Combined use of total metal content and size fractionation of metal biomolecules to determine the provenance of pine nuts (*Pinus pinea*)

J. L. Gómez-Ariza [*1], A. Arias-Borrego[1], T. García-Barrera[1]

J. L. Gómez-Ariza (corresponding author). [1]Departamento de Química y Ciencias de los Materiales, Facultad de Ciencias Experimentales, Universidad de Huelva, Campus de El Carmen, 21007 Huelva, Spain e-mail: ariza@uhu.es

Abstract Four essential elements (Mn, Ni, Zn, and Cu) and their molecular-size distribution patterns have been determined, for twenty four samples of pine nuts from eight areas in Spain and Portugal (Huelva, Cádiz, Badajoz, Cataluña, Castilla, Madrid, Faro, and Coimbra), by size- exclusion liquid chromatography (SEC) coupled on-line to UV and inductively coupled plasma mass spectrometric (ICP–MS) detection. The variability observed in total element content and the size-exclusion profiles of elements in samples from distant areas were considered as chemical descriptors for characterization of geographic origin. A pattern-recognition technique, the display method principal component analysis, was used as visualization technique to determine the provenance of the pine nuts collected. The results obtained confirmed that size fractionation profiles give more information for assessing the provenance of pine nuts than the total elements composition traditionally used for this purpose. Combination of these chemical descriptors was the most suitable choice for the samples studied.

Keywords Pine nuts · Metals · Multielement molecular-size fractionation · Size-exclusion chromatography· Inductively coupled plasma–mass spectrometry (ICP–MS) · Food provenance

Introduction

Pine nuts (*Pinus pinea*) are a very valuable traditional food with an exquisite flavor and high protein content and are frequently used for preparation of cakes, vegetables, and other foods [1, 2]. The nuts also have important health properties associated with reduction of the risk both of cor- onary heart disease and non-fatal myocardial infarction [2]. The presence of essential elements in this food is critical, because of their important biological roles, the potential connection with pollution, and the effect of soil element composition in forestry areas [3, 4]. The function of many biomolecules and, in particular, metalloproteins, critically depends on their interaction with elements, mainly transi- tion metals [5]. Some proteins (e.g. metallothioneins) are produced under heavy metal stress during pollution episodes, others, for example albumin and transferrin, are transporters of essential nutrient ions; finally, many ele- ments are enzyme activators [5]. In contrast, many small biomolecules that incorporate metallic or non-metallic heteroelements are also crucial for many biological pro- cesses related to cells, body fluids, tissues, and foods [5, 6]. Most of the problems arising in analytical characteriza- tion of these metallomolecules in food are related to their unknown nature [7]; this makes it mandatory to use multidimensional analytical techniques to obtain comple- mentary information (metallomics). The metallomics analytical approach [7, 8] usually involves use of one or several orthogonal chromatographic devices for species separation coupled with a sensitive atomic detector, for

example ICP–MS, that also has multielement capability and is easily interfaced with HPLC (high-performance liquid chromatography) and CE (capillary electrophoresis). Use in parallel of an instrumental technique for elucidation of species structure is also necessary. This combination of elemental and molecular mass spectrometry is an emerging and powerful tool in food and life science [9].

Many workers have studied the total element content of food in relation to nutritional value, toxicity, pollution processes, etc. [3, 4, 10]. In several reports the trace element content has been used as a marker for identification of product's geographical origin and authenticity [11], and several authors have proposed use of the mineral content to characterize the provenance of wines [12, 13], vinegars [14], coffees [15], potatoes [16], honey [17], teas [18], nuts [19], and other foods. Few have considered using metals bonded to biomolecules in foods, especially in nuts, for this purpose, however. Size-exclusion chromatography (SEC) coupled to ICP–MS has been proposed for separation and on-line detection of elements bonded to biomolecules in some nuts [19–21].

In the work discussed in this paper the total Mn, Cu, Ni, and Zn content and the relative abundance of these elements in fractions obtained by SEC have been quantified by use of ICP–MS and the results have been used to determine the provenance of pine nuts collected from eight forestry locations in the Iberian peninsula. These elements were selected because of their important biochemical roles, which are described elsewhere [22]. Briefly, Mn is a cofactor of several enzymes; it can be present in proteins, for example albumin and β_1-globulin, and is required for protein and fat metabolism, healthy nerves, a healthy immune system, and for sugar regulation [23]. Cu can be bound to several proteins (i.e. superoxide dismutase and cytochrome oxidase); it is also needed for transport of iron and is involved in the synthesis of connective tissue, lipid metabolism, and antioxidant pro- tection [19]. Ni is thought to play an important role in folate metabolism [24]. Zn is a constituent in more than 200 enzymes and proteins which participate in all major metabolic processes [25]. Zn also affects protein synthesis via gene expression [19].

Principal component analysis (PCA) has been used as a visualization technique; total element concentrations, the relative abundance of metal-biomolecules in size-exclusion fractions, or a combination of both have been used as chemical descriptors. Likewise, a statistical approach has also been applied to Mn separately, because it is the most abundant element in pine nuts [22].

The results described in this paper show that size fractionation profiles give more information for determi- nation of the provenance of pine nuts than the total element composition traditionally used for this purpose. Combination of these chemical descriptors is also a powerful tool for establishing differences between pine nuts in relation to the presence of elements in unknown samples.

Experimental

Standard solutions and reagents

Standard solutions were prepared by dilution of a multi- element standard (1000 mg l^{-1} in 1 M HNO_3) obtained from Merck (Darmstadt, Germany). Nitric acid (65%) of Supra- pur grade (Merck) was used for the mineralization of the samples. The mobile phase solution was daily prepared at
0.05 M of tris(hydroxymethylaminomethane) (Tris) pre- pared at pH 8.0 from Trizma base and Trizma hydrochlo- ride (Sigma-Aldrich, Steinheim, Germany). Copper, Manganese, Nickel and Zinc stock solutions (1000 mg l^{-1} in 1 M HNO_3) were also obtained from Merck. Ultrapure water (18 MΩ cm) from a Milli-Q Gradient System (Millipore, Watford, UK) was used throughout.

Samples

Samples of pine nuts (*Pinus pinea*) were obtained in different forestry zones from the most important production areas in Spain and Portugal. Twenty-four samples were collected from the Spanish locations (codes in parentheses): Huelva (Hu) and Cádiz (Cad) (southwest), Castilla (Cas) and Madrid (Ma) (center), Badajoz (Ba) (center–west), and Cataluña (Cat) (northeast). Samples from Portugal were collected from the south (Faro, Fa) and from the center to the north (Coimbra, Co). They were supplied by Frutos Secos Puig (Tarragona, Spain). All samples were washed with ultrapure water, ground by use of a conventional grinder Moulinette (Moulinex, Spain) and finally freeze- dried with a benchtop lyophilizer (Hucoa-Erlöss, Spain).

Instrumentation

Elemental analysis was performed with a Hewlett–Packard (USA) HP 4500 ICP–MS containing an ICP source with a plasma shielded torch. This instrument was fitted with a standard Babington nebulizer. The spray chamber was cooled to 2 °C. The

operating conditions were optimized, and are summarized in Table 1. The sensitivity of the ICP– MS instrument was optimized using a multielement standard solution containing 9.9±0.5 ng mL^{-1} Li, 10.01± 0.5 ng mL^{-1}, Y, 9.98±0.5 ng mL^{-1} Ce, and 10.00±0.5 ng mL^{-1} Tl, dissolved in 2% nitric acid.

Element distribution profiles for the pine nuts were determined by size-exclusion chromatography (SEC) with on-line simultaneous elemental detection by ICP–MS. The ICP–MS instrument was connected to the chromatograph by using Tygon capillary tubing to connect the size-exclusion column outlet to the inlet of the nebulizer. A Hiload 26/60 Superdex 30 Prep semi-preparative size-exclusion chromatography (SEC) column was used for separation of mole- cules in the range <10 kDa. An AKTA-Prime system (pump and UV detector at 280 nm) (Amersham), equipped with a 2-mL sample loop, was used as the mobile phase delivery system. The instrument settings used are listed in Table 1.

A Sigma (Spain) model 4-10 centrifuge and a Heidolph (Germany) Unimax 1010 constant orbital shaker operating at 105 rpm were also used, the latter to accelerate the phase- separation process during extraction of the compounds.

Procedures

Evaluation of the total element content of pine nuts

Samples were digested for metal analysis by ICP–MS by a procedure described elsewhere [22]. Briefly, residues (0.2 g, accurately weighed), previously defatted with a chloroform–methanol mixture, were digested in closed PTFE bombs with 10 mL nitric acid (65% *w*/*v*) by use of a domestic microwave oven. Three steps with decreasing energy input—heating at 800 W (3 min), 400 W (3 min), and 100 W (3 min)—were used successively for sample decomposition. Samples were cooled to room temperature for 10 min between each step to avoid overpressure. The final solution was filtered through a 0.20 μm surfactant-free cellulose acetate filter. The elements were measured by ICP–MS (conditions as listed in Table 1).

Metal species distribution patterns by SEC–UV–ICP–MS

Lipid-free pine nut samples were accurately weighted (0.2000 g) in PTFE centrifuge tubes to extract the metal species with 4 mL 0.1 mol L^{-1} sodium hydroxide by mixing for 10 min in a constant orbital shaker. NaOH has been proved to be a better extractant than HCl and hot water for high and low-molecular-weight species. HCl solutions mainly extract LMW compounds, because of the lower solubility of protonated compounds such as proteins, and hot water is used to much less an extent [19, 26, 27].

Element fractionation profiles for the pine nut samples were obtained by size-exclusion chromatography (SEC) using the column described in the section "Instrumentation". All instrumental operating conditions are listed in Table 1. The SEC column was calibrated by use of standards of bovine serum albumin (67,000 Da), metallothionein I (7,000 Da), rat gastrin I (2,126 Da), vitamin B_{12} (1,352 Da), and Gly_6 (360 Da). The standards were dissolved in the mobile phase and their chromatographic profiles were monitored by UV detection. The void volume was determined by use of bovine serum albumin.

Cleaning of SEC columns between consecutive injec- tions was achieved by passage of one column volume of 0.002 mol L^{-1} aqueous EDTA solution and then washing with a half to one column volume of 0.5 mol L^{-1} NaOH, to remove most of the proteins non-specifically adsorbed by the gel [22]. Finally, the column was equilibrated with at least two column volumes of mobile phase, until the UV baseline was stabilized, before injection of the next sample. To test the reproducibility of the SEC column each sample was chromatographed three times.

Chemometrics

Computations were performed by use of the statistical package Statistica version 6.0 (2001) (StatSoft, Tulsa, USA). Principal-components analysis was applied to determine the provenance of pine nuts from different forestry areas.

Results and discussion

Total metal composition and size exclusion fractionation profiles in pine nuts

The element composition of vegetables and fruits can be used to determine their geographical origin [3, 4]. This approach

is, however, “blind” to the metal species present in the sample, which are usually related to intake of essential elements or elimination of toxic elements, and are strongly affected by the conditions in which the plant grows. This loss of information from total metal analysis can be overcome by use of size-exclusion metal profiles, especially when the relative abundance of metals in the different peaks is used for this purpose. This increases the number of features suitable for statistical assessment and the general information obtained.

Table 2 shows the levels of the elements Cu, Zn, Ni, and Mn in twenty-four samples of pine nuts from the eight locations listed above. The variability in the concentrations of some of the elements is very marked, especially for manganese, which is the most abundant. One-way ANOVA revealed that means of these triplicate results were significantly different, ($p < 0.05$).

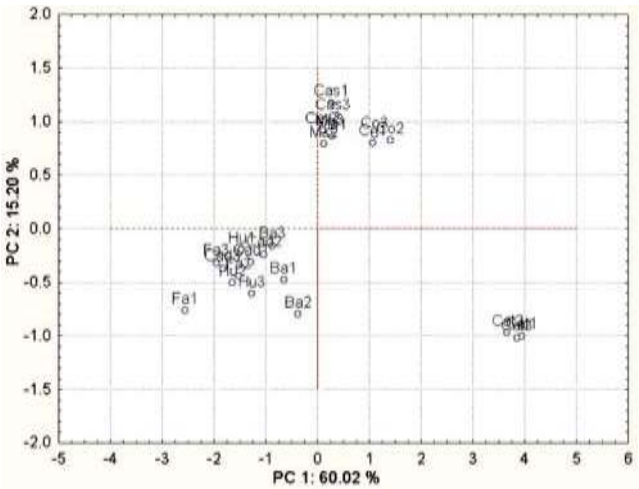


Fig. 1 Results from principal-components analysis for the variable total metal concentration (PC_1 versus PC_2)

The size-fractionation profiles for these elements reveal an important presence of metal species with molecular weights (MW) below 10 kDa. Table 3 shows the relative abundance of the different metal-fractions expressed as peak-area ratio (%) relative to the total area of the chromatogram [19].

Most of the manganese is usually bound to lower-MW species, although the exact distribution depends on sample location. For example, 90% of the Mn in the sample from Coimbra and 80% of that in the sample from Madrid is in the 7 to 10 kDa fraction. The samples from Huelva contain approximately 100% of the manganese in molecules in the range 7 to 2 kDa, whereas 75% of the Mn in Cádiz samples and 60% of that in Badajoz samples is in the 2 to 1.5 kDa fraction. Smaller MW manganese fractions (in the range 1.3 to 0.3 kDa) were detected in a few samples only, for example those from Castilla, in which this fraction contains 95% of this element. In the samples from Faro and Cataluña this element is distributed among the different fraction without any apparent trend (Table 3). For the other elements (Cu, Ni, Zn) several trends are apparent:

1. a low percentage of these elements is observed in the void volume (fraction >10 kDa; 20% and 30% of Zn in the samples from Cádiz and Badajoz, respectively);
2. the abundance of a metal in a particular fraction depends on the metal, thus the amount of Cu in the 10 to 7 kDa fraction ranges from 95% (Huelva) to 50% (Cataluña), the amount of Ni in the 2 to 1.3 kDa fraction ranges from 99% (Cádiz) to 90% (Faro, Coimbra, Badajoz, Cataluña), and the amount of Zn in the 7 to 2 kDa fraction is 95, 80, 60, and 50% for the samples from Castilla, Huelva, Cataluña, and Faro, respectively; and
3. samples from Castilla are the only ones with significant abundance of metals in the LMW fractions—95% of Mn in the 1.3 to 0.3 kDa fraction and 100% of Ni in the same fraction.

Statistical data analysis by principal-components analysis

The variability in the total element content and size- exclusion profiles for the elements in pine nut samples from different forestry areas in the Iberian peninsula (Tables 2 and 3) can be used as descriptors to establish differences between these samples. A pattern recognition technique, principal-components analysis, was used as display method for this purpose.

The data set were analyzed on the basis of the results listed in Tables 2 and 3. Several aspects were considered.

Total metal concentration

When PCA is applied to total element concentrations (Table 2), two principal components (PCs) were extracted. PC1, the first principal component, explained up to 60.02% of the total variance and PC2, the second principal

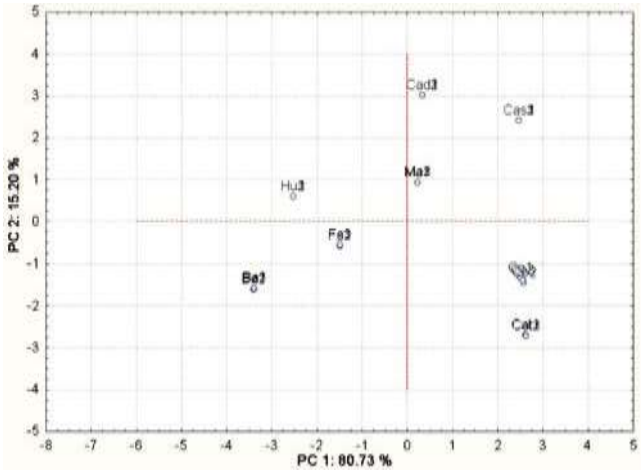
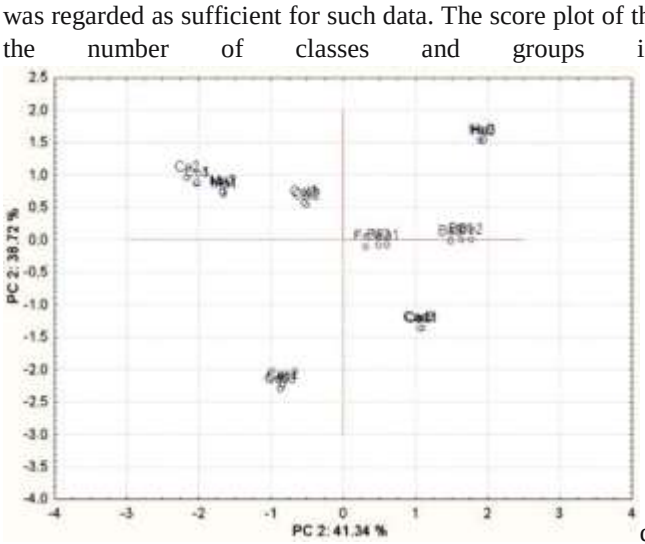
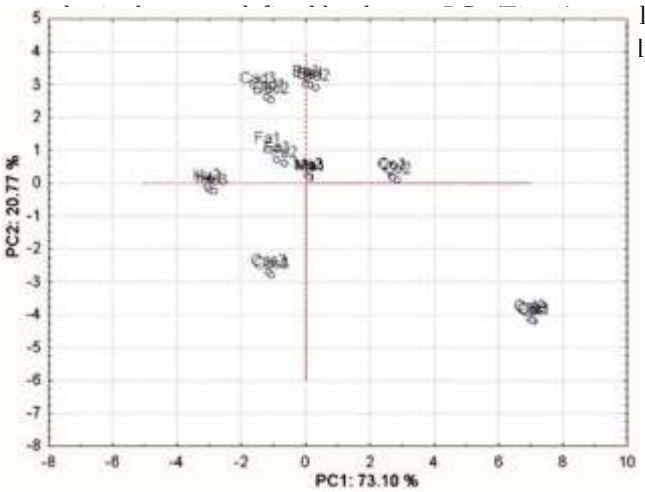


Fig. 2 Results from principal component analysis for the variables total metal concentration and metal molecular size distribution (PC_1 versus PC_2)

component, explained up to 15.20%. The first two principal components thus account for 75.22% of the total variance, which was regarded as sufficient for such data. The score plot of the ls the number of classes and groups is ly





distinction that can be

Fig. 3 Results from principal-components analysis for the variable metal molecular size distribution (PC_1 versus PC_2)

clearly achieved is that between samples from Cataluña (Cat) and the others. The samples from Castilla (Cas), Coimbra (Co), and Madrid (Ma) are in the same group, and this is separated from another group containing the samples from Huelva (Hu), Badajoz (Ba), and Faro (Fa). As shown in Table 4, the dominant variable is Mn in PC1. All the variables appear at negative values in PC1 which suggests they give the same kind of information. In PC2 the dominant variable is Zn, with Mn and Cu very close. In

Fig. 4 Results from principal-components analysis for the variable Mn molecular size distribution (PC_1 versus PC_2)

PC2, Mn and Ni appear at negative values but Cu and Zn appear at positive values.

Total metal concentrations plus metal molecular size distribution

PCA was also applied using both total metal content (Table 2) and metal distribution obtained by SEC-ICP– MS (Table 3) as chemical descriptors. PC1 now explained up to 80.73% of the total variance and PC2 up to 15.20%. Thus both components account for 95.93% of the total variance, much higher than that in Fig. 1. The score plot enables very clear recognition of the samples according to their forestry area location (Fig. 2). The loadings of the variables used in this section are shown in Table 5. The most important variable for PC1 is Mn fraction in the molecular weight range 10,000 to 7,000 Da, followed by Ni (2,126- 1,352 Da) and, very closely, by Mn (2,126–1,352 Da). In

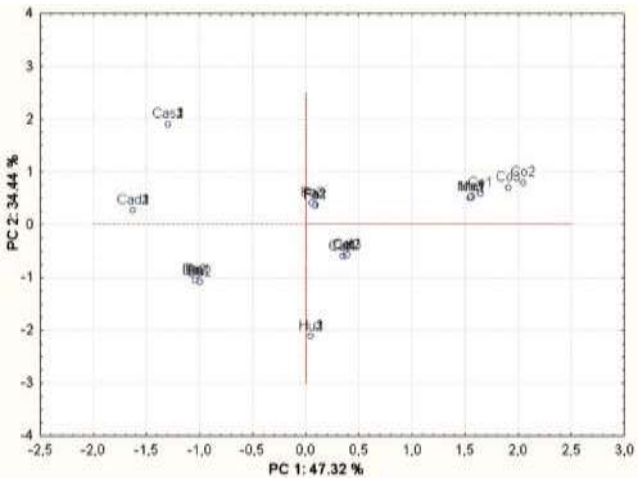


Fig. 5 Results from principal-components analysis for the variable total Mn concentration and Mn molecular size distribution (PC_1 versus PC_2)

PC2 the variables with higher loadings are different; the Zn fractions (7,000–2,126 Da) and (>10,000 Da) should be noted; the former is the only one with a negative sign.

Metal molecular size distribution

When PCA was applied to data from Table 3 only the origin of the samples could again be unequivocally recognized (Fig. 3). PC1 explained 73.10% of the total variance and PC2 up to 20.77%. Both components accounted for 93.87%. The loadings of the variables are collected in Table 6, in which we draw attention to PC1 for the molecular weight distributions Cu (10000 to 7000 Da), Cu (7000 to 2126 Da), and Mn (10000 to 7000 Da). It is important to remark that the most important variable has a negative sign relative to the other two variables. In PC2 the most important variable is Zn (>10000 Da), followed by Zn (10000 to 7000 Da), and Mn (2126 to 1352 Da); in this instance no differences between the loadings were observed. It is possibly the important differences between the main molecular size fractions in which the elements are present (especially Mn) that explain the exploratory power of this approach. For this reason a further study based on this aspect was accomplished.

Pattern recognition based on manganese data

Inspection of the results for Mn in Tables 2 and 3 shows that concentration of this element is most variable. Figures 4 and 5 show the results obtained by using, respectively, Mn molecular size distribution and the combination of this with element content as chemical descriptors. In this case, similarly to the results obtained for all the metals, combined use of Mn molecular size distribution and manganese concentration data enables better explanation of the total variance—81.76% compared with 80.06%. Although we cannot conclude that use of manganese data only is better than those from all the metals, it is evident that the variance explained by this element is high enough to be regarded as a simple option for this kind of study.

On the basis of these findings the best choice for determination of the provenance of pine nuts is the combined use of total element concentrations and their multielement fractionation profiles obtained by SEC. As we can conclude from PCA, the three cases mentioned above can be used because the variance of the data explained by use of all of them is high. Combination of both chemical descriptors gives the best results, however. The advantage of using only one chemical descriptor is that sample

throughput is higher, because these approaches can be used with few data only. In studies in which the classes are very similar or other elements must be considered, however, combined use of the chemical descriptors might be mandatory.

Conclusions

Results from multielement fractionation and total element analysis for *P. pinea* nuts are good chemical descriptors for differentiating samples of different geographic origin. This has been confirmed by applying unsupervised pattern recognition techniques to samples from several parts of Spain and Portugal. Several procedures were tried but the best for determination of the provenance of pine nuts was the combined use of total element concentrations and their multielement fractionation profiles. SEC–UV–ICP–MS is a powerful tool for distinguishing the provenance of these of samples from element fractionation patterns. Mn was the most abundant element in the samples; for this reason it was studied separately. Results obtained showed that this element explained a higher percentage of the total variance in the PCA. Use of Mn as a chemical descriptor is, therefore, sufficient to determine the provenance of the samples; this is a simpler alternative to considering all the elements, although better results are obtained by the latter procedure.

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Tables

Table 1 Operating conditions for the ICP–MS and SEC Conditions

SEC conditions	
Column	Hiload 26/60 Superdex 30 Prep
Resolution range	Mr < 10,000 Da
Mobile phase	Tris 50 mmol L^{-1} (pH 8.0)
Flow rate	2 mL min^{-1}
Injection volume	2 mL
UV–visible 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

Table 2 Total element concentrations[a] (μg g^{-1}) in the pine nut samples analyzed in this work. [a] Average from determination in triplicate ± SD

Code	Mn (μg g^{-1})	Ni (μg g^{-1})	Cu (μg g^{-1})	Zn (μg g^{-1})
Hu1	500±21	9.2±0.8	41±10	107±19
Hu2	512±23	11±1	39±11	106±17
Hu3	513±26	10.7±0.4	35±4	105±30
Co1	65±6	4.5±0.2	31±6	86±17
Co2	64±9	3.5±0.3	29±2	85±10
Co3	67±10	4.72±0.06	30±5	90±18
Cat1	26±7	2.1±0.3	8±1	25±9
Cat2	28±5	3.05±0.02	8±2	30±12
Cat3	25±4	2.6±0.2	8±1	26±10
Ba1	458±23	8±2	35±9	94±20
Ba2	498±20	7±1	30±10	93±10
Ba3	399±12	9±1	36±8	102±24
Ma1	87±6	7.1±0.2	34±12	97±8
Ma2	90±9	8±1	33±15	100±16
Ma3	88±10	7.8±0.5	34±9	98±10
Fa1	559±38	15±5	41±11	113±30
Fa2	523±35	10±4	38±16	109±20
Fa3	450±17	13±3	40±6	111±28
Cas1	72±9	6.8±0.2	35±9	104±24
Cas2	70±9	8±2	34±20	101±19
Cas3	73±8	6.5±0.7	33±9	104±22
Cad1	414±15	11±1	38±10	101±19
Cad2	398±11	10±1	37±18	100±47
Cad3	400±13	14±2	39±16	106±38
LOD	0.023	0.032	0.018	0.037
LOQ	0.077	0.107	0.060	0.123

Table 3 Molecular size distribution of Mn, Cu, Ni, and Zn in pine nut samples from different forestry areas (n=5)

Pine nut sample	Mn area (%)					Cu area (%)					Ni area (%)					Zn area (%)				
Fraction number[a]	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Cad	–	–	–	75	25	7	70	2.5	0.5	20	0.3	–	–	99	0.7	20	50	10	15	–
Cas	–	–	5	–	95	–	90	–	–	10	–	–	–	–	100	–	4	95	1	–
Fa	–	30	30	5	35	10	70	10	2	8	5	–	–	90	5	–	7	50	40	3
Hu	–	–	100	–	–	–	95	–	–	5	–	–	–	97	3	7	10	80	3	–
Co	–	90	7	2	–	4	60	14	2	20	2.5	2.5	–	90	5	3	–	2	95	–
Ba	–	–	40	60	–	10	70	10	2	8	–	3	–	90	7	30	20	5	45	–
Cat	–	35	40	25	–	–	50	20	5	35	–	2	–	90	7	–	20	60	15	5
Ma	–	80	10	10	–	–	80	5	–	15	–	2	5	90	3	10	30	30	30	–

[a] 1, >10 kDa MW fraction; 2, 10–7 kDa MW fraction; 3, 7000–2126 Da MW fraction; 4, 2126–1352 Da MW fraction; 5, 1352–360 Da MW fraction

Results for the different locations (i.e. Hu1, Hu2, Hu3) were averaged in this table

Table 4 Loadings of variables for the two PCs for the variable total metal concentration (Mn, Ni, Cu, Zn) for the eight locations studied

Variable	PC 1	PC 2
Mn	−0.752283	−0.4077
Ni	−0.511151	−0.2885
Cu	−0.524581	0.4027
Zn	−0.508900	0.5036

Table 5 Loadings of variables for the two PCs for the variables total metal concentrations and metal molecular size distribution for the eight locations studied

Variable	PC 1	PC 2
Mn	−0.546528	0.278990
Ni	−0.253440	0.212282
Cu	−0.304001	0.176694
Zn	−0.311537	0.141990
Mn-2[a]	0.701249	−0.018272
Mn-3	−0.053752	0.012429
Mn-4	0.632007	0.292407
Mn-5	−0.500589	−0.248593
Cu-1	−0.020142	0.324850
Cu-2	−0.629966	−0.084319
Cu-3	0.342646	0.012457
Cu-4	0.315120	−0.027215
Cu-5	0.403742	−0.085166
Ni-1	0.019330	0.070549
Ni-2	0.451885	0.105636
Ni-3	0.005847	0.013181
Ni-4	0.643977	0.328712
Ni-5	−0.153474	−0.325539
Zn-1	−0.051018	0.340468
Zn-2	0.002641	0.223551
Zn-3	−0.139560	−0.349636
Zn-4	0.167773	0.158392
Zn-5	0.224124	−0.116536

[a] 1, >10 kDa MW fraction; 2, 10–7 kDa MW fraction; 3, 7000–2126 Da MW fraction; 4, 2126–1352 Da MW fraction; 5, 1352–360 Da MW fraction

Table 6 Loadings of variables for the two PCs for metal molecular size distribution for the eight locations studied

Variable	PC 1	PC 2
Mn-2[a]	0.769290	−0.359546
Mn-3	−0.133723	0.001057
Mn-4	0.359382	0.719853
Mn-5	−0.750536	−0.237565
Cu-1	0.374029	0.396235
Cu-2	−0.879589	0.293333
Cu-3	0.831778	−0.496411
Cu-4	0.699385	−0.511417
Cu-5	0.580446	−0.365502
Ni-1	0.229420	−0.373777
Ni-2	0.702575	0.024913
Ni-3	0.035321	0.180029
Ni-4	0.731124	0.338782
Ni-5	−0.754329	−0.312908
Zn-1	0.231987	0.906299
Zn-2	0.213465	0.688338
Zn-3	−0.752777	−0.503984
Zn-4	0.645059	−0.104193
Zn-5	0.392631	−0.642856

[a] 1, >10 kDa MW fraction; 2, 10–7 kDa MW fraction; 3, 7000–2126 Da MW fraction; 4, 2126–1352 Da MW fraction; 5, 1352– 360 Da MW fraction