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Chemical and physical parameters of Andalusian honey: classification of *Citrus* and *Eucalyptus* honeys by discriminant analysis

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Abstract

The characterization of two types of Andalusian unifloral honey (*Citrus* spp. and *Eucalyptus* spp.) was carried out on the basis of their physicochemical properties: moisture, hydroximetylfurfural, diastase, pH, free acidity, lactone acidity, electrical conductivity, glucose, fructose, sucrose, proline, invertase, glucose-oxidase, water activity and insoluble solids. All the data were statistically tested using analysis of variance, principal factor analysis (PFA) and stepwise discriminant analysis (SDA) with the aim of classifying the honeys and identifying the most significant parameters in the classification.

Statistically, it was verified that the variables were different, depending on the type of honey. Of the six main factors obtained with a variance percentage of 78.95, it was the first one (free acidity, water, invertase, total sugars, electrical conductivity and solids) which explained the greater part of the variability (22.9%). The variables with the greatest discriminatory power were water activity and electrical conductivity with discrimination coefficients of −22.367 and 11.739, respectively. The overall proportion of accurately arranged samples was 96.6%.
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1. Introduction

The annual honey production of Andalusia is estimated at 4500 tons, which represents 14.5% of the annual production in Spain. The large variety of melliferous sources also enables Andalusia to produce characteristic uniforal nectar honeys. The main Andalusia unifloral honeys are thyme (*Thymus* spp.), sunflower (*Helianthus annuus* L), orange (*Citrus* spp.), broom (*Sarothamnus scorparius*), eucalyptus (*Eucalyptus* spp.), and rosemary (*Rosmarinus officinalis*). Of the types of honey produced in our region, *Citrus* honey and *Eucalyptus* honey acquire a special economic importance due to their high production. Each honey is unique on the basis of the number and combination of the various components that give it a specific individual note and often a characteristic flavor (Dustmann, 1993).

To ensure the authenticity of the honey, it is required to perform extensive honey compositional analyses.

Today, the characterization of the flavor and quality control of monofloral honeys is a subject of great interest in apiculture. Although it is possible to make a partition between the monofloral honeys, the classification between mono and polyfloral honey can sometimes be imprecise and ambiguous (Serra & Ventura, 1995).

It is necessary to determine some parameters that would unequivocally establish its origin and this calls for efforts to improve honey characterization (Mateo & Bosch-Reig, 1997).

Identification and pollen counts have been used for authentication, although there are difficulties in assuring a correct assignment of the origin (Maurizio, 1975). Many authors (Krauze & Zalewski, 1991; Mateo & Bosch-Reig, 1997; Persano, Piazza, Sabatini, & Accorti,

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1995; Accorti, Persano Oddo, Piazza, & Sabbatini, 1986; Salinas, Alvarez, Montero de Espinosa, & Lozano, 1994) have suggested the use of physicochemical criteria (pH, sugar content, electrical conductivity, proline, enzymatic activity, water content) analyses for the characterization of unifloral honeys. One of the objectives of this work is the application of a discriminant analysis to all the parameters studied to find the best combination of factors to characterize and classify two types of Andalusian unifloral honeys.

2. Materials and methods

2.1. Samples

Twenty nine reportedly unifloral honey samples were obtained from different beekeepers and dealers in southern Spain (Andalusia). Fourteen were *Citrus* honey samples (seven from beekeepers and seven normal commercial honey) and 15 *Eucalyptus* honey samples (seven from beekeepers and eight normal commercial honeys).

The frames of honey were processed as soon as they were obtained, and stored at 20 °C until fully analyzed. Enzyme (diastase, α-glucosidase, glucose-oxidase) and hydroxymethylfurfural analyses were performed first.

Analyses were carried out in duplicate. Their botanical origin was first ascertained by palynological analysis according to Louveaux, Maurizio, and Vorwohl (1978).

2.2. Analytical procedures

Water content (moisture) was determined following Chataway (1932) and Wedmore (1955), a method established by the Codex Alimentarius Commission (1969). We used an Abbe-type refractometer, obtaining the corresponding percentage of water from the Chataway table.

Measurement of pH was performed potentiometrically at 20 °C in a 15% (w/v) solution of honey in freshly boiled distilled water according to Bogdanov, Matin, and Lullmann (1997) using a Basic. 20. Crison pHmeter (Crison Instruments, Barcelona, Spain).

Total, free and lactone acidity were analysed by the titrimetric method using 0.1 M NaOH in accordance with AOAC method no. 962.19. Results were expressed as milliequiv. NaOH/kg.

Diastase activity. The procedure of Siegenthaler (1977) modified by Bogdanov (1984) was used. Adsorption was followed using a Pharmacia Biotech Ultrospec-3000 spectrophotometer (England). Results were calculated (as Gothe's degrees, °G) as ml of 1% starch hydrolysed by an enzyme in 1 g honey in 1 h.

Invertase activity. Was measured according to the method of Siegenthaler (1977), based on the spectrophotometric measurement of 4-nitrophenol, which is formed by the reaction of honey invertase with 4-nitrophenyl-α-D-glucopyranoside, used as a substrate. Results were expressed as invertase number (IN). IN indicates the amount of sucrose per gram hydrolysed in Ih by the enzymes contained in 100 g of honey under test conditions.

Glucose oxidase. Determined by screening for peroxide accumulation following Kerkvliet (1996), using Merckoquant test strip (no. 10011; Merck, Germany). Results are expressed in micrograms of hydrogen peroxide per gram honey per hour at 20 °C.

Hydroximethylfurfural. Determination made according to Winkler method (Winkler, 1955). A Pharmacia Biotech Ultrospec-3000 spectrophotometer was used. Results are expressed in HMF milligrams per kg of honey.

Electrical conductivity. Was measured at 20 °C in a 20% (w/v) solution of honey (dry matter basis) in deionised water using a Crison model 524 conductimeter (Crison Instruments, Barcelona, Spain), according to Vorwohl (1964).

Measurement of aw Determination was made by means of a Novasina IC.500 AW-LAB apparatus (Switzerland).

Glucose, fructose and sucrose were obtained by the enzymatic determination method using a sucrose/D-glucose/D-fructose UV test no 716260 (Boehringer Mannheim, Germany). A Pharmacia Biotech Ultrospec-3000 spectrophotometer was used.

Insoluble solids determined following Lord, Scotter, Whittaker, and Wood (1988) method. Results expressed in percentages.

Proline was measured according to the original method of Ough (1969). Proline and ninhydrin form a coloured complex. After adding 2-propanol, the extinction of the sample solution and a reference solution at a wavelength maximum is determined. The proline content is determined from the ratio. Results are expressed in proline milligrams per kilograms honey.

2.3. Statistical analysis

First, a descriptive analysis of the variables was carried out and the normality of the data was also verified by means of the Kolmogorov test.

Secondly, an analysis of variance was made (one way ANOVA) to detect if the factor ORIGIN (with *Citrus* and *Eucalyptus* levels) was significant, namely, if the means of the variables considered were different depending on the type of honey.

As a third step, we proceeded to carry out a study of the bivariate correlations between all the variables, detecting which of them were significant. The Bartlett test of sphericity and the KMO test (Kaiser–Meyer–Olkin measure of sampling adequacy) were also performed to

check whether or not the correlations matrix can be presumed to be the identity. This fact would indicate that the data matrix is suitable for proceeding with a factorial analysis.

The factorial analysis is the objective of the fourth step, in which the main factors identified will explain most of the variability existing in the data matrix.

Lastly, a stepwise discriminant analysis was made on the variables of the study with the aim of determining which of then discriminated best between the honey varieties analysed (*Citrus* spp. and *Eucalyptus* spp.), as well as establishing a mathematical model for this purpose.

Statistical package for Social Science (SPSS) was used for these objectives.

3. Results

The data analyzed for the different variables considered come from two types of unifloral Andalusian honey (*Citrus* spp. and *Eucalyptus* spp.).

Firstly, Table 1 shows the usual descriptive statistics of the variables considered, both total, and for each of the two types of honey analyzed. Also, from the results of the Kolmogorov test on the normality of sample distributions, it can be concluded that all of them were significantly normal (p-value>0.05).

The one-way analysis of variance, considering the factor ORIGIN (with *Citrus* and *Eucalyptus* categories) shows that, in general, this factor is significant. In particular it can be affirmed (p-value<0.008) that this occurs in the variables: free ac, proline, electrical conductivity, and diastase.

Moreover, the Levene test on the homogeneity of variances shows that these are different (between the factor levels) in the variables proline and HMF (p-value<0.001), i.e. that the variation existing between the data of these variables is significantly different according to whether one or other of the honey types is being considered.

The KMO and Bartlett tests show that even when the KMO index (0.382) is fairly low, the Bartlett sphericity test is significant ($p < 0.001$), therefore, there are significant intercorrelations between the variables. This fact indicates that the data matrix is suitable for proceeding with a factorial analysis.

Significant correlations (p-value<0.05) exist between pH and total sugar, between free ac. and proline, conductivity and fructose, between lac ac. and solids and invertase, between water and total sugar and aw, between proline and diastase and sucrose, between solids and fructose, between glucose oxidase and invertase and HMF, between invertase and diastase and HMF, between glucose and fructose, and finally between fructose and aw.

In this regard, the factorial analysis made indicates that the greater part (78.95%) of the variation existing in the data can be explained by the overall effect of six main factors, as shown in Fig. 1. (The number of factors to be considered coincides with the abscissa point corresponding to the point of inflexion).

The percentage of the variance explained for each factor is shown in Table 2.

The saturations of each variable in the six factors considered are those shown in Table 3.

These saturations establish six groups of variables. The first (saturated to a higher degree in the first factor) is constituted by that formed by the variables free acidity, water, invertase, total sugar, electrical conductivity and solids. The second group is formed by the variables diastase, fructose, glucose and *aw*. A third group is formed by glucose oxidase, proline and HMF.

The fourth, fifth and sixth are groups formed by the individual variables lactone acidity, sucrose and pH, respectively.

Lastly, a stepwise discriminant analysis was made on the variables of the study. In the first place, the M de Box test on population equality of the covariance matrices was significant (p-value>0.05) as the prior condition for using the discriminant analysis establishes.

The stepwise discriminant procedure showed that the variables which discriminated most were the electrical conductivity (EC) and aw (p-value<0.001 in both cases).

The high canonical correlation (0.897) is indicative of the discriminant analysis model being fairly acceptable. This fact is corroborated by the Wilks test being significant (p-value<0.001).

Even when the two variables discriminate well and should be in the discriminant analysis model, the electrical conductivity has more influence on this discriminatory function (the standardized canonical coefficient is higher in the variable electrical conductivity, 1.105 compared to −0.595). By means of an analysis of variance, it was noted that electrical conductivity proved to have the highest discriminatory power (Stefanini, 1984)

The canonical discriminant function is

$$D = 8.126 + 11.739\text{Conductivity} - 22.367\text{aw}.$$

So, a sample is therefore classified in one or other group (*Eucalyptus* or *Citrus*) depending on whether its discriminant score is nearer to the discriminant score of its means. Thus, the discriminant scores for eucalyptus (D_{eu}) and for citrus (D_{ci}) are

$$D_{eu} = 1.822\ yD_{ci} = -2.102.$$

Another classification rule, easily deducible from the previous one, would be to classify as *Eucalyptus* those samples whose discriminant score is over −0.14. ($D > -0.14$). According to this criterion, the percentage of well-classified cases is 96.6% (Table 4).

As an additional proof, the classification criterion for nine additional incomplete samples (some values of the variables considered as being unavailable) was used and

Table 1
Descriptive statistics

Parameter[a]		N	Mean	SD	SE	95% Confidence interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
pH	*Eucalyptus*	15	4.1067	0.2337	6.035E−02	3.9772	4.2361	3.72	4.64
	Citrus	14	4.0243	0.1855	4.958E−02	3.9172	4.1314	3.80	4.52
	Total	29	4.0669	0.2122	3.941E−02	3.9862	4.1476	3.72	4.64
Free Ac	*Eucalyptus*	15	26.9460	5.5945	1.4445	23.8479	30.0441	19.20	41.51
	Citrus	14	17.7107	5.8504	1.5636	14.3328	21.0887	9.20	29.20
	Total	29	22.4876	7.3211	1.3595	19.7028	25.2724	9.20	41.51
Lac Ac	*Eucalyptus*	15	13.7240	4.3391	1.1204	11.3211	16.1269	2.70	18.53
	Citrus	14	13.9950	5.4039	1.4443	10.8749	17.1151	6.94	27.50
	Total	29	13.8548	4.7949	0.8904	12.0309	15.6787	2.70	27.50
Water	*Eucalyptus*	15	16.6333	1.1721	0.3026	15.9842	17.2824	14.90	18.60
	Citrus	14	16.5429	1.0508	0.2808	15.9361	17.1496	14.90	18.40
	Total	29	16.5897	1.0962	0.2036	16.1727	17.0066	14.90	18.60
Proline	*Eucalyptus*	15	429.5267	285.0495	73.5995	271.6715	587.3818	112.08	986.63
	Citrus	14	185.4431	126.5551	33.8233	112.3723	258.5138	36.95	417.25
	Total	29	311.6932	251.9335	46.7829	215.8628	407.5236	36.95	986.63
Solids	*Eucalyptus*	15	3.5467E−02	5.9455E−02	1.5351E−02	2.5419E−03	6.8391E−02	0.000	0.240
	Citrus	14	3.2286E−02	4.6282E−02	1.2370E−02	5.5630E−03	5.9008E−02	0.000	0.186
	Total	29	3.3931E−02	5.2579E−02	9.7637E−03	1.393 1E−02	5.3931E−02	0.000	0.240
Glucose oxidase	*Eucalyptus*	15	4.8260	6.9504	1.7946	0.9770	8.6750	0.00	25.00
	Citrus	13	4.8077	4.8101	1.3341	1.9010	7.7144	0.00	10.00
	Total	28	4.8175	5.9440	1.1233	2.5126	7.1224	0.00	25.00
Conductivity	*Eucalyptus*	15	0.5299	0.1106	2.856E−02	0.4687	0.5912	0.36	0.68
	Citrus	14	0.2157	6.790E−02	1.815E−02	0.1765	0.2549	0.11	0.37
	Total	29	0.3782	0.1838	3.414E−02	0.3083	0.4482	0.11	0.68
Invertase	*Eucalyptus*	15	11.0840	13.4691	3.4777	3.6251	18.5429	0.20	50.05
	Citrus	14	4.9643	6.0739	1.6233	1.4573	8.4712	0.20	20.60
	Total	29	8.1297	10.8408	2.0131	4.0060	12.2533	0.20	50.05
Diastase	*Eucalyptus*	15	24.2960	11.1848	2.8879	18.1021	30.4899	1.47	49.42
	Citrus	14	13.9536	7.9978	2.1375	9.3358	18.5714	5.94	35.35
	Total	29	19.3031	10.9504	2.0334	15.1378	23.4684	1.47	49.42
Glucose	*Eucalyptus*	15	27.8987	7.0393	1.8175	24.0004	31.7969	15.98	45.25
	Citrus	14	25.8743	6.6079	1.7660	22.0590	29.6896	10.69	38.75
	Total	29	26.9214	6.7903	1.2609	24.3385	29.5043	10.69	45.25
Fructose	*Eucalyptus*	15	34.7233	9.0960	2.3486	29.6861	39.7605	17.00	45.00
	Citrus	14	33.1929	8.7679	2.3433	28.1304	38.2553	13.55	42.19
	Total	29	33.9845	8.8129	1.6365	30.6322	37.3367	13.55	45.00
Sucrose	*Eucalyptus*	15	3.3633	3.0384	0.7845	1.6807	5.0459	0.16	8.74
	Citrus	14	4.1886	4.1239	1.1022	1.8075	6.5696	0.14	11.49
	Total	29	3.7617	3.5620	0.6614	2.4068	5.1166	0.14	11.49
aw	*Eucalyptus*	15	0.5600	2.360E−02	6.094E−03	0.5469	0.5731	0.53	0.60
	Citrus	14	0.5714	2.852E−02	7.62 1E−03	0.5550	0.5879	0.53	0.63
	Total	29	0.5655	2.627E−02	4.877E−03	0.5555	0.5755	0.53	0.63
HMF	*Eucalyptus*	15	10.9893	7.2460	1.8709	6.9766	15.0020	0.96	28.52
	Citrus	14	16.5481	16.5754	4.4300	6.9778	26.1185	1.10	53.80
	Total	29	13.6729	12.7202	2.3621	8.8344	18.5114	0.96	53.80
Total sugar	*Eucalyptus*	15	81.9900	0.9804	0.2531	81.4471	82.5329	80.40	83.50
	Citrus	14	81.9286	0.9547	0.2552	81.3774	82.4798	80.00	83.30
	Total	29	81.9603	0.9512	0.1766	81.5985	82.3221	80.00	83.50

[a] Units: Free ac. and lac. ac. (miliequivalents/kg); water, solids and total sugars (%); proline (mg/kg); glucose oxidase (μg glucose, fructose and sucrose (% of H_2O_2 and hour); E.C. (Siemens/cm); Invertase (Invertase Number); Diastase (°Gothe); total sugar); HMF (mg/kg).

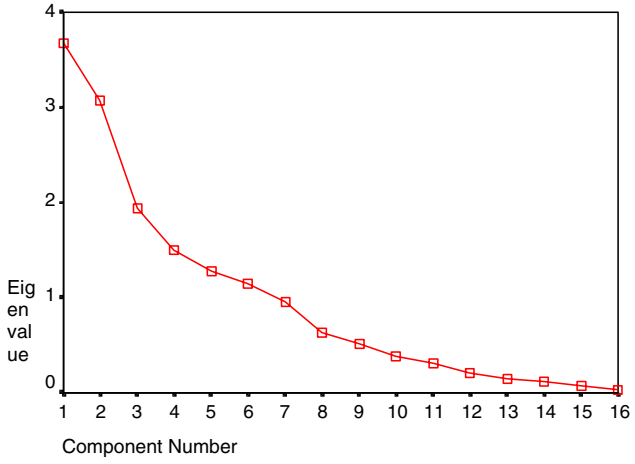

Fig. 1. Overall effect of six main factors.

the nine samples were classified correctly, so that the correct classification index rose to 97.36%.

4. Discussion

Comparing our results for *Citrus* honey with those of other authors (Mateo & Bosch-Reig, 1997; Persano et al., 1995; Thrasyvoulou & Manilas, 1995) we observed the coincidence of the data obtained with the exception of the values for hydroximethylfurfural (16.5 mg/kg) and proline (185.4 mg/kg). With respect to the former, the obtention of a higher value than 6 mg/kg (a normal value in *Citrus* honeys) is due to the influence of the commercial honeys which had high values, although they were within permitted limits. With regard to the

Table 2
Total variance explained

Component	Initial Eigenvalues			Extraction sums of squared loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.679	22.992	22.992	3.679	22.992	22.992
2	3.076	19.224	42.216	3.076	19.224	42.216
3	1.939	12.119	54.335	1.939	12.119	54.335
4	1.505	9.403	63.738	1.505	9.403	63.738
5	1.281	8.006	71.744	1.281	8.006	71.744
6	1.153	7.207	78.951	1.153	7.207	78.951
7	0.958	5.984	84.935			
8	0.638	3.990	88.925			
9	0.510	3.189	92.113			
10	0.375	2.346	94.459			
11	0.313	1.957	96.416			
12	0.204	1.273	97.689			
13	0.154	0.960	98.648			
14	0.113	0.704	99.352			
15	7.685E−02	0.480	99.833			
16	2.679E−02	0.167	100.000			

Extraction method: Principal component analysis.

Table 3
Component matrix

	Component					
	1	2	3	4	5	6
Free ac	0.698	−0.109	0.363	−0.137	0.481	
Water	0.671	−0.330	−0.136	0.369	−0.151	0.254
Invertase	0.652	0.365	−0.398			−0.218
Total sugar	−0.616	0.419		−0.397	0.326	
Conductivity	0.615	0.400	0.296		0.438	0.292
Diastase	0.550	0.538		0.254	0.133	
Solids	0.531	−0.310	0.235	−0.456	−0.326	−0.167
Fructose	−0.225	0.779	−0.101		−0.372	0.112
Glucose		0.726		0.221	−0.245	
a_w	0.394	−0.642	−0.229			0.391
Glucose oxidase	0.180	0.221	−0.791		0.225	0.281
Proline	0.501	0.385	0.578	0.140		−0.294
HMF	−0.401	−0.386	0.540	0.260	−0.143	0.211
Lac ac	−0.410	0.114	0.206	0.671	0.355	0.223
Sucrose	−0.363	−0.267	−0.196		0.443	−0.358
pH	−0.150	0.352	0.301	−0.565		0.599

Extraction method: Principal component analysis. Six components extracted.

Table 4
Classification results[a,b,c]

		Origin	Predicted group membership		Total
			Eucalyptus	*Citrus*	
Original	Count	*Eucalyptus*	14	1	15
		Citrus	0	14	14
	%	*Eucalyptus*	93.3	6.7	100.0
		Citrus	0.0	100.0	100.0
Cross-validated	Count	*Eucalyptus*	14	1	15
		Citrus	0	14	14
	%	*Eucalyptus*	93.3	6.7	100.0
		Citrus	0.0	100.0	100.0

[a] Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.
[b] 96.6% of original grouped cases correctly classified.
[c] 96.6% of cross-validated grouped cases correctly classified.

content in proline, because of its great variability and in agreement with Sancho, Muniategui, Huidobro, and Simal (1991), it was seen not to be a good parameter for finding out the origin of the honey. For *Eucalyptus* honeys, no differences were found to the data obtained by other authors (Costa et al., 1999; Mateo & Bosch-Reig, 1997; Persano et al., 1995).

With respect to the enzymatic content, the *Eucalyptus* honeys showed themselves to be richer than the *Citrus* honeys, which are naturally poor in enzymes. In this aspect we coincide with Huidobro et al. (1995) who, in *Eucalyptus* honeys, find a range of diastasic activity of 10.6–24.7° Gothe and for invertase activity 87.3–153.4 μm ρ-nitrophenyl glycoside hydrolysed/kg honey/min.

The low content in glucose oxidase (4.8 μg peroxide/g/h) may be due to honey samples with a high content in HMF and/or a low level of diastase, or to the low enzymatic content typical of some honeys (honey of *Robinia pseudoacacia*, *Citrus* spp. and honey of *Apis cerana*, Kerkvliet, 1996).

In the light of the results obtained by PFA, 78.95% of the variance between the *Citrus* and *Eucalyptus* types is explained. Of the six main factors obtained, the first factor explaining 22.9% of the variance includes the variables free acidity, water, invertase, total sugars, solids and electrical conductivity. We consider that these variables are clear parameters of a botanical origin for the honeys, except solids. This first group does not coincide with the results of Krauze and Zalewski (1991), who associate electrical conductivity, free acidity, proline and pH as being significant parameters in the classification.

Our second factor (19.22% of variance) is made up of the variables diastase, fructose, glucose and aw, which we interpret as indicators of the maturity of the honey.

The third factor is composed of glucose oxidase, proline and HMF, being both glucose oxidase and HMF indicators of honey deterioration. Even though proline is a parameter associated to botanical origin, its great variability has screened the association with the first factor, proving one more time it is not a suitable parameter in honey classification.

Our fourth, fifth and sixth factors correspond to lactone acidity, sucrose and pH, respectively. The possibility of including pH in the fourth factor with the lactone acidity (see saturation numbers in Table 3), should reduce the number of factors. This relation is moreover logical according with Terrab, Díez, and Heredia (2002), understanding the pH as measure of lactones in honey.

Sanz, Pérez, Herrera, Sanz, and Juan (1995) conclude that the determination of the geographical origin in honeys from la Rioja (Spain) is possible with 83% precision using a factorial analysis and a discriminant analysis with legal quality control parameters without it being necessary to carry out a polynic analysis for this purpose. However, they found acidity, pH, electrical conductivity, ash, HMF and diastase as classification factors. The aw, as a second discriminant variable coincides with Pena-Crecente and Herrero Latorre (1993), who establish moisture and acidity as classification factors in their study of the geographical origin of honeys from Galicia. However, Popek (2002) concludes that the water content is invariable in various honeys, although the concept of water activity differs from the concept of moisture. At any rate, also in our study the water activity is also an important variable capable of discriminating.

5. Conclusions

From the statistical study by PFA and SDA of the variables analyzed for the *Citrus* spp. and *Eucalyptus* spp., six main factors explain 78.95% of the total of the variance between both types. Of these variables, electrical conductivity and water activity reached the highest

discriminant power, the classification of the types being possible in 96.6% of cases.

Thus, the electrical conductivity is a parameter well known as proof of botanical origin for honeys. On the other hand, the water activity is a variable rarely described as discriminant for the different honey types. In the light of our results this water activity should be included as variable in the statistical studies for classification of honeys.

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