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Using an automatic pulse voltammetric electronic tongue to verify the origin of honey from Spain, Honduras and Mozambique

Short running title: Pulse voltammetric electronic tongue to verify the country origin of honey

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ABSTRACT

Background: The growing need to classify the origin of honey in a simple way, is leading to the development of affordable analytical equipment that is in-line and manageable enabling rapid on-site screening. Therefore, the aim of this work was to evaluate whether an electronic tongue (made of four metallic electrodes: Ir, Rh, Pt, Au) based on potential multistep pulse voltammetry with electrochemical polishing is able to differentiate between honey samples from Spain, Honduras and Mozambique.

Results: It has been demonstrated for the first time that an automatic pulse voltammetry, in combination with a PCA statistical analysis, is able to differentiate honey samples from these three countries. A PLS analysis predicted the level of certain physicochemical parameters; the best result being for conductivity and moisture with correlation coefficients of 0.948 and 0.879, whereas the weakest correlation was for the sugars.

Conclusion: The tool proposed in this study could be applied to ensure the country origin of the three types of multifloral honey here considered. It also offers promising perspectives for expanding the knowledge of the provenance of honey. All this could be achieved once a comprehensive database with the information generated by this electronic tongue has been created.

Keywords: honey; country-origin; pulse-voltammetric-electronic-tongue; physicochemical parameters

1. Introduction

Nowadays, consumers are increasingly interested in the possibility of choosing among different honeys with specific characteristics. The world market demands special honeys with different attributes such as geographical origin, organoleptic properties and/or attributable therapeutic properties.[1,2] In countries where the standard of living is precarious (such as Mozambique and Honduras) and where apiculture does not play an important role, but has the potential to increase the sustainability of communities, promoting the origin of honey could be a good option in protecting and marketing this traditional food.[3,4]

To date, melissopalynological analysis (based on the identification and quantification of the percentage of pollen) is the technique “of choice” used to classify honey according to botanical and/or geographical origin. Since this technique is very tedious and requires very skilful specialists, the new analytical tendencies to discriminate honeys are moving towards the development of more simple, environmentally friendly and inexpensive methodologies that permit real-time and on-site screening.[5] This is the case of the electronic tongue system, a qualitative analytical technique that allows the classification or identification of samples. Its usefulness depends on the composition of the sensor array in combination with a multivariate analysis tools (PCA, LDA, CA, etc.), due to the enormous amount of data generated.[6,7,8]

Electronic tongue systems have been widely used for classifying liquid and solid foodstuffs. In the specific case of honey, most of the reported papers dealt with the differentiation of samples according to their botanical origin.[5,7-15] Electronic tongue methodology was also useful in discriminating authentic honeys from adulterated ones.[16] However, the geographical origin has been considered in very few occasions and when used, in most cases was focused only on areas belonging to the same country.[8,10] Highlighting the few exceptions are works reported by Bougrini at al.,[5] and Hassani et al.,[3] where samples of honey from different countries were analysed using almost exclusively a cyclic voltammetric electronic tongue, but, as far it is known, pulse voltammetry electronic tongue has never been used for this purpose.

For all the above, the goal of the present study was to evaluate whether an electronic tongue based on potential multistep pulse voltammetry is able to differentiate between honey samples coming from Spain, Honduras and Mozambique. In addition, the recognition ability of the electronic tongue was correlated with classical analytical parameters.

2. Materials and methods

2.1. Samples

Sixty multifloral honey samples from three different countries were used in this study: 20 from Mozambique, 20 from Honduras and 20 from Spain. Mozambiquean samples came from the provinces of Nampula, Zambezia, Manica, Sofala, which were obtained using traditional beehives and harvesting methods (Nampula and Zambezia) or more modern methods (Sofala and Manica). Honduran samples were provided by Lencas communities located in the department of Comayagua municipality of Siguatepeque (central Honduras), in an area of environmental interest due to its proximity to Cerro Azul Meámbar National Park. Spanish samples were supplied by transhumant Valencian beekeepers that were collected in different regions of the country.

None of the samples exhibited signs of fermentation or granulation before initiating the analyses.

2.2. Analytical parameters

Pollen analysis

Pollen analysis was based on the extraction of pollen grains from 10 g of honey (Juan-Borrás et al., 2014). Each sample was dissolved in 20 mL of acidulated water (sulfuric acid, 5%) and after a first centrifugation the sediment was re-dissolved with distilled water and centrifuged one more time. The observation of pollen sediment slides was carried out using a light optical microscope (Zeiss Axiolab, Göttingen, Germany). A minimum of 600 pollen grains per sample were counted. To recognize the different types of pollen, general palynological databases,[17]as well as specific from Spain,[18]

Honduras,[19] and Mozambique[20, 21] were used together with the reference collection of the laboratory.

In Table 1 of Supplementary Material is shown the race of the honeybee from the three countries, as well as the percentage of pollen from the predominant botanical species, and the other pollen types identified in honey sample from the three countries. The abundance of each taxa (frequency of pollen appearance) was categorized as follows: dominant or predominant pollen (> 45%); secondary pollen (16-45%); important minor pollen (3-15%); minor pollen (1-3%) and pollen present (<1%).[21]

Moisture

Moisture content was obtained by using a refractometer (Abbe-type model T1; Atago, Bellevue, WA, USA) and the Chataway tables in accordance with the Harmonized Methods of the European Honey Commission.[22]

Electrical conductivity

Electrical conductivity was determined by conductimetry (Crison Instrument, Barcelona, Spain, model C830) in accordance with the Harmonized Methods of the European Honey Commission. [22]

Colour

Colour was measured using a millimetre Pfund scale C221 Honey Colour Analyzer (Hanna Instruments, Eibar, Spain).

Sugar content

Liquid Cromatograph (Agilent Technologies modelo 1120 Compact LC, Germany) with an Evaporative Light Scattering Detector (ELSD Agilent Technologies 1200 Series, Germany) and a Waters Carbohydrate column (4.6 x 250 mm, 4 µm) was used to analyse the major sugars (fructose and glucose). [22]

Water and acetonitrile (20/80), in isocratic mode, at a flow rate of 0.8 mL/min was used as mobile phase. The elution was finished in 14 minutes. The detector conditions were: 50 °C, 3.5 bars of gas pressure (N_2) and gain=6. EZChrom Elite system software was used for data processing. Calibration curves of the corresponding external standards were used for quantification. The limits of quantification of both sugars were 0.1 g/100 g honey.

2.3. Voltammetric electronic tongue analysis

A previous dilution in water (up to 50 mL) of the sample was only necessary to start the voltammetric analysis. For each iteration 8 g (in dry matter) of sample was weighed, being dry matter equal to 100 minus moisture (in percentage). The reason for this procedure was to standardize the quantity of sample used for each measurement, since the moisture content was different for all honey samples.

The pulse voltammetry measurements were carried out in an equipment, based on a potentiostat, designed at the *Universitat Politècnica de València* (Institute of Molecular Recognition and Technological Development-IDM).[23] In the present work, 40 pulses of 50 ms were applied. Figure 1 shows a typical distribution of voltages (similar to a stair case) in increasing or decreasing steps of 200 mV between +1 V and -1 V (to avoid water electrolysis). The potential was set to zero after each increment.

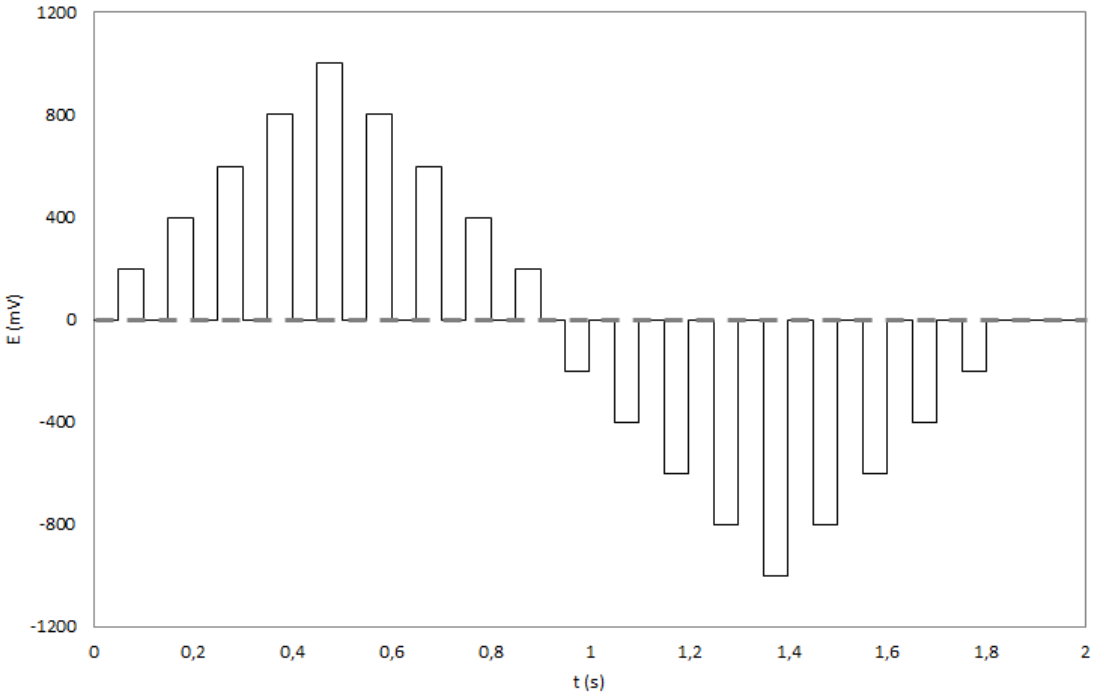

Figure 1. Typical voltammetric pulse pattern.

Four working electrodes (Ir, Rh, Pt and Au) were used in the voltammetry tests. The electronic tongue body consisted in these electrodes placed inside a stainless-steel cylinder. In all cases a calomel electrode was used as reference and a stainless steel circular piece as counter electrode. This device included an innovative electrochemical polishing of the working electrodes previously describe by Sobrino-Gregorio et al.[24]

An in-house specific software designed by Campos et al.,[23] permitted the controlling of both the pumping system and the measurement of the equipment. The described electronic tongue was patented by Bataller et al.[25]

All samples were analysed in triplicate.

2.4. Statistical analysis

An analysis of variance (ANOVA) using Statgraphics Centurion 16.1 was applied to study the influence of the country origin of honey on each the analytical parameter (moisture, electrical conductivity, colour fructose and glucose). Least Significant Difference (LSD) at significance level α=5% was used to analyse the differences between samples. A full residual analysis was previously carried out for checking the

assumptions of ANOVA, which indicated that this analysis was suitable for all the dataset. In this respect, independence (each sample was randomly selected and independent), homoscedasticity (by means of Levene's test) and normality (by means of a normal probability plot) were all tested.

Multivariate statistical analysis techniques were used to analyse the data generated by the electronic tongue response. Principal Components Analysis (PCA) was applied to discriminate between samples and Partial Least Square (PLS) to correlate the data of electronic tongue with each one of the analytical parameters. The PLS model was calibrated with 66% of the data set and validated with the remaining 33% and the assessment was carried out by comparing real versus predicted physicochemical analysis. The parameters considered in the model were: correlation coefficient (r^2), a, b (from the simplest linear model: $y = ax + b$) and the root mean square error of prediction (RMSEP), as the most common metric obtained to measure the accuracy of this methodology.[26]

The multivariate statistical analysis was performed with Solo 8.6 software (Eigenvector Research, Inc., Wenatchee, Washington, DC, USA).

3. Results and discussion

3.1. Analytical parameters

Table 1 shows the average and standard deviation values of the quantified analytical parameters (moisture, electrical conductivity, colour and, fructose and glucose content) in the honey samples from Spain, Honduras and Mozambique). In addition, this table illustrates the ANOVA results (F-ratio and significant differences) obtained for each analytical parameter considering the factor “country origin”. Significant differences were found between the three countries for all the parameters analysed. Considering the bigger the F-ratio is, the greater the effect the factor has over one variable, the electrical conductivity (688.8) was the parameter most affected for the country of origin, followed by colour (407.7), while the sugar contents (19.0 for glucose and 6.8 for fructose) were less affected. The biggest average values of electrical conductivity (1.254 mS/cm) and colour 129 mm Pfund) corresponded to samples from Mozambique whereas moisture (20.7 g100g) and fructose (42.8 g/100 g) to samples from Honduras. On the contrary, the smallest values of all the parameters analysed were found in Spanish samples (average of 16.5 g/100 g, 0.426 mS/cm, 68 mm Pfund and 38.8 g/100 g for moisture, electrical conductivity, colour and fructose, respectively), except for glucose, where it obtained the highest value (34.0 g/100 g).

Table 1. Mean and standard deviation values of moisture, electrical conductivity, colour, fructose and glucose found in honey from different countries (Mozambique, Honduras and Spain) and ANOVA results (F-ratio and significant differences).

	Moisture (g/100 g)	**Electrical conductivity (mS/cm)**	**Colour (mmPfund)**	**Fructose (g/100 g)**	**Glucose (g/100 g)**
Mozambique	19.5 (1.5)b	1.254 (0.157)c	129(18)c	41.2 (3.2)b	32.4 (2.1)a
Honduras	20.7 (1.3)c	0.496 (0.162)b	75(12)b	42.4 (4.6)c	33.4 (3.1)b
Spain	16.5 (0.7)a	0.426 (0.060)a	68 (6)a	38.8 (0.5)a	34.0 (0.9)c
ANOVA					
F-ratio	179.5***	688.8 ***	407.7 ***	6.8 **	19.0 ***

According to findings in other studies related to honey from different origins, moisture content values obtained herein (Table1) are in the same range as: Africa [Southern African, from 15.3 to 21.7 g/100 g;[27,28] North Africa, from 14.2 to 22.1 g/100 g];[29,30] Southern and Central American honey, from 16.4 to 19.4 g/100 g;[31] or European from, 15.2 to 19.9 g/100 g. [32] On the contrary, in other African honey, higher and lower values of electrical conductivity and colour were found: 4.18 mS/cm and 56 mm Pfund from Yemen; 1.98 mS/cm and 73 mm Pfund from Egypt; 0.67 mS/cm and 89 mm Pfund from Kashmir or 0.53 mS/cm and 113 mm Pfund from Saudi.[33]

The differences in the levels of moisture detected between countries could be due to the beekeeping practices: harvesting of honey must be done when honeybees have operculated honey. In this respect, the local climate could also have an influence.[21] The electrical conductivity and colour are related to the type of vegetation visited by the honeybees. In general, both parameters are higher when honey came from exudates of plants and lower in the case that honeybees collect nectar from flowers. In the same way, the concentration of sugars is influenced by the vegetation surrounding the hives. [4,11,21]

3.2. Voltammetric electronic tongue analysis

Differentiation of honey samples

Figure 2 shows, as an example, the pattern of the signal obtained by applying the corresponding potential pulse to the honey samples from the three countries considered in this work. There is a clear differentiation between the signals obtained for the three countries demonstrating that these are affected by the origin of samples. The highest signal corresponds to honey from Spain, followed by Mozambique and Honduras.

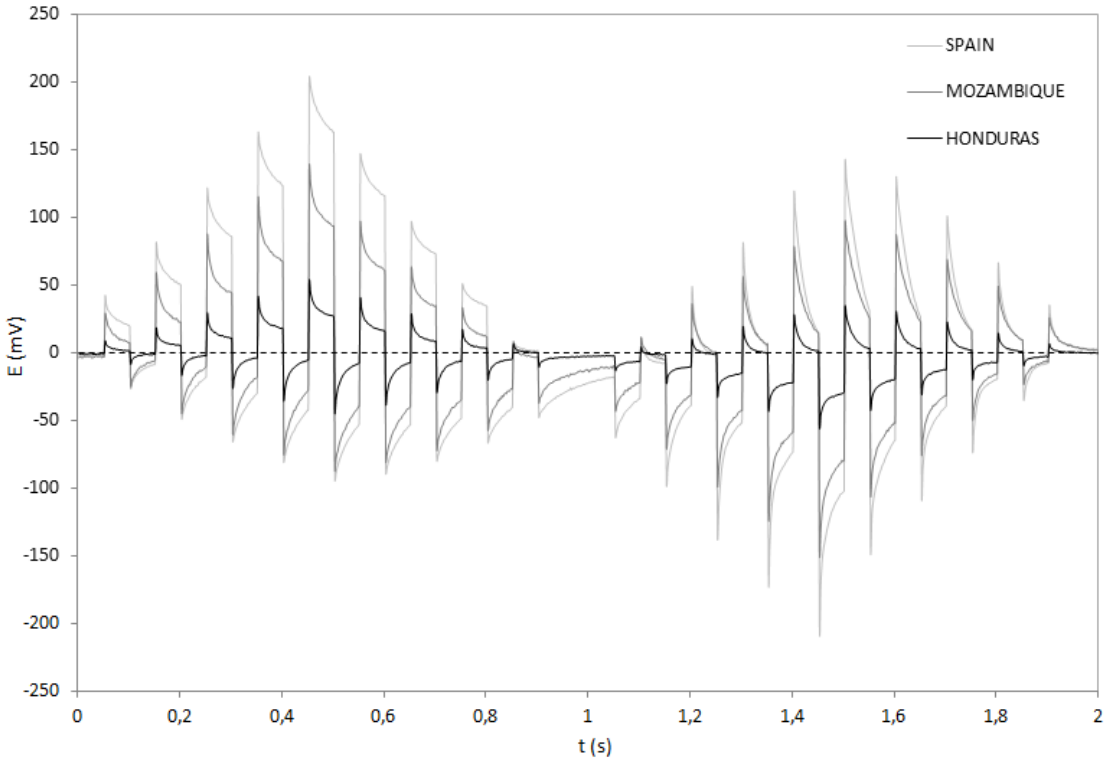

Figure 2. Electrochemical trace of the layered sequence of the potential for honey samples from three different countries: Mozambique, Honduras and Spain.

A PCA analysis, from the data generated by electronic tongue, was applied in order to show if there was a spontaneous differentiation of the samples studied. Figure 3 shows the score plot of this analysis, in which the first two principal components together explain 89.96% of the data variability, specifically 78.29% by PC1 and 11.67% by PC2. Discrimination between Mozambique samples and those from the other two countries is mainly determined by the X axis (PC1), where the honey samples of Mozambique are in the left of the score graph and the others, in the right. Since, proximity between samples indicates similar behaviour in terms of the electrochemical response of the sensors, small differences between Honduras and Spanish samples in the X axis (PC1) were found. However, these two origins are differentiated by PC2, where Spanish honey is in the upper half and Honduras honey in the lower half.

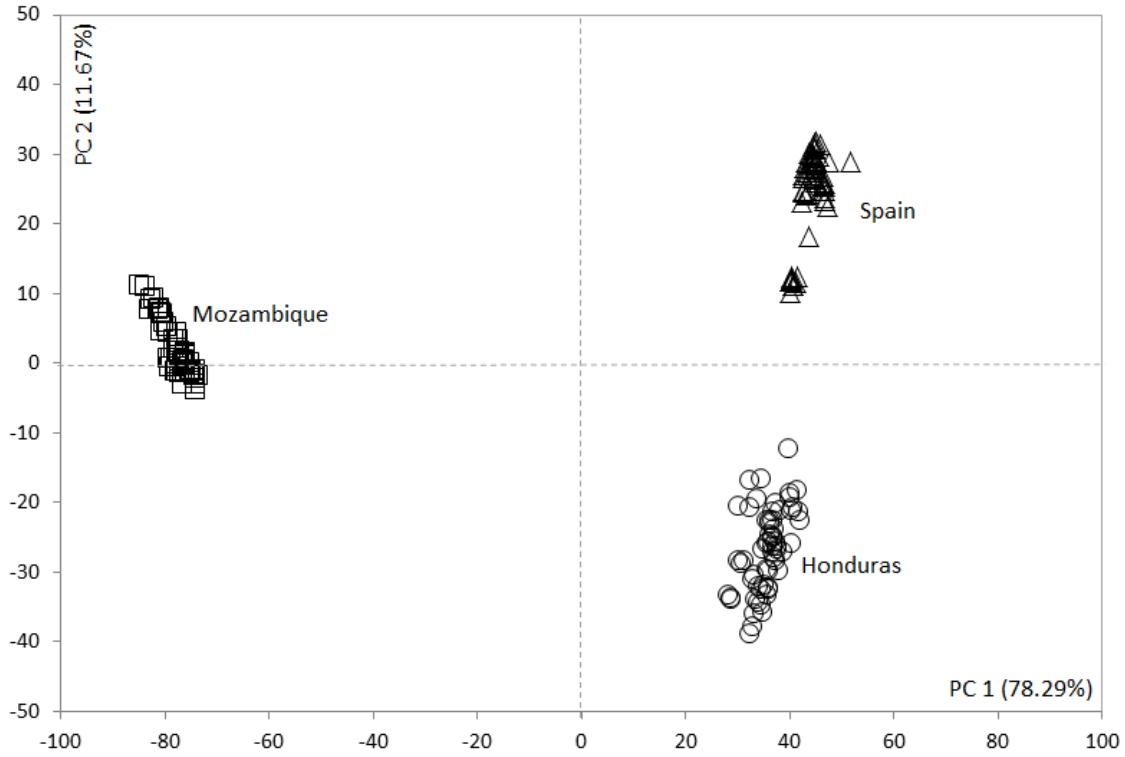

Figure 3. Score plot of the PCA performed on multifloral honey samples from three different countries: Mozambique, Honduras and Spain.

Similar results were reported by Hassani et al.,[3] where a cyclic voltammetry was used as an electronic tongue. Bougrini et al.[5] applying a cyclic voltammetry also obtained an acceptable discrimination of honey originated from different countries, but only explaining 63% of the variability of the data with the first two components.

PLS analysis

A Partial Least Square (PLS) analysis was applied in order to verify whether the data provided by the electronic tongue could be useful in predicting the analytical parameters: moisture, electrical conductivity, colour and sugars content. Five PLS models of prediction were created (origin x 5 physicochemical analysis) with the voltammetric experimental data obtained. Table 2 shows the PLS prediction results (number of latent variables, the regression coefficient, slope, intercept and RMSEP) for the five models obtained. The best results were for the conductivity, moisture and colour with correlation coefficients of 0.948, 0.879 and 0.864, respectively. The weakest correlation was for the sugars (fructose: 0.201 and glucose: 0.094). Hassani et al.,[3] obtained better results applying cyclic voltammetry but using different parameters: phenols (0.997), HMF (0.996), total acidity (0.991) and proteins (0.969). In order to quantitatively describe the accuracy in prediction, the slope and the intercept were calculated (Table 2). Moreover, parameter RMSEP (root mean square error of prediction) was also determined to evaluate the precision of the model. Thus, the PLS model is better when the slope value approaches 1 and when the RMSEP value comes

close to 0. The best results in terms of accuracy were for colour (0.954), moisture (0.886) and conductivity (0.874) and, in terms of precision: conductivity (0.097) followed by the moisture (0.772). In this case, the worst model was for colour (10.845). On the contrary, in terms of precision, Hassani et al.,[3] obtained a better result in colour (value of 0.103). In the same study, the errors ranged between 0.015 for sucrose and 0.184 for free acidity.

Table 2. PLS prediction results obtained from the validation data for the multifloral honeys with analytical parameters.

Analytical Parameters	No. latent variables	Correlation coefficient	Slope	Intercept	RMSEP
Conductivity	3	0.948	0.874	0.101	0.097
Moisture	6	0.879	0.886	2.226	0.772
Colour	5	0.864	0.954	5.150	10.845
Fructose	3	0.201	0.229	31.344	3.147
Glucose	2	0.094	0.096	29.143	4.618

4. Conclusions

This work successfully demonstrated that an automatic pulse voltammetry electronic tongue system (made of Ir, Rh, Pt, Au), with electrochemical cleaning and polishing, can be applied in a simple way to differentiate honey samples from Spain, Honduras and Mozambique. This tool, in combination with a PCA multivariate analyses offers encouraging perspectives for expanding the knowledge of the geographical origin of honey. The present findings might help to solve the necessity to have manageable and on-site analytical equipment that enables a rapid screening and also a more affordable instrument for the apiculture sector. However, future studies on the current topic are recommended, in order to create a wider and more comprehensive database of different types of honey from as many geographical origins as possible.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Supplementary material (Table 1). Melissopalynological results of the analyzed honey from Spain, Honduras and Mozambique.

	POLLEN CATEGORY				
COUNTRY ORIGIN AND HONEYBEE RAZE	**Dominant pollen (>45%)**	**Secondary pollen (16–45%)**	**Important minor pollen (3–15%)**	**Minor pollen (1-3%)**	**Present pollen (<1%)**
Spain *(Apis mellifera iberica)*	Non existent	*Echium sp.* *Onobrychis sp.* Brassicaceas Taraxacum type *Hypecoum sp.*	*Citrus sp.* *Rosmarinus officinalis* *Prunus dulcis* *Helianthus annuus* Vicia type *Trifolium sp.* *Ceratonia siliqua*	*Lavandula stoechas* *Erica sp.* Ulex type *Retama sphaerocarpa* *Centaurea cyanus* *Thymus sp.*	*Picris sp.* Liliaceae *Gleditsia sp.* *Pimpinella anisum* *Fumaria sp.* *Sideritis sp.*
Honduras *(Apis mellifera ligústica/ Apis mellifera scutellata*)	Non existent	Myrtaceae Leguminosae Asteraceae *Mimosa pudica*	Burseraceae Anacardiaceae Polygonaceae Verbenaceae	Tiliaceae *Ageratum sp.* *Quercus sp.* Ulmaceae	Combretaceae *Spondia purpurea*
Mozambique *(Apis mellifera scutellata*)	Non existent	Astragalus type Vicia type Anacardiaceae *Vernonia* sp. *Sigesbeckia* sp. *Acanthus* sp. Brassicaceae Unknown	Poaceae *Justicia* sp. Asteraceae Vicia type Myrtaceae Celastraceae	*Acacia* sp. Carex type *Brachystegia* sp. Combretaceae Caryophyllaceae Poaceae Euphorbiaceae	*Mimosa* sp. *Sesamum* sp. Nymphaeaceae Convolvulaceae Campanulaceae Cyperaceae