



## Analytical Methods

# Analysis of trace elements in multifloral Argentine honeys and their classification according to provenance

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## ARTICLE INFO

## Article history:

Received 14 April 2009

Received in revised form 8 December 2011

Accepted 19 February 2012

Available online 25 February 2012

## Keywords:

Pattern recognition

Discriminant analysis

Fingerprints

Honey

## ABSTRACT

The concentrations of 14 trace elements (Br, Ce, Co, Cr, Cs, Eu, Fe, La, Rb, Sb, Sc, Sm, Th and Zn) have been determined in 120 samples of light-coloured honeys from the provinces San Luis and La Pampa, middle Argentina, using instrumental neutron activation analysis. The elemental composition was used in multivariate statistical analysis to discriminate the honeys according to geographical origin. Results indicated that element analysis provides a good prospect for discriminating honeys by regions, even if the element composition is not dependent on the year of harvest. Eight key variables (Ce, Cr, Cs, Fe, La, Sb, Sc and Zn) were identified by LDA as providing the maximum discrimination between samples according to their provenance. It was concluded that the methodology in its current state can be used to provide reliable origin information.

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## 1. Introduction

Bee-honey plays a very important role in the economy of several countries and among these Argentina is one of the most renowned exporters worldwide. The two main features of natural honeys from this country are quality and geographical origin. In addition, Argentine honeys count with many comparative and competitive advantages, some of them are important flower offer, variety of climates for production, vast uncontaminated fields, good quality of honey and beehive products (Vázquez, 2007). The authenticity determination of honeys is necessary since certain countries have strict compositional standards that must be applied for labelling honeys according to their contents (Anklam, 1998).

Generally, honey composition is closely associated with its botanical origin and, to some extent, also the geographical area in which it originated because soil and climate characteristics determine melliferous floral (Rashed & Soltan, 2004). Therefore, trace element (including rare earth elements, or lanthanides) contents in honey samples could give an indication of environmental pollution and herewith also an indication of the geographical origin of honey (Ajtony, Bencs, Haraszi, Szigeti, & Szoboszlai, 2007; Conti, Stripeikis, Campanella, Cucina, & Tudino, 2007; Terrab, Hernanz, & Heredia, 2004). Chemometric techniques appear to be the most powerful tools for characterise and classifying honeys

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and other bee-products by geographical source (Berrueta, Alonso-Salces, & Heberger, 2007; Cantarelli, Camiña, Pettenati, Marchevsky, & Pellerano, 2011; Chudzinska & Baralkiewicz, 2011; Kelly, Heaton, & Hoogewerff, 2005; Popek, 2002).

Instrumental neutron activation analysis (INAA) generally meets these requirements, thus this technique has been applied for the quantitative determination of mineral composition in Turkish (Sevimli, Bayulgen, & Varinlioglu, 1992) and Portuguese honey samples (Almeida-Silva et al., 2011). It could be considered as a powerful tool, not only because of its well-known high sensitivity, but also because it allows multielement analysis for each single sample of a complex matrix. In addition INAA is currently a method of choice in trace analysis in environmental and food matrices (Galinha et al., 2011; Orvini, Speziali, Salvini, & Herborg, 2000; Singh & Garg, 2006), including characterisation and certification of standard reference materials SRMs or certified reference materials CRMs (Zeisler et al., 2006). Besides several nuclear properties of lanthanides, such as large thermal neutron cross-section and abundance of radionuclides with unique gamma radiation of convenient half-life, make them excellent candidates for analysis by INAA.

Mineral composition of honey has been the subject of many studies. Absorption and emission spectrometries (Pohl, 2009), X-ray fluorescence spectroscopy (Golob, Dobersek, Kump, & Necemer, 2005), inductively coupled plasma atomic emission (Camiña et al., 2008; Ioannidou, Zachariadis, Anthemidis, & Stratis, 2005) and inductively coupled plasma-mass spectrometry (Chudzinska & Baralkiewicz, 2010; Madejczyk & Baralkiewicz, 2008) are commonly used. But honeys are particularly difficult to

analyse for their trace metal contents since some of them are present at low concentrations. Also sample preparation methods prior to analysis can have large effects on the results. In general wet, dry or microwave digestions are required in order to eliminate the organic matrix follow by a dissolution step with acids, it could be consider an important source of troubles. INAA does not require sample dissolution and depends on the nuclear rather than atomic properties of the element of interest so that any sources of uncertainty or bias in INAA are independent of those associated with most other techniques of chemical analysis.

Finally, the purpose of the present study was to characterise multifloral honeys from Argentine Pampas applying the INAA as a method for the quantitative analysis of 14 trace elements and lanthanides (Br, Ce, Co, Cr, Cs, Eu, Fe, La, Sb, Sc Sm, Rb, Th and Zn). These experiments aim at providing tools for the identification of the geographical origin as a function of the production area.

## 2. Materials and methods

### 2.1. Honey samples

The present study was conducted using 120 light-coloured honey samples from the central region of Argentina. This region is called Argentinean pampas and corresponds to a characteristic South American grassland biome. Samples were collected directly from beekeepers from the provinces of La Pampa and San Luis (between parallels  $-32^\circ$  and  $-38^\circ$  latitude,  $-62^\circ$  and  $-65^\circ$  west longitude), it was divided into three phytogeographic regions: Province of the Espinal, one district: Caldén (La Pampa and the portion south of San Luis province: SO) and Province Chaqueña, with two districts: Chaqueño occidental (Northeast of San Luis province: NE) and Chaqueño Serrano (corresponding to the central portion of San Luis province: SL). Honeys, 40 for each region, were produced in the years 2007 and 2008. The botanical origin was certified by Official Laboratories. All samples analysed were classified as multifloral with the exception of two samples from La Pampa (collected in 2007) that were *Melilotus albus* unifloral. Samples were collected in glass bottles and stored in a freezer prior to analysis.

Before analysis, about 10 g were weighed into ignited crucibles, and placed under an IR lamp with variable voltage input. Voltage was slowly increased until samples were black and dry and then they were incinerated in a furnace a  $600^\circ\text{C}$  to constant weigh. Finally, the resulting white ash was put in polyethylene boxes, for further analyses.

### 2.2. Analytical determinations

Instrumental neutron activation analysis (INAA) was used for trace-element determination. The honey ashes together with comparative standards and suitable control materials, were put in polyethylene boxes, wrapped in aluminium foil and packed in aluminium capsules for irradiation. They were irradiated for 4 h in the RA-3 reactor (8.5 Mw,  $6 \times 10^{13}$  thermal neutron fluxes) at Ezeiza Atomic Centre, National Commission of Atomic Energy, of Argentina. The samples, standards and control materials were measured in a hipper pure germanium detector (HPGe, 30% efficiency, 1.8 keV resolutions for the 1332.5  $^{60}\text{Co}$  peak); Gamma Vision software was used for data acquisition. The element concentrations were calculated using software developed at the laboratory. A reference material was used for analytical quality control, IAEA V-10 (Hay Powder). The characteristic nuclides, half-life and gamma ray used for element concentration determination are listed in Table 1.

**Table 1**

Nuclides, half-life and gamma ray energy used for element concentration determination.

Element	Nuclides	Half-life	Gamma ray energy (keV)
Br	$^{82}\text{Br}$	1.47 days	776.5
Ce	$^{141}\text{Ce}$	32.5 days	145.4
Co	$^{60}\text{Co}$	5.26 years	1332.5
Cr	$^{51}\text{Cr}$	27.8 days	320.1
Cs	$^{134}\text{Cs}$	2.05 years	795.8
Eu	$^{152}\text{Eu}$	12.7 years	1408.1
Fe	$^{59}\text{Fe}$	45.6 days	1291.5
La	$^{140}\text{La}$	1.68 days	328.8
Rb	$^{86}\text{Rb}$	18.7 days	1076.8
Sb	$^{56}\text{Sb}$	2.8 days	564
Sc	$^{46}\text{Sc}$	83.9 days	1120.5
Sm	$^{153}\text{Sm}$	46.2 days	103.2
Th	$^{233}\text{Pa}$	27 days	312.0
Zn	$^{65}\text{Zn}$	243 days	1115.5

### 2.3. Data analysis

An initial explorative analysis was performed, using box-plots based on the median and the quartiles, to allow the anomalous samples to be removed. Then, data were log transformed to reduce asymmetry of data before analysis when assumption of normality was not achieved. Normality of the transformed data was verified by means of the Kolmogorov–Smirnov test and equality of the variance between groups was evaluated by the Bartlett test. Secondly, an analysis of variance was made (one way ANOVA), to determine, for each element, the main effect of the region and of the year of harvest on the multielemental composition of samples. Afterwards, data were processed using multivariate chemometric techniques involving principal component analysis (PCA) and linear discriminant analysis (LDA). The aim of PCA is dimension reduction, in which the main factors (PCs) identified will explain most of the variability existing in the data matrix. PCA considers all variables and accommodates the total data structure; it is a method for exploratory data analysis (unsupervised learning). Lastly, LDA, as a supervised technique, provides a discriminant model with respect to the descriptors previously defined (geographic origin), as well as to study variable discriminate best between the honey geographic provenance. Then, the probabilities of correct classification were estimated using cross-validation methods. At first, we applied the holdout method. The holdout method is the simplest cross validation method. The data set is randomly separated into two sets, called the training set and the testing set. A training data set (2/3 of total observations) was used to create a classification model whose predictive ability was estimated using a test data set (1/3 of total observations). The procedure was repeated 30 times to get a better estimation of error. The errors it makes were accumulated as before to give the mean absolute test set error, which was used to evaluate the model. Finally, leave-one-out cross validation method was applied; the average error was calculated and used to evaluate the model.

Both, univariate and multivariate analyses were performed using R software version 2.8.1 (R Development Core Team, 2008) with ade4 package for PCA and DA (Chessel, Dufour, & Thioulouse, 2004).

## 3. Results

The detection power of the INAA technique was able to give good data for determination of elements of interest in honey samples from the three regions under study. In order to check the sensitivity and reproducibility of the calcinations procedure, three honey samples were treated three times. The relative standard deviations of the elements obtained from the analysis of the

samples were between 0.8% and 1.7%. For Co the value was up to 4.1% whereas, Sb and Sm gave a value up to 5.0%.

Duplicate portions (250 mg) of a certified reference material (IAEA V-10, hay powder) were analysed together with multiple empty bags to determine blank levels for later blank correction, to verify the accuracy of the elemental calibrations as well as to monitor and verify the overall analytical system performance. Agreement between duplicates for the controls was good, indicating the analytical system was in control. The results for the measurements of minerals in reference material are summarised in Table 2.

The levels obtained in honeys under study were similar to those found by other authors in honey samples from Argentina (Baroni 2009) and also in light-coloured honeys samples from Italy (Pisani 2008). The variability of results for all metals in both groups is large, reflecting the wide variation in the composition of honey samples. Descriptive statistics of all data for trace elements and lanthanides analysed in honey samples are presented in Table 3. The concentration distributions are characterised by the arithmetic mean value, standard deviation at 95% confidence interval and range. Only the iron contents distributions shows a remarkable symmetry, proved by the low value expressed by skewness statistic, consequently log transformation were applied to reduce asymmetry of all data before analysis.

The one-way analysis of variance showed that for 85% of the 14 elements considered, there were highly significant differences in the composition of honeys of different geographical origin, while no significant differences among the Eu and Sm content of honeys by region were recorded. Furthermore, ANOVA showed that the year of harvest did not differentiate honeys of the same origin from the 2007 and 2008, according to the elemental composition considered.

### 3.1. Exploratory data analysis

The log-transformed data set of parameters analysed was subjected to PCA, in order to decrease the number of descriptors retaining the maximum amount of variability present in the experimental data. Prior to performing PCA, the suitability of the data for factor analysis was checked. From the results of the Kolmogorov–Smirnov test on the normality of sample distributions, it can be concluded that all of them were significantly normal ( $p$ -value  $>0.05$ ).

On the basis of eigenvalues  $>1$ , PCA evolved three principal components explaining about 67.8% of the total variance. According to the eigenvalue criterion, only the PCs with eigenvalues greater than one are considered important. This criterion is based

**Table 2**  
Quality control parameters. Reference material IAEA V-10: Hay Powder.

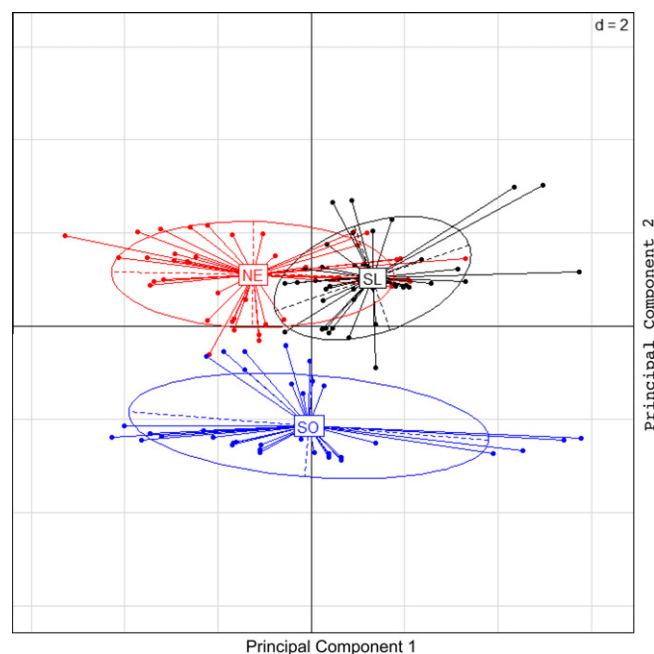
Element	IAEA V-10 ( $\mu\text{g g}^{-1}$ )	
	Found (mean $\pm$ SD)	95% Confidence interval
Br	8.76 $\pm$ 0.15	7–11
Ce	ND (<0.2)	NR
Co	0.157 $\pm$ 0.015	0.11–0.14
Cr	6.33 $\pm$ 0.38	5.6–7.1
Cs	0.015 $\pm$ 0.03	0.016–0.017
Eu	0.0025 $\pm$ 0.0001	0.0023–0.0032
Fe	184 $\pm$ 11	177–190
La	0.083 $\pm$ 0.024	0.06–0.09
Rb	7.60 $\pm$ 1.45	7.3–7.8
Sb	0.0198 $\pm$ 0.0045	0.018–0.02
Sc	0.01334 $\pm$ 0.00082	0.012–0.015
Sm	0.0144 $\pm$ 0.0030	NR
Th	ND (<0.01)	NR
Zn	25.7 $\pm$ 1.4	23–25

ND: non-detectable (<LOD); NR: non-reported.

**Table 3**  
Descriptive statistics of data in honeys.

	N	Range		Mean	SD	Skewness
		Min	Max			
Cr ( $\text{mg kg}^{-1}$ )	120	0.01	2.65	0.47	0.54	1.703
Fe ( $\text{mg kg}^{-1}$ )	120	1.00	13.20	6.44	3.45	0.258
Rb ( $\text{mg kg}^{-1}$ )	120	0.06	4.54	0.46	0.57	4.830
Zn ( $\text{mg kg}^{-1}$ )	120	0.06	2.78	0.85	0.60	0.819
Br ( $\mu\text{g kg}^{-1}$ )	120	4.30	156.40	46.32	34.00	1.139
Ce ( $\mu\text{g kg}^{-1}$ )	120	7.40	196.70	50.22	37.58	1.906
Co ( $\mu\text{g kg}^{-1}$ )	120	1.70	46.90	12.88	8.64	1.506
Cs ( $\mu\text{g kg}^{-1}$ )	120	0.30	15.30	3.97	3.20	1.909
Eu ( $\mu\text{g kg}^{-1}$ )	120	1.60	59.40	13.73	11.50	2.240
La ( $\mu\text{g kg}^{-1}$ )	120	2.60	370.52	28.84	44.92	5.873
Sb ( $\mu\text{g kg}^{-1}$ )	120	0.80	14.90	6.90	11.75	3.840
Sc ( $\mu\text{g kg}^{-1}$ )	120	0.80	61.10	8.30	8.27	2.863
Sm ( $\mu\text{g kg}^{-1}$ )	120	0.70	11.10	3.21	2.33	1.675
Th ( $\mu\text{g kg}^{-1}$ )	120	1.20	1.90	9.35	7.38	1.699

on the fact that the average eigenvalues of the autoscaled data is just one. The first principal component explains 38% of total variability, the second one 20.8% and the third one 8.9%. Fig. 1 represents the graphic distribution of the samples according to their components scores, this simply do not represent enough variation to confidently differentiate between the geographical origins of all analysed honeys. But, one cluster formed by samples corresponding to the South region with regard to the other zones could be distinguished. This tendency on scores distribution related to PC2 mainly could be related with the fact that the measured contents of microelement concentrations (Cr, Fe and Zn) on samples from the south region were lower than the rest of samples. Table 4 shows the factor loadings for each variable on the unrotated principal components. Each number represents the correlation between the item and the PCs. The first PC was principally correlated (loading  $>0.70$ ) with six ultra-trace elements (Sc, Th, La, Co, Sb and Rb), all of them are present at very low concentrations in honeys ( $<1 \text{ mg kg}^{-1}$ ). Chromium, caesium and zinc are the dominating variables in the second principal component, although iron is also



**Fig. 1.** Score plots of the two first principal components used to discriminate geographical origin of honeys. NE: Northeast San Luis – SL: San Luis – SO: South San Luis and La Pampa.

**Table 4**  
Principal component-loading matrix obtained from the data matrix.

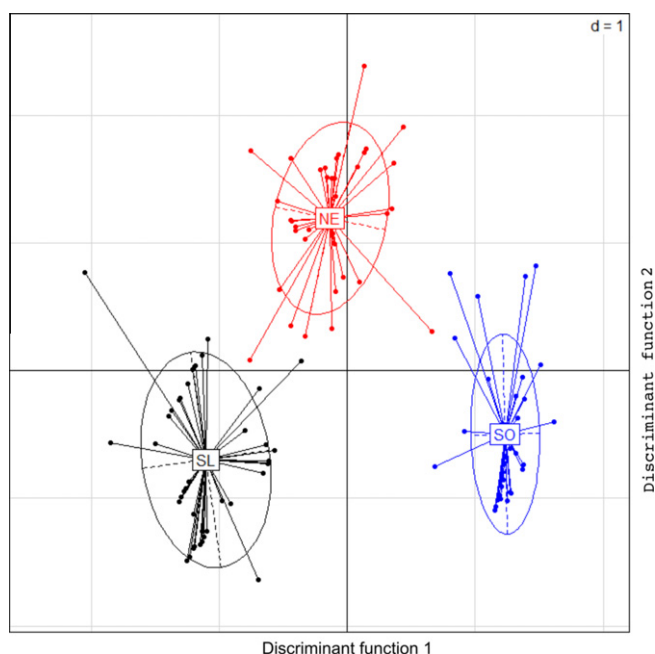
Loadings	PC-1	PC-2	PC-3
Sc	-0.844	-0.120	-0.149
Th	-0.843	-0.007	-0.216
La	-0.803	0.138	-0.070
Co	-0.792	-0.304	-0.123
Sb	-0.759	0.019	-0.373
Rb	-0.757	0.400	-0.062
Br	-0.636	0.238	0.211
Sm	-0.409	-0.338	0.047
Cs	-0.257	0.774	0.425
Cr	-0.391	-0.725	-0.019
Zn	-0.404	0.704	0.014
Ce	-0.507	-0.650	0.310
Fe	-0.064	-0.631	0.482
Eu	-0.499	0.152	0.687

correlated to a lower extent. The variability of these elements contents could be a result of geographical variation.

### 3.2. Linear discriminant analysis

The next step was to determine which elements were the most important in discriminating honeys belonging to different regions. LDA successfully designed element functions that separated the honeys based on geographical origin. This technique allows classifying unknown samples after checking of possible differentiation of samples of known origin.

Stepwise LDA in forward direction was applied to the concentration of 14 elements of each single sample, and using the groups of geographical origin as classification factor. The stepwise analysis (Probability of F to remove = 0.10) has eliminated five variables from the model (Br, Co, Eu, Sc and Sm). The bidimensional plot (Fig. 2) of the first two roots shows three cluster for the three production zones of central region of Argentina, respectively (Fig. 2). In the graphic, the covariances of each group could be visualised as ellipses in the plots. Here all three groups have the same prior



**Fig. 2.** Plot of the first two principal component scores for three geographic provenance groups. NE: Northeast San Luis – SL: San Luis – SO: South San Luis and La Pampa.

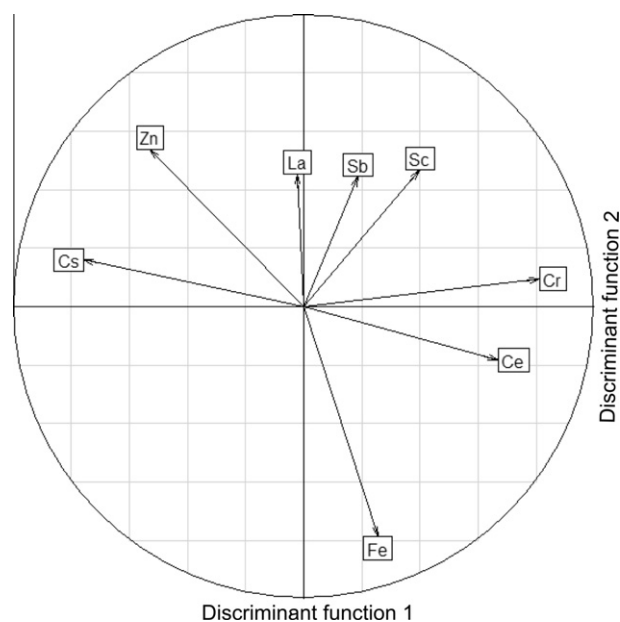
probability, but their covariance matrices are not equal (different shape and orientation of ellipses), this confirm the results obtained from Barlett's test about the equality of the variance–covariance matrix of the predictors ( $p$ -value  $< 0.05$ ). But this is not a limitation because, in general, the linear discriminant function works very well even for unequal variance–covariance matrices (Lee, Herrman, & Jones, 2009). LDA allows visualisation of data in graphical representations, simplifying the observation and interpretation of information. A correlation circle plot of loadings for selected elements by stepwise process (Fig. 3) shows the associations obtained between elements, in order to visualise the discriminating efficiency of the proposed model.

The LDA shows significant differences among groups; in fact the Wilks Lambda values are near zero (0.023), indicating a good discriminating power of the model. Besides, the chances of a correct classification are very high, because the  $p$ -levels are lower than 0.05 ( $p$ -value =  $2.2 \times 10^{-16}$ ). From the results of the LDA and cross-validation we obtained that, original grouped cases (97.5%) were correctly classified and 95.9% of cross-validated grouped cases were correctly classified.

In order to verify the reliability of the model, the method has been tested using known samples as unknown objects. We select a training data set randomly (2/3 of the objects) and use the derived classification rule to predict the group membership of the remaining data (test data). Since the result will depend on the choice of the test data, the procedure is repeated 30 times. According to this criterion, the mean of well-classified cases was 96.2%. Finally, other classification methods like quadratic discriminant analysis  $k$ -nearest neighbor algorithm were applied, but the correct classification rate of honeys into predetermined individual groups according to its geographic provenance was much greater in a linear discriminant analysis.

### 3.3. Summary

The differentiation displayed in the INAA data illustrates clear separation of honeys based on inter-honey chemical composition. After the differentiation among the tested honeys was achieved, we attempted to determine if any particular elements may be observably driving the differentiation of the three origins. The



**Fig. 3.** Correlation circle of variables included in stepwise discriminant analysis.

**Table 5**  
LDA structure matrix.

	Function	
	1	2
Cr	0.812	0.094
Cs	0.758	0.159
Ce	0.670	-0.183
Co	0.413	0.283
Sm	0.233	-0.078
Fe	0.256	-0.788
Zn	-0.531	0.539
Rb	-0.131	0.498
Sc	0.400	0.468
La	-0.024	0.455
Br	-0.002	0.449
Sb	0.187	0.445
Th	0.179	0.359
Eu	-0.038	0.073

LDA structure matrix (Table 5) provided the correlation for any given individual element in defining the variability among honey samples. According to the structure matrix, no single element significantly contributed to the variability for any of the two discriminant functions. The analyses of the generated functions show that the differentiation is determined above all by two microelements such as Cr and Cs, additionally to one lanthanide Ce, on the first discriminant function. In addition, two microelements (Fe and Zn) determine the second discriminant function. These elements alone could not differentiate between all three analysed honeys. Considering this data, it appears that the combination of multiple trace and lanthanides is necessary to differentiate among the selected honey samples.

#### 4. Conclusions

Using compositional data generated with INAA in combination with chemometric techniques, we can confidently differentiate these honeys from three producing zones, based on their chemical variability. INAA combined with pattern recognition techniques resulted in clear honey-level differentiation of all analysed samples. Although successful in differentiating the honeys, data compiled in this project suggests that the chemical composition of the honeys analysed offers a minute amount of inter-honey variability. The detectable variability appears limited, but this project clearly displays that intra-sector honey signatures are definable by sensitive methods of analysis such as INAA.

The method can be extended to build up a set of data to trace the geographical origin of honeys based on reliable clues. Future studies should test the success of this strategy for larger and more variable data sets including different honey types.

#### Acknowledgement

The authors wish to thank the Consejo Nacional de Investigaciones Científicas (CONICET) for their financial support.

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