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Multielemental Analysis and Classification of Amaranth Seeds According to Their Botanical Origin

Elba G. Aguilar,[†] Miguel A. Cantarelli,[‡,§] Eduardo J. Marchevsky,[†,‡] Nora L. Escudero,[†] and José M. Camiña*[,§,∥]

[†]Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera (5700) San Luis, Argentina
[‡]Instituto de Química de San Luis (INQUISAL), Chacabuco y Pedernera (5700) San Luis, Argentina
[§]Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151 (6300) Santa Rosa, La Pampa, Argentina
[∥]Instituto de las Ciencias de la Tierra y Ambientales (INCITAP), Av. Uruguay 151 (6300) Santa Rosa, La Pampa, Argentina

ABSTRACT: The characterization of amaranth seeds (*Amaranthus* spp.) was developed for *Amaranthus hypochondriacus*, *Amaranthus cruentus*, and *Amaranthus dubius*. The elemental concentrations were determined by inductively coupled plasma optic atomic spectroscopy. Pattern recognition methods were used for the characterization of seed samples: nonsupervised methods included principal components analysis and cluster analysis; supervised methods were linear discriminant analysis and partial least squares discriminant analysis (PLS-DA). Informed are the concentrations of the following elements: Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, V, Zn, and Zr. The lowest mineral content was found in *A. hypochondriacus*, and the highest one was found in *A. dubius*. For the classification, selected variables for all multivariate methods were Ba, Cr, Li, Mn, Ni, S, and Sr. Nonsupervised methods allowed us to distinguish between the three species of amaranth; however, PLS-DA supervised methods showed the best prediction ability.

KEYWORDS: Amaranth, seeds, *A. hypochondriacus*, *A. cruentus*, *A. dubius*, ICP-OES, multivariate, pattern recognition

INTRODUCTION

Amaranth (*Amaranthus* spp.) is a plant that was used by pre-Spanish original populations of America. After the Spanish conquest, the consumption of this crop decreased.[1] During the 1960s and 1970s, amaranth was reconsidered as a food due to its high level of protein and its amino acid profile. Recently, it has been proved that amaranth seeds contain proteins whose peptides have several biological functions, such as *Amaranthus hypochondriacus*, which has lunasin, a peptide with anticarcinogenic activity.[2] In other works,[3−8] numerous unknown properties of amaranth have been reported, including the ability of this plant to bioaccumulate metals in contaminated soils. In this way, the content of Cd in amaranth, in both aerial and root parts of the plant, was reported. It was also discovered that the root of *Amaranthus hybridus*, *Amaranthus spinosus*, *Amaranthus viridis*, and *Amaranthus rudis* can accumulate from 50.8 to 73.9 mg/kg of Cd, which can be useful for phytoremediation in polluted zones.[3] Similar conclusions were obtained by other authors.[4−6] On the other hand, there are only a few reports about the elemental content in amaranth from noncontaminated soil: iron, lead, and zinc concentrations were studied in amaranth leaves from Kenya,[7] and the contents of several metals, such as Al, Fe, Ni, Cr, Pb, etc., were determined in amaranth leaves from South Africa.[8] However, the elemental content of amaranth edible seeds has not been previously reported.

On the other hand, the chemometric classification made use of multivariate calibration methods,[9,10] which are divided in nonsupervised and supervised methods. Nonsupervised methods involve the use of multivariate models whose prediction ability can not be confirmed; however, they can be useful as a previous step to the use of a supervised method. The nonsupervised methods most frequently used are the following:

(a) Principal components analysis (PCA), in which the original data are reduced to a few new variables, known as principal components (PCs). This approach permits one to detect several characteristics that cannot be seen in the original data, which can help to identify the differences within the original data and to obtain groups or families.
(b) Cluster analysis (CA), which is based on the ability to establish relationships between data, using different criteria of similitude. The use of amalgamation and distance criteria allows for the construction of a tree plot or dendogram, which shows the possible groups and their differences.

The supervised methods included in this work were as follows:

(a) Linear discriminant analysis (LDA): It helps to confirm the classification made through PCA or CA as unsupervised methods. The LDA method constructs a model based on discriminant equations, which can mathematically classify different groups within the samples, producing a table that shows the grade of fit of a model based on the evaluation of the samples known in the prediction step, as well as graphical classification through the canonical functions score plot.

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(b) Partial least-squares discriminant analysis (PLS-DA): This works by building a PLS multivariate calibration model by means of partial least-squares regression, which is then used to predict unknown samples. As LDA, when samples are known, it is possible to obtain the grade of fit of a model, and it offers the possibility to obtain a graphical classification by means of a PLS score plot.

Recently, these multivariate classification methods were successfully used for the classification of different samples based on their multielemental content, that is, among others, in propolis,[11] herbs,[12] and honeys.[13−15] Because of the scarce information about the elemental content of edible amaranth seeds and the importance of using multivariate methods for the classification of raw materials, this paper discusses the mineral composition of *A. hypochondriacus* (*H*), *A. cruentus* (*C*), and *A. dubius* (*D*), including the concentration of Ag, Al, Ba, Ca, Co, Cr, Cu, Fe, K, La, Li, Mg, Mn, Mo, Na, Ni, P, S, Sr, V, Zn, and Zr, which were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES). On the other hand, classification of three plants was obtained by means of the multivariate PCA, CA, LDA, and PLS-DA methods.

MATERIALS AND METHODS

Seed Sampling. Twenty-five seed samples from each species of amaranth (*H*, *C*, and *D*) were obtained from the same nonpolluted agricultural region of La Pampa province (Argentina) from November 2009 to March 2010. The botanical characterization of seeds was carried out by an expert botanist.

Reagents. Nitric and hydrochloric ultrapure acids were acquired from Sigma (St. Louis, MO). All standard solutions were prepared using reagents of spectroscopic grade supplied by Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was used to prepare all standard and samples solutions.

Apparatus. Mineralization was carried out using an Anton Paar MW3000 microwave system (Graz, Austria). Elemental analysis was performed through the use of a Varian ICP-OES model ICP-OES Vista Pro (Palo Alto, CA) with a Czerny-Turner monocromator, holographic diffraction grid, and a VistaChip charge coupled device (CCD) array detector. Ultrapure water was obtained using a Barnstead Easy-Pure RF compact water system (Dubuque, IA).

Preparation of Seed Samples. Ten grams of each of the three plants' seed samples was individually ground and passed through a sieve (0.50 mm diameter). Two grams of the obtained powder was transferred into a microwave vessel and mixed with 6 mL of HNO_3 and 1 mL of HCl; then, 5 mL of indium solution (500 mg L^{-1}) was added as an internal standard to evaluate the degree of recovery in the mineralization step. The mixture was mineralized at 230 °C during 20 min, according to the list of applications provided by the manufacturer. Later, the clean solution was transferred to a 50 mL volumetric flask and completed to mark with deonized water.[16,17]

Analytical Procedure. The concentrations of the 22 elements and internal standard were determined by direct nebulization. Twenty-five seed samples from each amaranth species were analyzed in triplicate. Calibration models for each element were obtained using five different concentration levels in triplicate. The regression coefficient (r^2) values of such straight ranged from 0.989 to 0.999.

Multivariate Analysis Software and Statistical Analysis. To obtain the PCA and PLS-DA models, The Unscrambler 6.11 software package (Throndheim, Norway) was used. CA and the LDA model were carried out using the InfoStat software package (Córdoba, Argentina). For each element, the concentration and standard deviation were obtained on the basis of 25 samples. On the other hand, every sample was analyzed in triplicate to obtain reliability of results from each seed sample.

Table 1. Composition of 22 Elements in *A. dubius*, *A. cruentus*, and *A. hypochondriacus*

	A. dubius[a]	*A. cruentus*[a]	*A. hypochondriacus*[a]
Ag	0.11 ± 0.03	0.04 ± 0.02	0.10 ± 0.03
Al	191 ± 67	689 ± 67	525 ± 53
Ba	21.7 ± 8.5	15.6 ± 1.85	8.34 ± 0.96
Ca	14600 ± 3200	2910 ± 150	4670 ± 140
Co	0.78 ± 0.28	0.24 ± 0.09	0.16 ± 0.06
Cr	9.79 ± 1.60	1.52 ± 0.81	1.52 ± 0.93
Cu	41.9 ± 6.9	13.1 ± 6.2	143 ± 95
Fe	8690 ± 770	253 ± 85	196 ± 69
K	22100 ± 9200	4320 ± 890	7110 ± 630
La	0.31 ± 0.18	0.08 ± 0.02	0.09 ± 0.06
Li	68.3 ± 0.6	8.55 ± 0.11	23.8 ± 2.8
Mg	12900 ± 1500	3070 ± 410	6790 ± 530
Mn	186 ± 24	51 ± 4	95.5 ± 4.5
Mo	3.50 ± 1.20	0.87 ± 0.64	1.01 ± 0.37
Na	603 ± 60	168 ± 53	169 ± 51
Ni	8.60 ± 1.57	17.1 ± 4.5	4.05 ± 0.10
P	20600 ± 6900	4100 ± 750	7210 ± 1090
S	1420 ± 80	464 ± 45	74.1 ± 7.2
Sr	33.7 ± 6.28	8.69 ± 1.23	15.5 ± 1.56
V	0.53 ± 0.14	0.16 ± 0.03	ND
Zn	129 ± 28	108 ± 25	51.5 ± 5.2
Zr	0.49 ± 0.22	0.12 ± 0.03	0.55 ± 0.10

[a] Expressed in μg g^{-1} of dried seed. Mean ± standard deviation (n = 25).

RESULTS AND DISCUSSION

Validation and Recovery. Mineralization is a critical step in the analysis, due to the possibility of analytes loss during the process; in this case, the method of standard addition is considered as an adequate validation method.[18,19] In a recovery test, a known amount of standard is added to the sample, and the analysis is performed after addition so that the amount recovered can be calculated.[20] The addition of indium as internal standard was used to evaluate the recovery percentage in the mineralization step.[21] In the present work, known quantities of indium were added prior to the microwave mineralization. After that, indium was analyzed as another analyte by ICP-OES, obtaining a mean recovery value of 96.8 ± 4.2% (n = 75), similar to results previously reported.[16]

Mineral Composition Analysis. Table 1 shows the results of the mineral analysis obtained for the three amaranth species. Potassium was the most abundant element in *D*, and its concentration was similar to the one previously reported for Hungarian plants with a mean of 16276 μg g^{-1};[22] however, for Ca, Mg, P, and S, the concentrations in *D* were higher (mean: 6281, 1472, 2730, and 2085 μg g^{-1}, respectively). The table shows the presence of Cr in *H*, *C*, and *D*: The concentration of this element was lower than that of the standard accepted limit in food of 120 μg g^{-1}.[23] Also, the concentrations of Cr were lower in comparison with other plants from Egypt, with a reported mean of 16.01 μg g^{-1},[16] but similar to those found in Hungarian plants with 3.47 μg g^{-1}.[22] The accepted limits of Zn, Ni, Cu, and Fe in edible plants are 60, 10, 40, and 45 μg g^{-1}, respectively.[17]

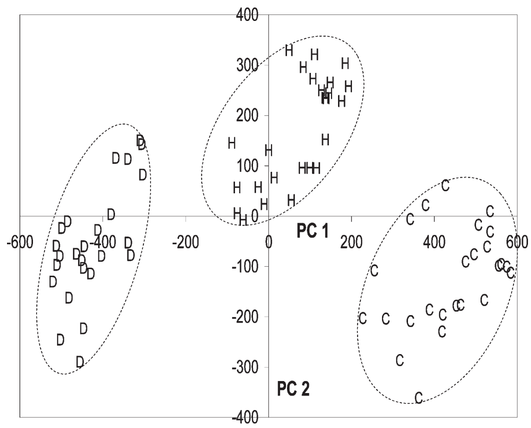

Figure 1. PCA 2D score plot for 75 samples of amaranth species: *A. cruentus* (*C*), *A. hypochondriacus* (*H*), and *A. dubius* (*D*).

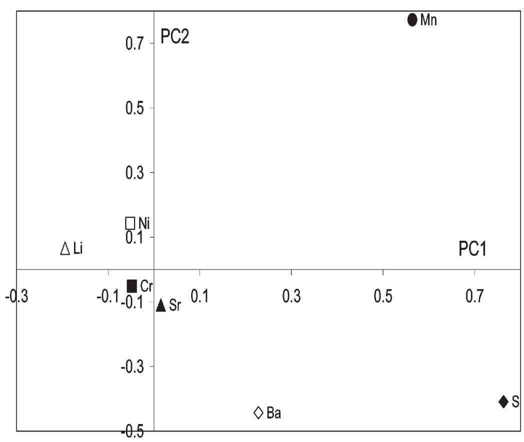

Figure 2. Loading plot showing the influence of elements in the PCA model.

The concentrations of Fe found in the three amaranth species were higher than the accepted limit; however, the contents of Fe and Zn in *H* were similar in comparison to amaranth plants from Kenya (Fe = 180 and Zn = 55 μg g^{-1}), indicating that amaranth can contribute to the provision of micronutrients.[7] In *D*, the Zn concentration was twice that of the accepted limit, while the Cu concentration was close to the limit. The Ni concentration was higher than the accepted limit only in *C*.

Nonessential elements (Al, Ba, and Sr) were found in the three amaranth species. The concentration of Al, Ba, and Sr in other plants, such as herbal tea, has been reported,[24] whose ranges were higher than those found in this study (mean: 1124, 59.65, and 60.29 μg g^{-1}, respectively). On the other hand, the Al concentration was high in *H* and *C* in comparison to red and green leaf amaranth from South Africa[8] (mean: 263 μg g^{-1}). The concentration of Co found in *D* was three times higher than that reported previously for plants from Thailand (mean: 0.260 μg g^{-1})[24] and Turkey (mean: 0.23 μg g^{-1}),[21] but these values are similar to values in *H* and *C* in this work. The concentration of V in *D* and *H* was lower than that found in plants from Argentina and Thailand (mean: 1.29 and 0.84 mg/kg, respectively).[19,24] The concentration of La in *D* was similar to that in other plants from Turkey (mean: 0.37 μg g^{-1})[21] but lower than in *H* and *C*. The concentration of Mo in *D* was 70 times higher than that reported for herb plants (mean: 0.05 μg g^{-1}, respectively); in *C*, it was 20 times higher and 17 times higher in *H*.[22]

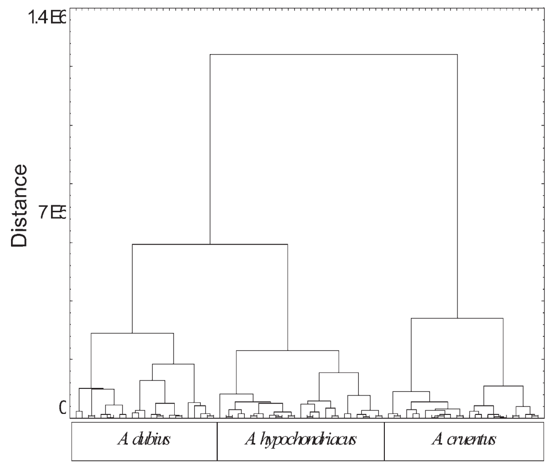

Figure 3. Dendogram plot of amaranth species samples.

Table 2. Results of Classification Obtained by LDA

training	C^a	H^b	D^c	total	correct (%)
C	20	0	0	20	100
H	0	19	1	20	95
D	0	0	20	20	100
prediction	C^a	H^b	D^c	total	correct (%)
C	5	0	0	5	100
H	0	5	0	5	100
D	0	0	5	5	100

[a] *A. cruentus*. [b] *A. hypochondriacus*. [c] *A. dubuis*.

Classification of Amaranth Seeds According to Their Botanic Origin. *PCA.* The definitive PCA model was obtained using five PCs, which can explain 99.3% of the original information on the variables. As it can be seen in Figure 1, which shows the score plot, there are three groups of samples: the *C* group is on the right, the *H* group is in the middle, and the *D* group is on the left. The original variables used in the definitive model were Ba, Cr, Li, Mn, Ni, S, and Sr due to their high influence in comparison to the other elements. Figure 2 shows a loading plot, which explains the influence of the selected variables onto the PCA model. S has the highest influence on PC1 and PC2, followed by Mn and Ba; the rest of the elements (Cr, Li, and Ni) had a minor influence in both PCs. Comparing the score plot (Figure 1) with the loading plot (Figure 2), the *C* group is influenced principally by S and Ba; the *H* group is influenced by Li, Ni, Cr, and Sr, and the *D* group has a negative influence from S and Mn in PC1 and a positive influence of Ba in PC2.

CA. CA was carried out using the same variables that were used in the PCA model. The amalgamation criterion was complete linkage, and the selected distance was the Euclidean one, with which the best results were obtained.[12] Figure 3 shows the dendogram obtained by CA for the classification of amaranth. Seed samples were grouped in the three species: *D*, *H*, and *C*. Also, the dendogram shows that the *C* group is different from the *H* and *D* ones: This situation cannot be seen in Figure 1, which indicates similarities between *H* and *D*, adding new information to the multivariate study.

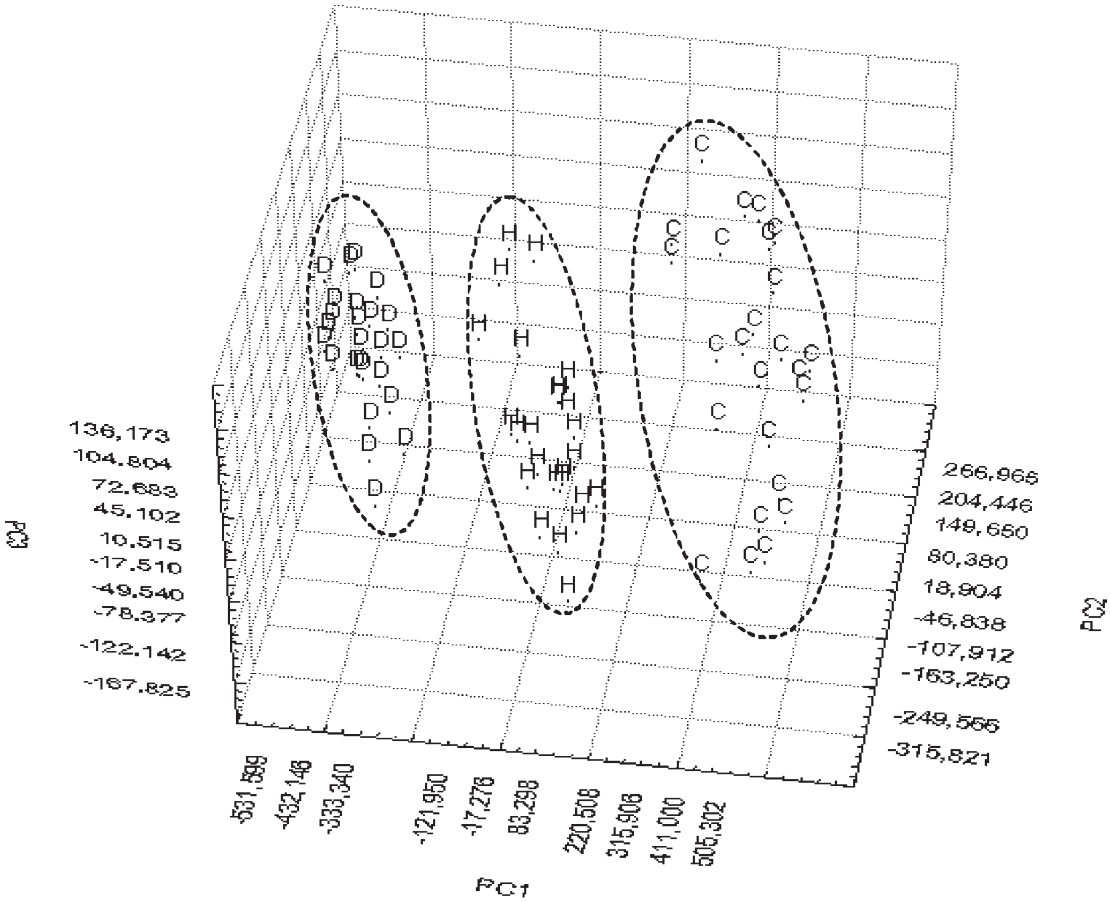

Figure 4. PLS-DA 3D score plot for 75 samples of amaranth species: *A. cruentus* (*C*), *A. hypochondriacus* (*H*), and *A. dubius* (*D*).

LDA. Once again, Ba, Cr, Li, Mn, Ni, S, and Sr were used as variables to confirm the results obtained by means of the PCA and CA. The LDA classification of amaranth is shown in Table 2. For the training step, 20 random samples of each species were used, while for the prediction step, five samples of each species were used. All samples were predicted for the three species in the correct form with one error in the training steps but without error in the prediction step. The canonical discriminant function[10] obtained using the variables above-mentioned is shown in the following equation:

$$D = 1.2 \times 10^{2} - 2.4 \times 10^{-3}\ \mathrm{Ba} + 1.0 \times 10^{-2}\ \mathrm{Cr} + 2.5 \times 10^{-3}\ \mathrm{Li} - 1.3 \times 10^{-3}\ \mathrm{Mn} - 9.5 \times 10^{-4}\ \mathrm{Ni} + 1.7 \times 10^{-3}\ \mathrm{S} + 4.4 \times 10^{-3}\ \mathrm{Sr}$$

PLS-DA. PLS is an important multivariate tool, which was used as a calibration tool some years ago. More recently, PLS has had new applications, for example, as a classification tool, by means of PLS-DA, from which a supervised model is obtained to classify groups from different categories. Also, the method gives the possibility to obtain graphical classifications as those obtained by PCA, as well as to assess the predictive ability as LDA.[25]

In the present work, PLS-DA was used as supervised discriminant analysis. For the selection of variables, the PCA served as a variable preselecting tool.[26] The PLS model was obtained using only three PLS components, which had 98.6% of the original information in the calibration step. To build the model, a cross-validation method was used, in which one sample is left out to build the model, and then, this sample is used to obtain the prediction. When all samples are left out, the root-mean-square of error prediction (RMSEP) can be obtained, whose value was low (0.16) for this model.

In Figure 4, the 3D classification score plot obtained with this model can be seen, where the three species of the plant are distinguished. *D*, *H*, and *C* samples are enclosed into three ellipses, and they are separated by the first PC, which is strongly influenced by Mn, S, and Ba as in the PCA model: These elements were the principal basis for the classification. On the other hand, different grades of dispersion on each species can be perceived as follows: *D* being the lowest and *C* the highest. The dispersion occurs on the third PC, which is also strongly influenced by Mn, S, and Ba. From this plot, it can be determined that the dispersion of every amaranth species could be associated to the botanical origin, since the samples had the same geographical origin.

After that, a PLS-DA table was obtained by the use of 20 random samples of every species in the calibration set and five samples used in the test set. Table 3 shows the results of the PLS-DA classification, where 100% of correct classification was obtained in the calibration (training) and validation (prediction) test sets.

The present work represents a contribution to the field, regarding the elemental composition in seeds of three amaranth species: *A. cruentus*, *A. hypochondriacus*, and *A. dubius*, which were consumed in South America by pre-Spanish populations and

Table 3. Results Obtained by PLS-DA

calibration	C[a]	H[b]	D[c]	total	correct (%)
C	20	0	0	20	100
H	0	20	0	20	100
D	0	0	20	20	100
validation	C[a]	H[b]	D[c]	total	correct (%)
C	5	0	0	5	100
H	0	5	0	5	100
D	0	0	5	5	100

[a] *A. cruentus*. [b] *A. hypochondriacus*. [c] *A. dubuis*.

then extended to the rest of the population. In general, the high content of several elements found in amaranth seeds was in agreement with previous studies. *A. dubuis* had the highest mineral content, while *A. hypochondriacus* had the lowest. From the point of view of multivariate classification methods, nonsupervised methods such as PCA and CA showed good ability as graphical classification tools and were useful as variable preselection tools. However, the PLS-DA supervised method showed better ability for graphical and discriminant analysis; in this respect, LDA had one mistake in comparison to PLS-DA. On the basis of the results shown and compared to the other multivariate methods, PLS-DA was the best tool for the botanical classification of amaranth species. For these reasons, the reported method can be applied in the quality control of seeds to evaluate the botanical origin of seed raw materials of amaranth in the food industry. Because this plant has the ability of preconcentrating several elements, new studies about it must be performed to evaluate the influence of geographical origin or other effects in the composition of seeds in nonpolluted soils, as well as its consequence on the proposed models.

AUTHOR INFORMATION

Corresponding Author

*Tel: +54-2954-425166. Fax: +54-2954-432535. E-mail: jcaminia@yahoo.com or jcaminia@gmail.com.

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