EVOOs samples, F = 4.76, $p = 0.09$ ($F_{crit} = 7.70$, $p = 0.05$); and proteic As concentration in EVOOs samples, F = 2.22, $p = 0.21$ ($F_{crit} = 7.70$, $p = 0.05$).

Recoveries for both EVOOs samples ranged from 3.28 to 6.85%, compared with total As determination, in proteic and non proteic samples. Recent studies have reported presence of arsenolipids in edible oils, from 17 to 42% (Sele et al., 2014). The existence of arsenolipids and the low protein concentration in EVOOs leads to low As recoveries when proteins are extracted compared to total As determinations.

3.4. Arsenic species determination in proteic fractions

The limits of detection (LOD) of AEC-ICP MS were calculated according to 3σ criteria and correspond to 0.01, 0.014, 0.025, and 0.017 ng kg^{-1} for As^{3+}, As^{5+}, MMA and DMA respectively. The limit of quantification (LOQ) calculated according to 10σ criteria corresponds to 0.09, 0.12, 0.17 and 0.3 respectively. These limits are comparable to other techniques applied to arsenospecies analysis in edible oils (Chu and Jiang, 2011; Wang et al., 2011), however do not provide information regard As distribution in proteic fractions. Precision presented as relative standard deviation correspond to 13.7% (n = 5).

Due to the absence of olive oil reference materials with certified concentrations of arsenospecies associated to proteins, a recovery study was performed in the proteic fraction of EVOOs. To this end, arsenospecies (As^{3+}, As^{5+}, MMA and DMA) were added in three concentrations level to five aliquots of protein extracts of EVOOs. Afterwards they were directly analyzed by AEC-ICP MS, and average recoveries for the 3 aliquots were of 101, 97, 99 101%, for As^{3+}, As^{5+}, MMA and DMA respectively.

In EVOOs samples #7 and #10, As^{3+} and DMA were determined (Fig. 3). In order to check if As signals effectively corresponds to arsenospecies or arsenic associations with proteins or peptides, and not to As artifacts with proteins, sulphur signal was monitored and no associations were detected. Concentrations found can be observed in Table 1. Inorganic As^{3+} was determined in EVOOs while As^{5+} was not detected (0.014 ng kg^{-1}). It has been reported that As^{5+} concentrations decreases from non-aerial to aerial organs of plants as olives (Sadee et al., 2016). In addition, As^{3+} presence in proteic fraction is correspondent with its affinity for sulfhydryl groups of proteins. ANOVA analysis showed no significant variation (CI = 95%) in As^{3+} concentration between EVOOs samples, F = 0.06, $p = 0.81$ ($F_{crit} = 7.70$, $p = 0.05$).

Organic arsenic species as DMA was determined in EVOOs by the techniques developed in this study. ANOVA analysis showed significant variation (CI = 95%) in DMA concentration between EVOOs samples,

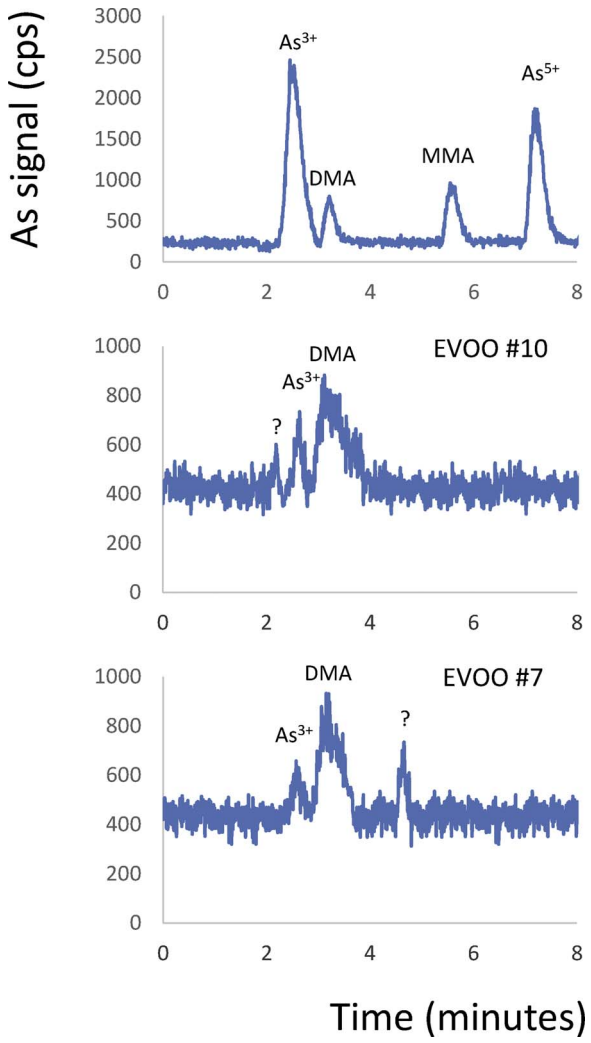

Fig. 3. Chromatogram from AEC–ICP MS corresponding to Arsenic species present in proteic fractions from EVOOs #7 and #10. Volume injected: 200 uL. As species concentration 10 μg L^{-1}.

$F = 10.18$, $p = 0.03$ ($F_{crit} = 7.70$, $p = 0.05$), according to the different areas. Different DMA concentrations can be attributed to the different procedence of EVOOs and different varieties. Beyond reduction of As^{5+} to As^{3+} in plants, a further reduction has been observed in terms of methylation inside plants, suggesting the phenomenon of biomethylation of As in plants (Bentley and Chasteen, 2002), explaining the presence of DMA in vegetal oils as EVOOs.

4. Conclusion

A technique based on multidimensional chromatography and preconcentration procedures allowed determination of As species distribution in proteic and non proteic fractions of EVOOs. A first screening approach by MAD followed by ICP MS analysis succesfully identified EVOOs with elevated As concentrations from As endemic areas.

EVOOs with elevated As concentrations were selected for protein extraction by the cold acetone method. The extracted proteins were identified and purified by SEC and MWCO filters. ETAAS was introduced according to the μL range of collected volume of extracted proteins, and after optimization of pyrrolysis temperature, an improved sensibility allowed As determination in proteic and non proteic fractions of EVOOs.

Since As was determined in the proteic fractions of EVOOs, these findings encourage As species analysis by AEC-ICP MS. As^{3+} and DMA were found associated to proteins. These As species distribution has been established for the first time in EVOOs, contributing to a better understanding of the toxicological risks of EVOOs consuption produced in Arsenic endemic areas.

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