Multielemental fractionation in pine nuts (*Pinus pinea*) from different geographic origins by size-exclusion chromatography with UV and inductively coupled plasma mass spectrometry detection

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Abstract

Pine nuts (*Pinus pinea*) from different geographical origin in Spain and Portugal have been investigated concerning total element content and metal-biomolecules size distribution patterns Mn, Zn, Ni and Cu. All the studied metals were at the highest concentration in pine nuts from Faro and at the lowest from Cataluña. The most abundant element in samples was Mn at concentrations in the range of 26 $\mu g\,g^{-1}$ (Cataluña) to 559 $\mu g\,g^{-1}$ (Faro). Zn was also present at high concentration in samples, from 25 $\mu g\,g^{-1}$ (Cataluña) to 113 $\mu g\,g^{-1}$ (Faro). To a deeper insight to obtain classification rules for samples, pine nuts were analyzed by size-exclusion chromatography (SEC) with UV detection and inductively coupled plasma mass spectrometry (ICP-MS). Two columns were used covering the molecular weigh range from <10 to 70 kDa that allowed the discrimination of the studied samples. Data reveal that the most differential UV-profile with low molecular weight (LMW) column was obtained with pine nuts from Huelva. This column allows good discrimination in the range of 2126–1352 Da in which a lot of peaks can be used to differentiate samples. The UV profiles obtained with the high molecular weight (HMW) column allows a poorer differentiation of samples, but pine nuts from Huelva, Castilla and Madrid are clearly distinguished to the others. In relation to fractionation patterns of metals, Mn allows a good discrimination between samples (LMW column), Cu was the only one associated to fractions at MW > 70 kDa in sample from Cádiz, and profiles of Ni and Zn are clearly different in terms of abundance of peaks. All these chromatographic profiles for elements give valuable information about the geographical origin of the studied samples and the differences found are discussed in this work.

Keywords: Pine nuts; Metals; Multielemental fractionation; Size-exclusion chromatography; Inductively coupled plasma mass spectrometry; Geographic origin distinction

1. Introduction

Pinus pinea has been widely planted in Spain, Portugal, Italy, Greece, Albania and Turkey where it is one of the most important ingredients of the Mediterranean diet [1]. Pine nuts, raw or roasted, are included as ingredients in a great variety of traditional dishes, such as breads, candies, sauces and cakes, as well as vegetable and meat dishes. It is an edible nut with an exquisite flavor and high protein content which makes them a good source of nutrients. The seeds of the *P. pinea* have a complex chemical composition (5.6% moisture, 31.1% protein, 47.4% fat, 10.7% carbohydrate and 4.3% ash) [2], they contain Vitamins, particularly B1 (thiamine) and also minerals [1]. In addition, the consumption of nuts has health benefits since they reduce risk of both coronary heart disease and non-fatal myocardial infarction [2].

The elemental composition of plant foods plays important biological roles and can also be associated with the toxicity, pollution, geographic origin of plants, etc. [3,4]. Therefore, the content of trace elements can be very useful as markers for the identification of product's geographical origin and authenticity [5]. Several authors have proposed the use of the mineral content to characterize wines [6,7], vinegars [8], coffees [9], potatoes [10], honey [11], teas [12] and so on. Likewise, multielement analysis can be a valuable tool for the authentication and characterization of foodstuffs.

Moreover, the bioavailability, effects and toxicity of the elements are highly associated with their chemical form [13]. In

addition, metals and metalloids can be found as part of biomacromolecules that represent a valuable information about their differential actions and behaviors [13–16] Therefore, the information about trace elements speciation in biological and food samples is today mandatory to understand the biochemistry of metals and semi-metals [16]. Consequently, the development of speciation studies gives valuable information rather than total elements determinations [13].

The elements studied in this work exhibit important biochemical roles, for example, Mn is a cofactor of the enzyme pyruvate carboxylase and it seems to act also as a non-specific activa- tor for several enzymes, such as superoxide dismutase, glycosyl transferases, arginase and other. Other proteins in which Mn can be present are albumin and ß$_1$-globulin [17]. Moreover, Mn is required for protein and fat metabolism, healthy nerves and healthy immune system as well as for sugar regulation [18]. Cu can be bound to several proteins such as superoxide dismutase and cytochrome oxidase [17]. This element is also needed for the transport of iron and it is involved in the synthesis of connective tissue, lipid metabolism and antioxidant protection [19]. Ni is thought to play an important role in folate metabolism [20]. Zn is a constituent in more than 200 enzymes and proteinswhich participate in all major metabolic processes [17]. Further- more, Zn also affects proteins synthesis through gene expression [19]. Co is a cofactor of several enzymes like cytochrome oxi-dase and superoxide dismutasa, and can also be the source of goiter disease. Moreover, Co is a constituent in vitamin B_{12} and it is absorbed as cobalamine that interacts with iron and Mn [21].

Along the last decade high attention has been paid on the development of analytical methods for the determination of elements bound to biomolecules in foods mainly based on ICP-MS coupled to some separation device. In connectionto this Mn compounds have been determined by capillary electrophoresis (CE) coupled to ICP-MS in liver [22] and in human milk by SEC/anion-exchange-ICP-MS [18]. Selenium speciation has been carried out in nuts by high-performance liquid chromatography (HPLC) coupled to ICP-MS [23] and electrospray mass spectrometry (ESI-MS) [24] and also in yeast by means of SEC-CE-ICP-MS [25], SEC-HPLC-ICP-MS and MALDI-TOF-MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) [26], using a metallomic approach. In addition, the multielemental fractionation of Ni, Cu, Zn, and Mn in nuts [19] and soybean flour [27] by SEC-ICP-MS has been performed. Other multielemental fractionation studies have also been performed with this coupling in premature human [28,18] and whey milk [13].

As can be concluded from these studies, SEC-ICP-MS is a very suitable technique to get distribution patterns of elements along different molecular weight fractions and provides valuable information about the association of elements with the different compounds in the sample [29,30]. Many distribution studies based on SEC-ICP-MS have been focused on Se in nuts [25,26]. However, other metals of nutritional and toxicological importance exist in nuts, which have less been studied [19] and under our knowledge, distribution patterns of elements has not yet been performed in pine nuts (*P. pinea*).

The aim of this work is to determine the total content of some of these metals (Cu, Mn, Ni and Zn) as well as metal-binding biomolecules fractionation in pine nuts (*P. pinea*) for geographic origin assessment. The analytical methodology for distribution patterns of these elements was based on size-exclusion liquid chromatography (SEC) on-line coupled to UV and inductively coupled plasma mass spectrometry (ICP-MS).

2. Experimental

2.1. Standard solutions and reagents

The mobile phase solution was daily prepared at 0.05 M of tris(hydroxymethylaminomethane) (Tris) prepared at pH 8.0 from Trizma base and Trizma hydrochloride (Sigma–Aldrich, Steinheim, Germany).

Hydrochloric acid (37%), nitric acid (65%) and perchloric acid (70–72%) were of Suprapur grade (Merck, Darmstadt, Germany). Copper, manganese, nickel and zinc stock solutions (1000 mg l^{-1}) were also obtained from Merck. Ultrapure water (18 M▲ cm) from a Milli-Q System (Millipore, Watford, UK) was used throughout. Commercial chemicals were of analytical reagent grade and were used without further purification. The presence of trace elements was not detected in the working range.

2.2. Samples

Samples of pine nuts (*P. pinea*) were obtained from different areas in Spain (Huelva, Cádiz, Badajoz, Cataluña, Castilla, Madrid) and Portugal (Faro and Coimbra) and were supplied by Frutos Secos Puig (Tarragona, Spain). All samples were washed with ultrapure water, freeze-dried using a Benchtop lyophilizer (Hucoa-Erlöss, Spain) and finally ground using a conventional grinder Moulinette (Moulinex, Spain).

2.3. Instrumentation

Semi-preparative size-exclusion chromatography (SEC) was carried out in both Hiload 26/60 Superdex 30 Prep for separation range <10 kDa (low molecular weight, LMW) and Superdex 75 Prep for separation range 3–70 kDa (high molecular weight, HWM) (all from Amersham Biosciences, Uppsala, Sweden). An AKTA-Prime system (pump and UV detector at 280 nm) (Amersham) was used as the eluant delivery system, equipped with a 2 ml sample loop.

Elemental detection was performed using a model 4500 ICP-MS system (Hewlett-Packard, USA). A model Sigma 4-10 centrifuge (Spain) and a constant orbital shaking of 105 rpm (Heidolph, Unimax 1010, Germany) was used to accelerate the phase separation process in the extraction of the compounds.

2.4. Procedures

2.4.1. Quality control of the analyses

To overcome problems related to contaminations, losses, stability of the species during analysis as well as identification

difficulties, several considerations were taken into account:

(1) The use of metal pieces (stainless steel) such as spatulas, injection syringes and syringe needles were avoided since this increases signal noise and causes contamination. All the instruments used were previously washed with a 10% (v/v) HNO_3 water solution and then with ultrapure water.
(2) The column must be cleaned properly [17] to prevent proteins absorption on the SEC matrices caused by hydrophobic interactions. Between two consecutive injections the SEC columns were cleaned with one column volume of 0.002 M EDTA solution. After that, the SEC columns were washed with one-half to one column volume of 0.5 M NaOH to remove most non-specifically adsorbed proteins to the gel. After cleaning, the columns were equilibrated with at least two column volumes of mobile phase until the UV baseline stabilizes before applying next sample.

    For a more rigorous cleaning the columns were washed with four column volumes of 1 M NaOH (removal of hydrophobic proteins and lipoproteins) followed by four column volumes of Milli-Q water. Finally, one-half column volume of 30% isopropyl alcohol (removal of lipids and very hydrophobic proteins) followed by two column volumes of Milli-Q water were passed.
(3) In order to obtain an accurate measurement of the rela- tive MW obtained in the pine nuts extracts, both columns were calibrated with appropriate calibrants. For LMWcolumn, the calibration standards were: bovine serum albumin (67 000 Da), metallothionein I (7000 Da), gas- trin rat I (2126 Da), vitamin B_{12} (1352 Da) and Gly_6 (360 Da). For the HMW column, the calibration stan- dards were: bovine serum albumin (67 000 Da), ovalbumin (43 000 Da), chymotrypsinogen A (25 000 Da) and ribonu- clease A (13 700 Da). The relation between retention time (determined by peak maxima in UV detection) and MW (Da) for both columns is shown in Fig. 1a (LMW) andFig. 1b (HMW). The void retention time was determined inthe LMW and HMW columns using bovine serum albumin(67 kDa) and Blue Dextran 2000 (2000 kDa), respectively.

2.4.2. *Total elements determination in pine nuts*

First of all, lipids were extracted from 5 g of previously freeze-dried pine nuts with 25 ml chloroform/methanol (2:1) mixture by mixing those together for 30 min in a constant orbital shaking and centrifugation at 10 000 rpm for 20 min. Afterward, the organic layer was discarded and the remaining residue was dried at room temperature.

Samples without lipids were exactly weighted (0.2 g) into PTFE bombs and digested in a domestic microwave oven placed inside a fumed cupboard. Nitric acid (65%, 10 ml) was addedand the vessels were tightly closed. The microwave program con-sisted in three steps as follows: heated at 800 W (3 min), 400 W (3 min) and 100 W (3 min). The bombs were left at room temper-ature for 10 min between each step to avoid overpressure. This solution was filtered through a 0.20 μm surfactant-free celluloseacetate filter. Cu, Mn, Ni and Zn were measured by ICP-MS (conditions as stated in Table 1).

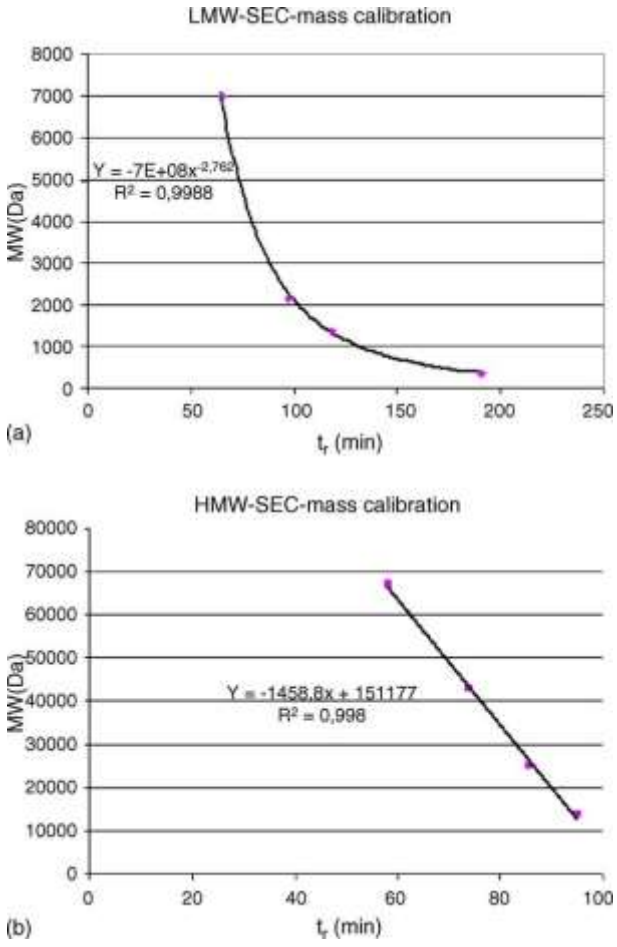

Fig. 1. Mass calibration of SEC columns. Relation between retention time (determined by peak maxima in UV detection) and MW (Da) for both columns: (a) LMW and (b) HMW.

2.4.3. *Extraction of elemental species and distribution patterns by SEC-UV-ICP-MS*

Freeze-dried samples (without lipids) were accurately weighted (0.2 g) in PTFE centrifuge tubes. The extraction of

Table 1
ICP-MS and SEC instrumental conditions

SEC conditions	
Columns	Hiload 26/60 Superdex 30 Prep Hiload 26/60 Superdex 75 Prep
Resolution range	Mr < 10 000 Da; 3000–70 000 Da
Mobile phase	Tris 50 mmol l^{-1} (pH 8.0)
Flow rate	2 ml min^{-1}
Injection volumen	2 ml
UV–vis wavelength	280 nm
ICP-MS conditions	
Forward power	1350 W
Plasma gas flow rate	15.0 l min^{-1}
Auxiliary gas flow rate	0.87 l min^{-1}
Carrier gas flow rate	0.975 l min^{-1}
Sampling depth	6 mm
Sampling and skimmer cones	Nickel
Dwell time	0.1 s per isotope
Isotopes monitored	^{63}Cu, ^{55}Mn, ^{58}Ni, ^{68}Zn

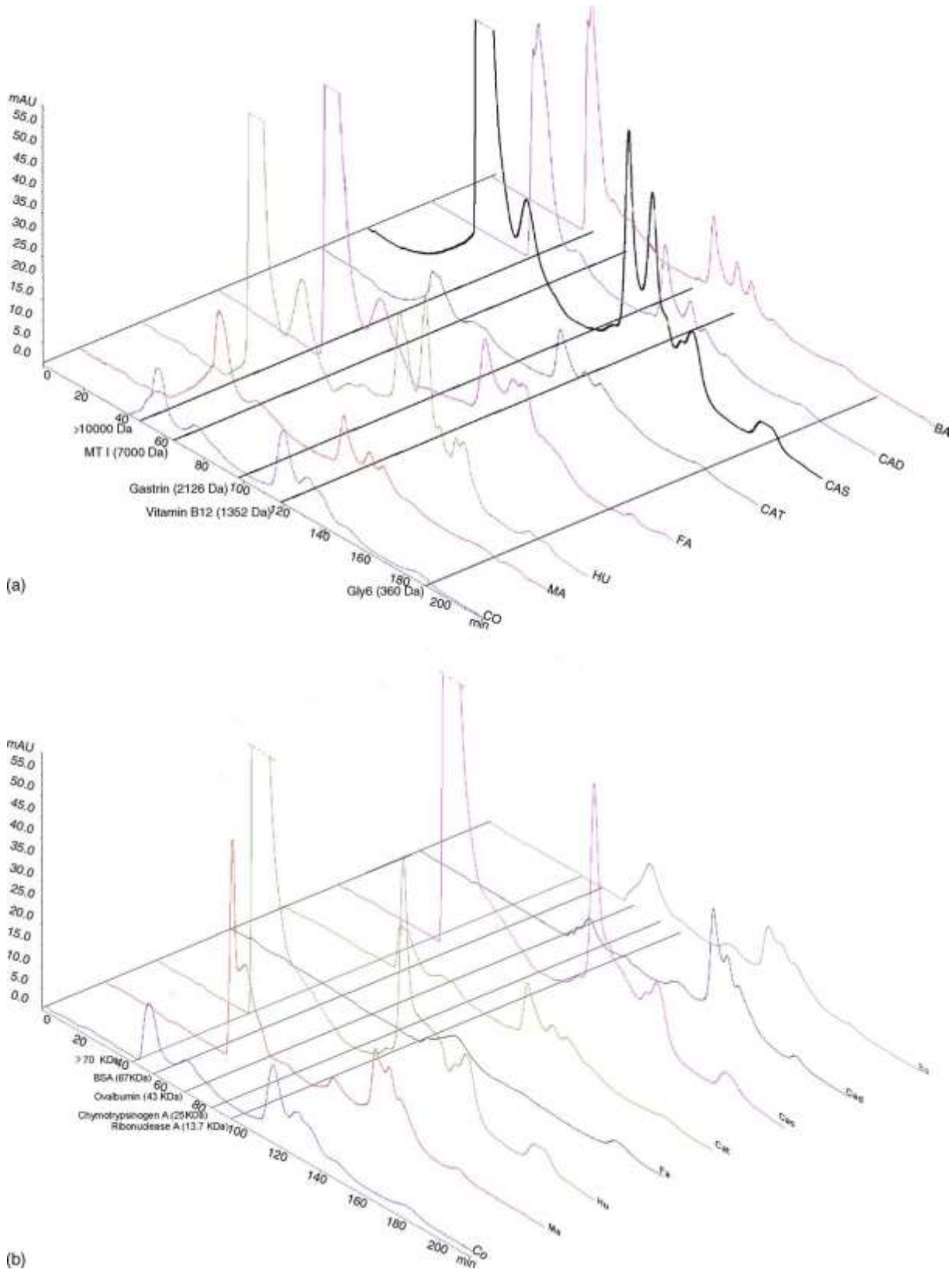

Fig. 2. UV profiles of the SEC separations from pine nuts samples: (a) LMW and (b) HMW. Codes in parenthesis: Coimbra (CO), Madrid (MA), Huelva (HU), Faro (FA), Cataluña (CAT), Castilla (CAS), Cádiz (CAD) and Badajoz (BA).

elemental species from pine nuts was performed with 4 ml of 0.1 M sodium hydroxide by centrifugation at 10 000 rpm for 20 min after mixing for 10 min in a constant orbital shaking. Generally, the extraction conditions for elemental species are based in the use of NaOH, HCl [19] or in a less extent hot water [31]. It has been reported that NaOH solutions extract both LMW and HMW elemental compounds with higher recov-eries while HCl solutions mainly extract LMW compounds dueto the lower solubility of protonated compounds such as proteins [19,32,33]. Therefore, NaOH solutions were used in the present study.

Elemental fractionation profiles of the pine nut samples was performed by SEC using the two columns described in Section 2.3.

Elemental detection was carried out using an ICP-MS. The sensitivity of the instrument was optimized using a multielemental standard solution containing the following elements: 10 μg l^{-1} Li, Y, Ce and Tl, of each element and dissolved in 2% nitric acid. The analytical conditions are shown in Table 1.

The SEC-UV-ICP-MS coupling was performed connecting the outlet of the UV detector to the nebulizer inlet of the ICP-MS. The instrumental operating conditions are given in Table 1.

3. Results and discussion

3.1. Optimization of total elements determination

Several digestion procedures were tested for the analysis of total elements in pine nuts by adding 1000 μg $l^{-1}$of each element. Wet-digestion with concentrated nitric acid in open PTFE vessels until nearly dryness and later treatment with 2:1 (HNO_3/$HClO_4$) mixture has been proposed in the literature for elemental analysis in honey [11] and plant samples [34]. However, this procedure was time-consuming and did not provide good recoveries. Therefore, other two different procedures based in closed vessel and microwave digestion were assayed. One of them based on the successive attack with concentrated nitric and perchloric acid, was reported for metal analysis in tea leaves [35],

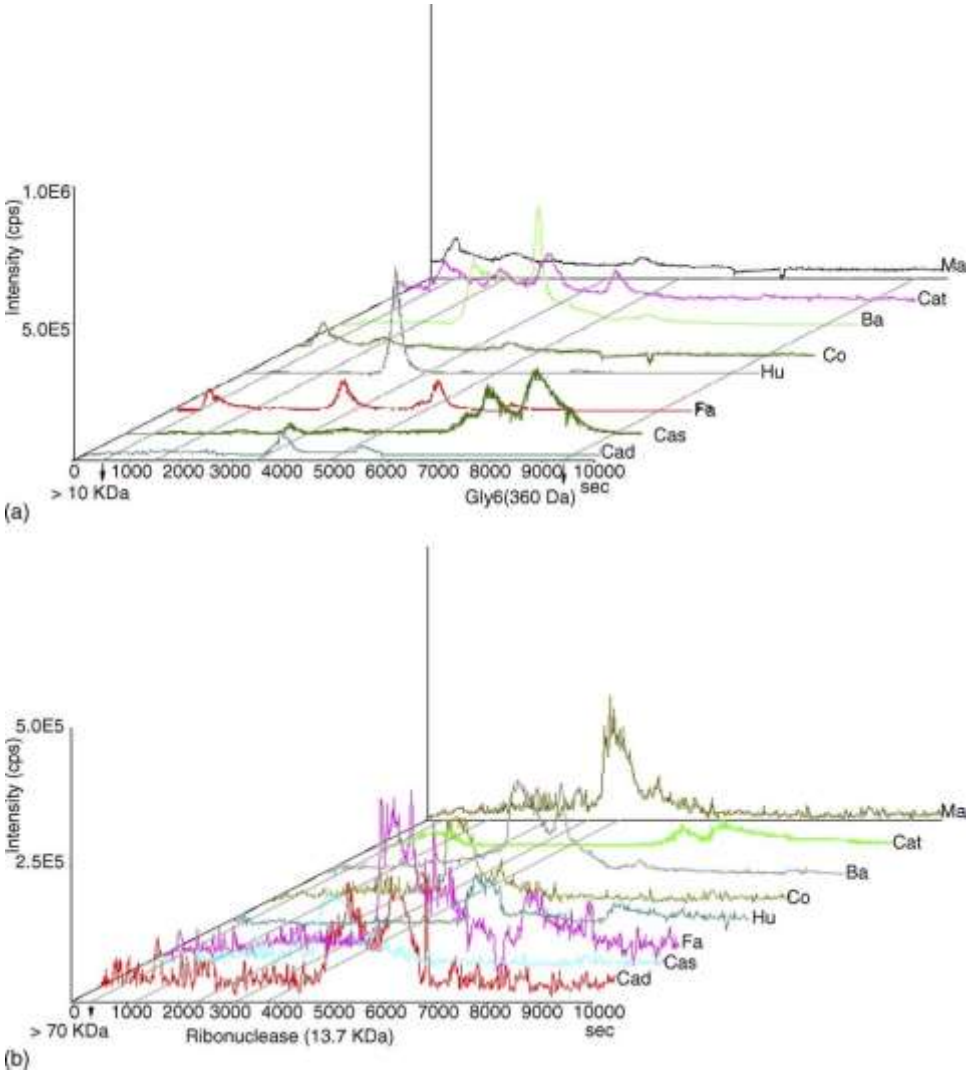

Fig. 3. Mn chromatograms of the SEC separations from pine nuts samples: (a) LMW and (b) HMW. Codes in parenthesis: Coimbra (CO), Madrid (MA), Huelva (HU), Faro (FA), Cataluña (CAT), Castilla (CAS), Cádiz (CAD) and Badajoz (BA).

with recoveries in the range of 80–91%. Other approach optimized in our laboratory considers the digestion with 65% nitric acid with a domestic microwave oven (see Section 2.4.2) providing higher recoveries in the range of 91–96%. With this later procedure the analysis time was considerably reduced (from 24 h to 29 min) and it was selected for the further studies.

Seven pine nuts samples from different geographic origins were analyzed for total element content (as described in Section 2.2). The results are shown in Table 2. As can be seen, all the metals were at the highest mass fraction in pine nuts from Cataluña and at the lowest in Faro. The most abundant element was Mn at mass fractions in the range of 26 $\mu g\,g^{-1}$ (Cataluña) to 559 $\mu g\,g^{-1}$ (Faro). Zn was also present at high mass fraction from 25 $\mu g\,g^{-1}$ (Cataluña) to 113 $\mu g\,g^{-1}$ (Faro).

Table 2
Total element mass fractions in the analyzed pine nut samples (n =3)

	Mn ($\mu g\,g^{-1}$)	Ni ($\mu g\,g^{-1}$)	Cu ($\mu g\,g^{-1}$)	Zn ($\mu g\,g^{-1}$)
Coimbra	65 ± 7.14	4.5 ± 0.49	31 ± 9.45	86 ± 9.45
Cataluña	26 ± 2.87	2 ± 0.19	8 ± 2.81	25 ± 2.81
Badajoz	458 ± 50.29	8 ± 0.85	35 ± 10.37	94 ± 10.37
Madrid	87 ± 9.58	7 ± 0.81	34 ± 10.70	97 ± 10.70
Faro	559 ± 61.41	15 ± 169	41 ± 12.42	113 ± 12.42
Castilla	72 ± 7.89	6 ± 0.65	35 ± 11.40	104 ± 11.40
Cádiz	414 ± 45.48	11 ± 1.21	38 ± 11.06	101 ± 11.06
Huelva	75 ± 8.26	5 ± 0.60	37 ± 11.73	107 ± 11.73

3.2. *Fractionation profiles by UV detection*

As previous step for trace elements fractionation analysis in pine nuts the eluate from the SEC column was passed trough the UV detector set at 280 nm and the corresponding chromatograms were registered. The absorbance was initially studied in the 200–500 nm wavelength range to detect the different compounds present in the extraction solutions. It was observed that all the fractions showed good response at 280 nm. The superimposed UV chromatographic profiles of pine nuts from the different geographic origins are shown in Fig. 2a (for LMW

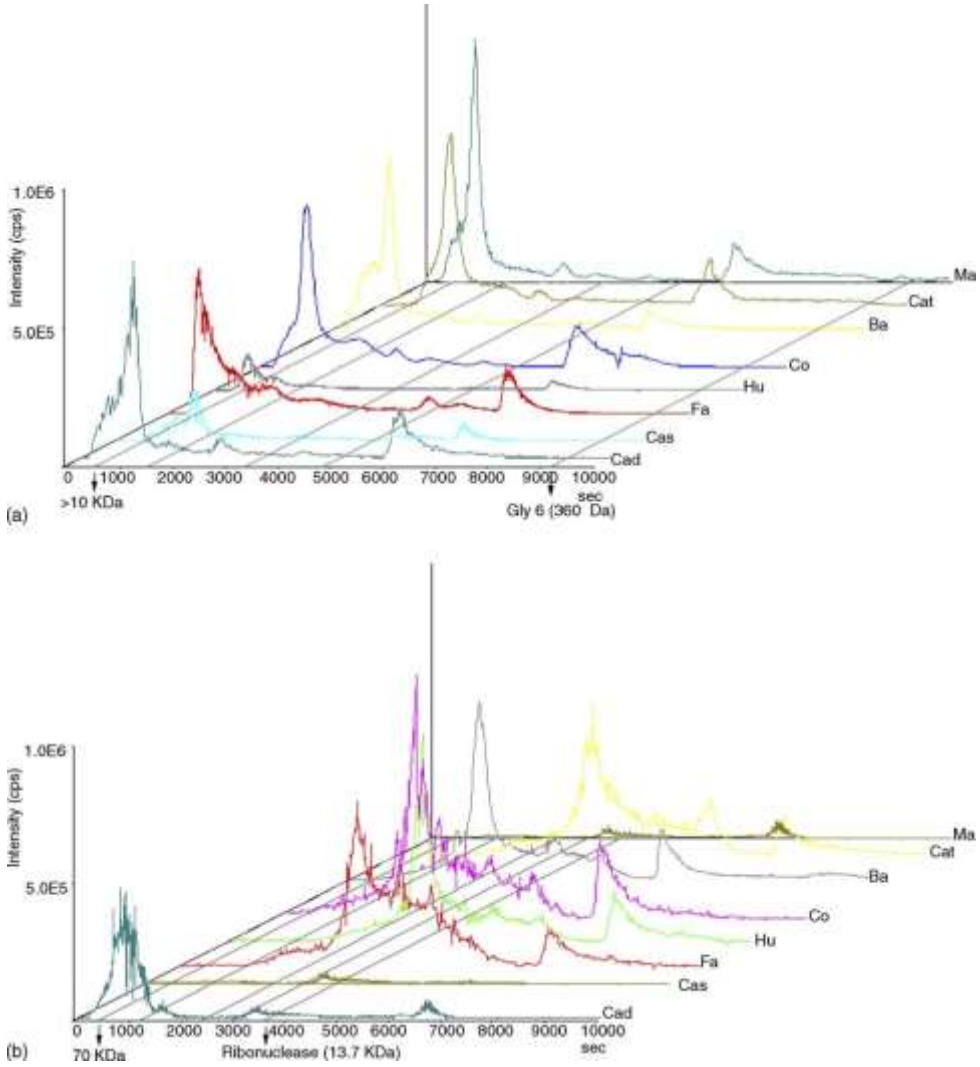

Fig. 4. Cu chromatograms of the SEC separations from pine nuts samples: (a) LMW and (b) HMW. Codes in parenthesis: Coimbra (CO), Madrid (MA), Huelva (HU), Faro (FA), Cataluña (CAT), Castilla (CAS), Cádiz (CAD) and Badajoz (BA).

column) and Fig. 2b (for HMW column). The combined use of both columns allowed a clear separation of compounds in the range of 360–70 000 Da.

3.2.1. Low molecular weight UV profiles (<10 kDa)

The chromatographic run time was extended to 4 h to ensure the elution of all fractions. However, no peaks were observed at retention times higher than 190.52 min that corresponds to Gly_6.

As can be seen in Fig. 2a, all the analyzed pine nuts present fewer peaks in the fraction corresponding to the MW range of 1352–360 Da and 7000–2126 Da. In the last range the Huelva samples present a characteristic profile with two peaks which are absent in the other samples. Profiles from Castilla and Huelva samples show some peaks at low intensity in the 1352–360 Da range. On the other hand, highly similar profiles can be found between sample from Coimbra, Madrid and Cataluña in the MW range of 2126–1352 Da and also between Cádiz and Badajoz samples. However, samples from Huelva do not present any peaks in this range and that from Castilla exhibits two prominent peaks of high intensity. Finally, highly abundant peaks can be observed for all the samples (except that from Faro) in the fraction >7000 Da with low resolution and they will be considered in more detail in Section 3.2.2 for HMW column. Therefore, in spite of somewhat similar patterns were found in the samples studied, the pine nuts UV-profile are depending of their geographic origin.

3.2.2. High molecular weight UV profiles (3–70 kDa)

As can be seen in Fig. 2b, molecules with MW > 7000 exhibit very similar profiles for pine nuts from Coimbra and Cataluña, Cádiz and Badajoz and also between Castilla and Huelva. It is significant the absence of peaks in the chromatogram of Faro that is in good agreement with data obtained from LMW column. In Fig. 2b it can be observed some peaks that discriminate samples from Madrid, Huelva and Castilla. Likewise the chromatogram of Madrid presents two highly abundant peaks at about 70 and 68 kDa that are absent in the rest of pine nuts. On the other hand, Huelva and Castilla present similar chromatograms with a peak at about 16 kDa that clearly distinguish these samples from the others.

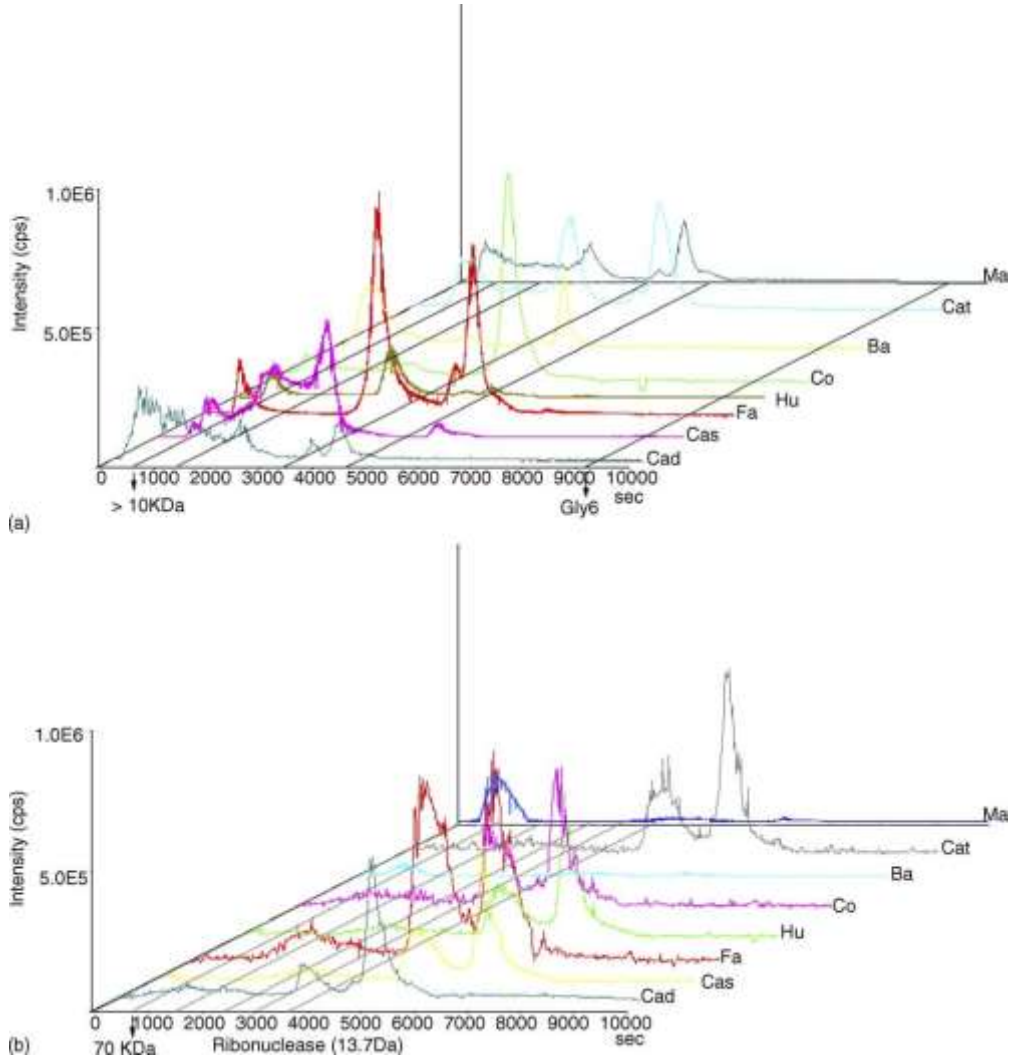

Fig. 5. Zn chromatograms of the SEC separations from pine nuts samples: (a) LMW and (b) HMW. Codes in parenthesis: Coimbra (CO), Madrid (MA), Huelva (HU), Faro (FA), Cataluña (CAT), Castilla (CAS), Cádiz (CAD) and Badajoz (BA).

Poor resolution can be observed in the chromatograms of pine nuts from Castilla, Huelva and into lesser extent from Coimbra, since the most abundant peak elutes in the void volume (retention time of Blue Dextran 2000, 49.7 min). However, the molecular weight range covered with both columns was suitable for most of the compounds present in the analyzed pine nuts.

3.3. Elemental fractionation patterns of pine nuts

The fractionation profiles of the metals in pine nuts are shown in Figs. 3–6.

3.3.1. Manganese fractionation profiles

3.3.1.1. LMW (<10 kDa). Typical chromatographic profiles obtained for ^{55}Mn are shown in Fig. 3a. In pine nuts from Cádiz, Cataluña and Madrid this element was absent. However, it is clear the presence of this element in the chromatographic profile of Huelva, in which it is mainly associated to a MW fraction of 7000–2126 Da. In the sample from Castilla, only one ^{55}Mn-containing fraction was observed in the range of 1352–360 Da. Finally three peaks were detected in the sample from Faro one of them associated to the fraction of MW > 10 kDa.

3.3.1.2. HMW (3–70 kDa). Fig. 3b shows the chromatographic profiles of ^{55}Mn in which it can be observed that the intensity of peaks is very low. However, this element allows the discrimination between samples fairly well as can be also concluded from the LMW profiles.

3.3.2. Copper fractionation profiles

3.3.2.1. LMW (<10 kDa). The chromatographic profiles of ^{63}Cu (Fig. 4a) were almost the same in all the samples witha peak outside the calibration range. So, they were further stud-ied with the HMW column. Only the fractionation profiles of Huelva and Castilla were slightly different in terms of the peaks intensity.

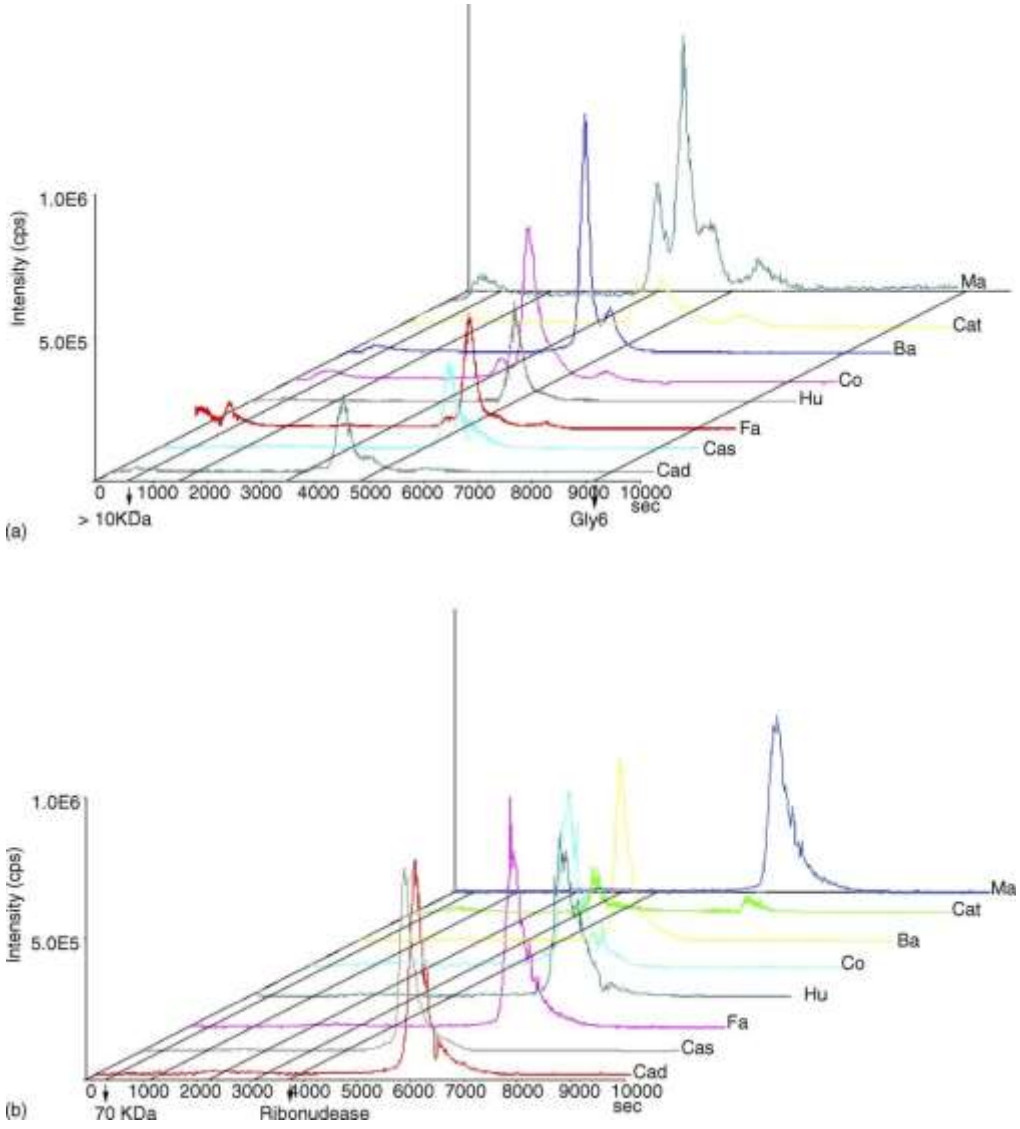

Fig. 6. Ni chromatograms of the SEC separations from pine nuts samples: (a) LMW and (b) HMW. Codes in parenthesis: Coimbra (CO), Madrid (MA), Huelva (HU), Faro (FA), Cataluña (CAT), Castilla (CAS), Cádiz (CAD) and Badajoz (BA).

3.3.2.2. HMW (3–70 kDa). Copper showed an especial behaviour since as can be seen in Fig. 4b, it was the only one associated to fractions at MW > 70 kDa in sample from Cádiz. For this reason this element can be used to discriminate samplesfrom this origin.

3.3.3. Zinc fractionation profiles

3.3.3.1. LMW (<10 kDa). This element shows low abundance in pine nuts from Cádiz and Madrid but the opposite situa- tion was observed in Faro in which two high abundant ^{66}Zn-containing fractions were detected at about 2186 and 993 Da(Fig. 5a). A similar profile can also be observed for samples from Cataluña. The second profile that contains Zn at high abundance is Coimbra. The profile of the samples from Coimbra also shows a high abundance of Zn.

3.3.3.2. HMW (3–70 kDa). Zn is manly associated to com- pounds of MW < 10 kDa. For this reason was considered in the previous section. However, samples from Madrid exhibit a dif- ferent profile with a prominent peak at about 70 kDa (Fig. 5b).

3.3.4. Nickel fractionation profiles

3.3.4.1. LMW (<10 kDa). This element shows highly similar behavior in pine nuts from all the analyzed samples except for Madrid, in which the abundance is clearly higher. For these sam- ples, the most abundant ^{58}Ni-containing peak was in the range 2126–1352 Da. On the other hand, the chromatographic pro- file obtained for the sample from Cádiz was clearly different with a peak at 1570 Da (Fig. 6a). Because of this element is mainly associated to the above mentioned fraction, that was bet- ter resolved with the LMW column, the profiles obtained withthe HMW column are not discussed (Fig. 6b).

4. Conclusions

The multielemental fractionation in pine nuts from *P. pinea* allows a good discrimination between samples from different geographic origins. The use of SEC-UV-ICP-MS represents a powerful tool to distinguish them using the fractionation patterns of elements.

In some cases, the analyzed elements were associated to LMW fractions, indicating the possible linkage of these elements to proteins. Further studies could be focused on the identification and quantification of these metallobiomolecules to their use as markers of the geographic origin of this food. In addition, clas- sification of samples could be performed by applying pattern recognition methods to a set of samples by using the proposed approach.

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