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Effect of country origin on physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys

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Summary

The aim of this study was to evaluate the influence of country (Spain, Romania, and Czech Republic) and botanical origin, on the physicochemical (HMF, diastase activity, moisture content, electrical conductivity), colour (Pfund scale and CIEL*a*b*), principal sugars (glucose, fructose and sucrose) and volatile composition of acacia, sunflower and tilia honeys. PCA analyses considering these variables showed that honey type had a far greater influence on the differentiation of samples (above all due to the presence of certain volatile compounds such as carvacrol and α-terpinene for tilia honey;α-pinene and 3-methyl-2-butanol for sunflower honey, and cis-linalool oxide for acacia honey) than geographical origin. Discriminant models obtained for each kind of botanical honey (classified 100% for acacia and tilia honeys and 93.8% for sunflower of the cross-validated cases) confirmed that differentiation of honeys according to their country was mainly based on volatile compounds

(For instance: 2-methyl-2-butenal and 2-methyl-2-propanol, for acacia honeys; 1-hexanol and α-pinene, for sunflower honeys and 3-methyl-1-butanol and otrienol, for tilia honey) and to a lesser extent on certain physicochemical parameters such as diastase, sucrose and conductivity, respectively. Correct classification of all samples was achieved with the exception of 10% of the sunflower honeys from the Czech Republic. The results suggest that the presented models are potentially useful tools for the classification of acacia, sunflower and tilia honeys according to the country of origin.

Keywords

Acacia honey; sunflower honey; tilia honey; country origin; physicochemical parameters; volatile compounds

1. Introduction

Consumers appreciate the possibility to choose between different unifloral honeys as they have specific organoleptic characteristics and different attributable therapeutic properties. Since these unifloral honeys are part of the import-export market, they offer beekeepers and the industry the opportunity to obtain higher prices in comparison to those without a determined botanical origin. Physicochemical properties and colour are taken into account when the market price of honey is fixed, and they can be measured to classify and typify the raw batches before entering the packaging process. Specifically, colour is one of the most valuable attributes since it is considered to represent the preferred honey flavour, and therefore directly contributes to consumer acceptability (Visquert, Vargas & Escriche, 2014).

The physicochemical properties of honeys with the same floral source can vary to some extent as a consequence of different climatic conditions or different geographical origins

(Anklam, 1998). The use of botanical appellation of honey together with geographical origin is becoming a good option to protect and promote this traditional food in different countries.

Melissopalynological characterization is commonly used for the classification of honey according to its uniflorality, and sometimes its geographical origin. However, in some cases the percentage of pollen is not always decisive because the production of pollen and nectar by flowers is not always simultaneous, varying between countries and even within the same country according to the geographical area (Feás, Pires, Iglesias & Estevinho, 2010a; Feás, Pires, Estevinho, Iglesias, & Pinto de Araujo, 2010b). For this reason, in addition to the quantification of pollen, the combination of multi-component analysis and chemometric techniques is now the most efficient approach to guarantee the authentication of honey (Anklam, E., 1998, Terrab, Gustavo-González, Díez & Heredia, 2003, Ruoff, et al., 2007). Kropf, et al., 2010). Among these procedures, physicochemical (electrical conductivity, diastase activity, moisture, etc.), colour and chemical analyses (such as sugars, among others) have been widely used in the characterization of unifloral honeys (Persano-Oddo & Bogdanow, 2004, Ruoff, et al., 2007, Escriche, Kadar, Juan-Borrás & Domenech, 2011, Oroian, Amariei, Escriche, & Gutt, 2013).

However, the discriminative power of the physicochemical properties and colour varies according to the botanical origin, and the geographical and climatic conditions as a consequence of their influence on the flowering or secretions of plants. For this reason, as suggested by Persano-Oddo & Bogdanow, 2004, the broader the analytical scope considered, the more accurate the classification of a specific honey.

Hence, considering that the flavour and aroma of honey are directly related to its volatile compounds, it is reasonable to consider that volatile fraction analysis could be of great

68 importance to reach a better understanding of the intrinsic characteristics of honey (Cuevas-
69 Glory, Pino, Santiago, & Sauri-Duch, 2007; Aliferis, Ttarantilis, Harizanis, & Alissandrakis,
70 2010). The importance of this analytical determination on its own or as a complement to the
71 information provided by other methodologies is reflected in different studies published in the
72 last decade (Radovic, Careri, Manglia, Musci, Gerboles, & Anklam, 2001; Serra-Bonvehí &
73 Ventura-Coll, 2003).

74 There are many works focused on the characterization of honey from different botanical or
75 geographical origins. However, to our knowledge there are no publications about the
76 combined use of physicochemical, sugar and volatile composition for this purpose, nor the
77 comparison of specific unifloral honeys (with the same botanical origin), from different
78 countries. For this reason, the aim of this study was to determine the influence of the country
79 (Spain, Romania, and Czech Republic) on the physicochemical, sugar and volatile
80 composition of acacia, sunflower and tilia honeys.

81 **2. Materials and methods**

82 *2.1. Honey samples and their classification*

83 A total of 80 raw unifloral honey samples (collected from beekeepers in 2011) with
84 different botanical origins: Acacia (*Robinia pseudoacacia*), sunflower (*Helianthus annuus)*
85 and tilia or lime (*Tilia sp*), and from different countries (Spain, Romania, and the Czech
86 Republic) were analysed. The acacia and sunflower honeys came from the three countries
87 mentioned above, whereas tilia honey was only from Romania and the Czech Republic since
88 it is practically inexistent in Spain. In summary, of the 80 raw samples, 30 came from
89 Romania (10 acacia, 10 sunflower and 10 tilia, all of them from the Transylvanian region);
90 another 30 came from the Czech Republic (10 acacia, 10 sunflower and 10 tilia, all of them

from the Central Bohemian region) and 20 from Spain (10 acacia from northern Spain and 10 sunflower from central Spain).

In order to guarantee the botanical origin of the samples, the percentage of pollen was measured for each one, following the recommendations of the International Commission for Bee Botany (Von Der Ohe, Persano-Oddo, Piana, Morlot & Martin, 2004). A light microscope (Zeiss Axio Imager, Göttingen, Germany) at a magnification power of x 400 with DpxView LE image analysis software attached to a DeltaPix digital camera was used in this analysis. According to this analysis, a honey was considered to be from acacia trees if the pollen from *Robinia pseudoacacia* L. was not lower than 45%; from sunflower, if the pollen from *Helianthus annuus* L. was not lower than 60% and from tilia trees if the pollen from *Tilia spp*. was not lower than 45% (Sainz-Laín & Gómez-Ferreras, 1999; Gómez-Pajuelo, 2004; Von Der Ohe, et al., 2004, Persano-Oddo & Piro, 2004). Samples were classified on arrival at the laboratory and were preserved at 12ºC until they were analysed. None of the samples exhibited signs of fermentation or granulation before initiating the analyses.

2.2. Physicochemical and colour analyses

Diastase activity (*Phadebas method*), 5-Hydroxymethylfurfural content "HMF" *(White method)*, electrical conductivity (by *conductimetry*), and moisture content (by *refractrometry*) were analyzed in accordance with the Harmonized Methods of the European Honey Commission (Bogdanov, 2002). Colour was determined using a millimetre Pfund scale C 221 Honey Color Analyzer (Hanna Instruments) and a spectrocolorimeter Minolta CM-3600d (Osaka, Japan). Translucency was determined by applying the Kubelka-Munk theory for multiple scattering of the reflection spectra (Hutchings, 1999). Colour coordinates were

obtained from R∞, between 400 and 700 nm for D65 illuminant and 2º observer. All tests were performed in triplicate.

Chromatic parameters Chroma (eq. 1) and hue (eq. 2), were calculated from L*, a* and b* coordinates.

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$h^*_{ab} = arctg \frac{b^*}{a^*} \quad (2)$$

2.3. Sugar determination

Sugar (fructose, glucose and sucrose) analysis was carried out as described by Bogdanov, Martin, & Lüllman, 1997. Separation of carbohydrates took place in a HPAEC-PAD high-resolution ionic chromatograph with a pulsed amperometric detector (PAD) (Bioscan, Methrom, Switzerland) and a Metrosep Carb chromatographic column (styrene divinylbenzene copolymer, 4.6 x 250 mm). Carbohydrates were eluted with NaOH 0.1N at a flow rate of 1 mL min^{-1}. Quantification of sugars was carried out using external standards. The corresponding calibration curves were constructed covering the values of the three sugars which were expected to be found in the honey samples. For fructose, glucose and sucrose, respectively, the correlation coefficients (R^2) were: 0.995, 0.996 and 0.996; the LODs (limit of detection) were: 0.01g/100g, 0.01g/100g and 0.05g/100g and the LOQs (limit of quantification) were: 0.05g/100g, 0.05g/100g and 0.1g/100g.

All analyses were carried out in triplicate.

2.4. Volatile compounds analysis

2.4.1. Extraction

Volatile compounds were extracted by purge and trap at 45ºC for 20 minutes and trapped in a glass tube packed with Tenax TA (20-35 mesh), bubbling purified nitrogen (100 mL min^{-1}) through the sample (Escriche et al., 2011). Next, the compounds were thermally desorbed at 220ºC for 10 minutes (at 10 mL min^{-1} helium flow) (TurboMatrix TD, Perkin ElmerTM, CT-USA), then cryofocused in a cold trap at -30ºC and transferred onto the capillary column by heating the cold trap to 250ºC (at a rate of 99ºC/s).

2.4.2. GC-MS analysis

A GC-MS (Finnigan TRACETM MS, TermoQuest, Austin, USA) with a DB-WAX capillary column (SGE, Australia) (60 m length, 0.32 mm i.d., 1.0 µm film thickness) was used to separate the volatile compounds. The carrier gas was Helium at a flow rate of 1 mL min-1. The temperature programme was: from 40ºC (2-minute hold time) to 190ºC at 4ºC min^{-1} (11-minute hold time) and finally to 220ºC at 8 ºC min^{-1} (8-minute hold time). Electron impact mass spectra were recorded in impact ionization mode at 70 eV, with a mass range of m/z 33-433. A total of 3 extracts were obtained for each sample.

2-pentanol was used as an internal standard. The identification of isolated volatile compounds was performed by comparing their mass spectra, retention times and linear retention indices against those obtained from authentic standards: acetic acid (ethanoic acid); nonanal; decanal; benzaldehyde; 6-methyl-5-hepten-2-one (6-methyl-hept-5-en-2-one); 2-methyl-3-buten-2-ol (Sigma-Aldrich, San Louis, Missouri and Acros Organics, Geel, Belgium); 2-methyl-1-propanol (2-methylpropan-1-ol); 3-methyl-3-buten-1-ol; octane; 3-hydroxy-2-butanone (3-hydroxybutan-2-one); 2-furanmethanol (furan-2-ylmethanol); furfural (furan-2-carbaldehyde); dimethyl sulphide; β-linalool (3,7-dimethylocta-1,6-dien-3-ol) (Fluka Buchs, Schwiez, Switzerland). The compounds for which it was not possible to find authentic

157 standards) were tentatively identified by comparing their mass spectra (m/z values of the most
158 important ions) with spectral data from the National Institute of Standards and Technology
159 2002 library as well as retention indices and spectral data published in the literature
160 (Kondjoyan & Berdagué, 1996; Radovic et al., 2001; Soria, Gonzalez de Lorenzo, Martinez-
161 Castro, & Sanz, 2004; De la Fuente, Martinez-Castro, & Sanz, 2005; Bianchi, Careri, &
162 Musci, 2005; Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2005). A mixture of a
163 homogenous series of alkanes (C8-C20 by Fluka Buchs, Schwiez, Switzerland) was injection
164 into the Tenax in the same temperature-programmed run, as described above in order to
165 determine the Kovàts retention indices of all the compounds. Due to fact that was not possible
166 to obtain authentic commercial standards for all the identified compounds, the variables used
167 in the statistical analysis for differentiation between honeys corresponded to semiquantified
168 compounds. These data were calculated (μg/100 g of honey) using the amount of internal
169 standard, the relative area between the peak areas of each compound and the peak area of the
170 internal standard, assuming a response factor equal to one (Castro-Vazquez et al., 2009).

171 *2.5. Statistical analysis*

172 A multifactor analysis of variance (ANOVA) (using Statgraphics Centurion for Windows)
173 was carried out to study the influence of the type of honey and the country of harvesting on
174 the physicochemical parameters, colour, sugars and volatile compounds. The method used for
175 multiple comparisons was the LSD test (least significant difference) with a significance level
176 $\alpha = 0.05$. In addition, data were analyzed using a Principal Component Analysis (PCA)
177 applying the software Unscrambler X.10 and a Stepwise Linear Discriminant Analyses
178 (SLDA) using "forward" procedure (SPSS 16.0). This analysis selects the variables that allow
179 differentiation between honeys. The classification functions corresponding to each group of

honeys were calculated. The statistical F function was used as a criterion for variable selection.

3. Results and discussion

3.1. Physicochemical, colour and sugar analyses

Table 1 shows the results of the analysis of the three types of honey harvested in the different countries: the average values and standard deviation of the physicochemical parameters (HMF, diastase activity, moisture content, electrical conductivity); colour (Pfund and CIEL*a*b*); the percentage content of the principal sugars (glucose, fructose and sucrose) and the fructose/glucose ratio. In addition, this table shows the ANOVA results (F-ratio and significant differences) obtained for the factors "type of honey" and "country". For the country factor, each type of honey was considered separately.

Although raw honey was used, hydroximethylfurfural (HMF) was evaluated to corroborate the freshness. All the analyzed honeys complied with the international maximum limit of 40 mg/kg (Council Directive 2001/110 relating to honey, 2002). Acacia honey had the lowest average values (from 3.3 to 7.2 mg/kg), and sunflower honey, especially from Romania, and the Czech Republic, had surprisingly high average values (23.4 and 21.9 mg/kg, respectively), taking into account that they were fresh, non-thermally treated samples. These values are in accordance with Kádár, Juan-Borrás, Hellebrandova, Doménech & Escriche (2010) and Oroian, M. (2012).

Diastase is one of the most important enzymes in honey. Its concentration varies not only according to its botanical origin, but also due to aging and extreme temperatures (Fallico, Arena, Verzera, & Zappalà, 2006). In this study samples ranged from 8.7 ºGoethe in acacia honey from the Czech Republic to 19.1 ºGoethe for Spanish sunflower honey. All the samples

complied with the Council Directive 2001/110 relating to honey (2002), which stipulates that these types of honeys should have a value higher than 8ºGoethe. The only exception is acacia honey for which a minimum of 3.1ºGoethe is admitted, as it is considered to have low enzyme content,. However, in this paper such low values were not found in any of the analyzed acacia honeys.

Honey moisture content, which can vary from year to year, depends not only on environmental conditions, but also beekeeping practices (Acquarone, Buera, & Elizalde, 2007). Taking into account the fact that the moisture content of honey has to be lower than 20 g/100g (Council Directive 2001/110 relating to honey, 2002), the values obtained in this work were satisfactory as they ranged from 15.3 g/100g in sunflower honey from Spain to 17.5 g/100g in tilia honey from Romania. Spanish acacia and sunflower honeys showed the best moisture values, lower than 16%.

As expected, tilia honey had the highest levels of conductivity, with average values of 0.80 and 0.50 mS/cm from the Czech Republic and Romania, respectively. Values higher than 0.80 mS/cm are not acceptable for floral honeys in general; however there are some specific honeys that can exceed this value. This is the case of tilia honey and others such as Calluna, Erica or Arbustus, because of the mineral content of these honeys. On the contrary, the low level of minerals in acacia honey is reflected by its low electrical conductivity (0.17-0.19 mS/cm) (Feás et al., 2010a). Nosignificant differences were observed between countries.

With regard to colour, semi-qualitative Pfund scale and colour coordinates CIEL* a* b* were measured (Table 1). CIEL* a* b*colour coordinates, and chromatic parameters (hue and chroma), are not commonly regulated. However, they are often used in research studies to supplement the information provided by the Pfund scale. In this work Pfund values ranged

from 4.3 mm for the acacia honey from the Czech Republic to 66.7 mm for sunflower honey from Spain. Acacia honey is characterized by a very light colour together with low conductivity. This is logical as honey colour is mainly related to mineral content. Light coloured honeys usually have low mineral levels, while dark coloured honeys normally have high mineral content (Al, Daniel, Moise, Bobis, Laslo, & Bogdanov, 2009).

In relation to CIEL* a* b* values, acacia honey had the highest lightness (especially from Romania: L*= 56.6), a yellowish hue (average value of 91.3) and the lowest chroma (average value of 17.3) which is associated with the lowest colour purity. On the contrary, sunflower honey was the darkest (lowest L*, with an average value of 44.9), with the same chroma as tilia honey and the lowest hue of the three types of analyzed honeys: 74.0, 81.6 and 91.3 for sunflower, tilia and acacia, respectively. In general, the tilia honey had intermediate L* values (average= 48.6), lower than those found by Kropf, et. al., 2010 (between 60.3 and 62.3), who analyzed this type of honey from three different geographical regions of Slovenia.

In general, the colour values obtained with the Pfund scale as well as CIEL*a*b, were as expected for these varieties of honey (Persano-Oddo, Piazza, Sabatini, & Accorti, 1995; Piazza, & Persano-Oddo, 2004).

The sugar composition of honey depends of the type of flowers used by the bees, and therefore varies according to the type of honey and geographical and climatic conditions (Mateo & Bosch-Reig, 1998; Al, et al., 2009; Kaskonienè, Venskutonis & Ceksterytè, 2010). For this reason, the level of some sugars and even the ratios between them are used to ascertain honey authenticity (Nozal et al., 2005). As expected, fructose was the most dominant sugar followed by glucose in all cases (Persano-Oddo & Piro, 2004). Acacia had high fructose (49.2g/100g for acacia from the Czech Republic) and low glucose content

(26.8g/100g for the acacia honey from Spain). Acacia honeys showed the highest sucrose content, as reported by Persano Oddo, et al. in 1995. In this study the Spanish acacia had the highest sucrose level: 2.2 (g/100g). Sunflower had a very high glucose level (average=36.3 g/100g) compared to both the other honeys and therefore a very low F/G ratio (average=1.06).

In respect to the fructose-glucose ratio F/G ratio, acacia and tilia honeys are characterized by high F/G values in contrast to sunflower honeys, as reported in previous works (Persano-Oddo et al., 1995) and as established by European legislation (Council Directive 2001/110 relating to honey, 2002). The values obtained in the present work (averages=1.6, 1.3 and 1.06 for acacia, tilia and sunflower) are in accordance with these .

Besides that, it is important to point out that the fructose-glucose ratio (F/G) indicates whether a honey will granulate; the lower the ratio, the quicker the crystallization. Accordingly, the order of crystallization of the three types of honey analyzed in this study is: sunflower honey (F/G= 1.06), tilia honey (F/G= 1.3), and acacia honey (F/G= 1.6).

Almost all the physicochemical, colour and sugar parameters differed significantly between the three botanical types of honey studied. However, considering each type of honey separately, significant differences between countries were only found for diastase activity (for acacia and sunflower honeys), conductivity (for tilia honey) as well as for some sugars (glucose for acacia and tilia, fructose for acacia, and sucrose for sunflower). In the same way, the F/G ratio differed significantly between countries for acacia and tilia.

In order to evaluate the global effect of the type of honey on the physicochemical parameters, colour, and F/G ratio from a descriptive point of view, a principal component analysis (PCA) was performed. Figure 1 shows the PCA bi-plot of scores and loading obtained considering the eight analysed honeys and the different parameters. The values of

HMF and moisture were not taken into account, as both are mainly related to the quality of honey and not to the botanical origin, and therefore are not useful for differentiation between honeys. This analysis was carried out considering the average values of each parameter obtained from each type of honey and country (the code for each point in the figure corresponds to: kind of honey-country). In the score plot, proximity between samples reflects similarity in relation to the analysed parameters.

Two principal components explained 74% of the variations in the data set. PC1 (55%) and PC2 (19%). The first principal component differentiates the three kinds of honeys to a certain extent. Acacia located on the left was differentiated clearly from the others, while sunflower and tilia on the right, are not so obviously separated from each other. This indicates that although the botanical origin of honey has an influence on the parameters studied, these are not sufficient for differentiation between the three varieties studied here. On the other hand, the country seems to imply a minor effect on the analysed parameters as the samples were principally grouped according to type of honey.

3.2. Volatile compounds

The average values and standard deviation of the volatile compounds analyzed in the three types of honey harvested in the different countries are showed in Table 2. Of the 51 identified compounds, only 17 compounds in acacia honey, 9 in sunflower and 8 in tilia honeys, showed significant differences between countries. However, considering the type of honey as a factor, significant differences were found for 45 volatile compounds.

Another PCA (Figure 2) was conducted to evaluate the global effect of the type of honey and country, but in this case for the volatile compounds. The distribution of the samples was similar but clearer than the previous bi-plot obtained from the FQ parameters. In this case, the

first principal component clearly differentiates acacia honey (bottom left quadrant) from tilia (bottom right quadrant) and the second principal component differentiates quite well between sunflower (upper quadrants) and acacia and tilia honeys (lower quadrants).

The loading plot shows that certain compounds are to some extent responsible for this differentiation. This is the case of compounds such as carvacrol (Lusic, Koprivnjak, Curic, Sabatini, & Conte, 2007; Plutowska, Chmiel, Dymerski, & Wardencki, 2011) and α-terpinene (Radovic et al, 2001) which were attributed as markers for tilia honey, and were only found in this kind of honey in this work. Plutowska et al. (2011), also only identified α-terpinene in tilia honeys, when analyzing 7 varieties of honeys. The same occurs for other compounds, such as α-pinene and 3-methyl-2-butanol in the case of sunflower, and cis-linalool oxide in the case of acacia which were essential in this work to differentiate these honeys. This is in line with (Radovic et al., 2001) for these two varieties of honey.

The aforementioned authors reported phenylacetaldehyde as a typical volatile compound for acacia honeys and phenylethyl alcohol for tilia honey, though this is not consistent with this study, nor with others (Plutowska et al., 2011).

As observed before for physicochemical parameters, volatile compounds seem to contribute more to the differentiation of honey according to botanical origin, than country of origin.

However, it is logical that honeys with the same botanical origin (*Robinia pseudoacacia* in the case of acacia honeys, *Helianthus annuus* in the case of sunflower honeys and *Tilia* sp in the case of tilia honeys), but from different countries are relatively similar. Nevertheless, there are obvious differences between the geographic sources which could be attributed to climatic conditions, but above all to the surrounding flora. The nectar of other plants may contribute to the variability of the analysed parameters: physicochemical, sugar and volatile compounds.

318 This should not be considered a negative aspect; instead it confers a certain singularity to the
319 same type of honey with different geographical origins.

320 *3.3. Identification of the variables with the highest discriminant power*

321 The information provided by both ANOVA and PCA analyses carried out for
322 physicochemical parameters and volatile compounds, shows that certain variables are to some
323 extent more important in the differentiation of honeys. To discern which variables contribute
324 the most to the differentiation of honeys from different countries but from the same botanical
325 origin a discriminant analysis was applied separately for every botanical type of honey
326 (acacia, sunflower and tilia).

327 Only the variables with significant differences between countries (in ANOVA results)
328 were included in the models. These models, obtained using the physicochemical and volatile
329 compound variables jointly and applying a stepwise method, permitted the classification of
330 100% of acacia and tilia honeys and 93.8% of sunflower for the cross-validated cases. Kadar
331 et al. in 2011 reported that a discriminant model obtained with volatile compounds and
332 physicochemical parameters used jointly and applying a cross-validated procedure was
333 effective for the differentiation of two types of honeys (between lemon blossom honey and
334 orange blossom honey).

335 Table 3 shows the standardized canonical discriminant function coefficients obtained in the
336 selected models for every type of honey. In the construction of the two discriminant functions,
337 different variables were used in each case. Specifically, 7, 6 and 3 volatile compounds and 1
338 physicochemical parameter (diastase activity, sucrose and conductivity) for acacia, sunflower
339 and tilia honeys, respectively.

The higher the absolute value of a standardized canonical coefficient, the more significant a variable is. The first canonical function was the one that discriminated best between honey groups, given that it represented the highest variability. Accordingly, the variables that most contributed to the discrimination of honeys according to their country of origin were: for acacia honeys (which function 1 explained 88.2% of the total variance), 2-methyl-2-butenal, 2-methyl-2-propanol and acetic acid butyl ester; for sunflower honeys (function 1 explained 94.3%), 1-hexanol, sucrose and α-pinene; and for tilia honey (function 1 explained 100%), 3-methyl-1-butanol, hotrienol y 2-butanone. It should be highlighted that despite the appearance of a physicochemical variable in each model, this was not the one which contributed the most in any case.

The classification results (expressed as percentages) of the discriminant analysis carried out by cross validated procedure demonstrated a very good classification of the acacia and tilia honeys according to their country (Table 4). This was also true of sunflower honey from Spain and Romania. However, 10% of sunflower honey from the Czech Republic was incorrectly classified as sunflower honey from Romania.

4. Conclusion

The information obtained about physicochemical parameters and volatile compounds is a useful complement to that provided by the percentage of pollen to distinguish acacia, sunflower and tilia monofloral honeys, with subsequent benefits for beekeepers and the industry. Although it was found that the country (Spain, Romania, and the Czech Republic) may lead to significant variations in the levels of certain parameters and compounds, it is the type of honey that has by far the greatest influence on the differentiation of honeys, above all due to the presence of certain volatile compounds such as carvacrol and α-terpinene in the

363 case of tilia honey, α-pinene and 3-methyl-2-butanol in sunflower honey, and cis-linalool
364 oxide in acacia honey. Discriminant models obtained for each kind of botanical honey
365 confirmed that the differentiation of honeys according to their country of origin was
366 principally based on volatile compounds (2-methyl-2-butenal, 2-methyl-2-propanol, acetic
367 acid butyl ester, etc., for acacia honeys; 1-hexanol, α-pinene, etc., for sunflower honeys and
368 3-methyl-1-butanol, hotrienol, 2-butanone, etc., for tilia honey) and to a lesser extent on
369 certain physicochemical parameters such as, diastase, sucrose and conductivity, respectively.

370 A correct classification of all the samples was achieved with the exception of 10% of the
371 sunflower honeys from the Czech Republic. The main advantage of the model presented is to
372 support the classification of the acacia, sunflower and tilia honeys according to the country of
373 origin. In order to be totally conclusive , it would be advisable to check the predictive
374 capacity of the proposed classification model with additional batches with the same botanical
375 and country origin but from different years

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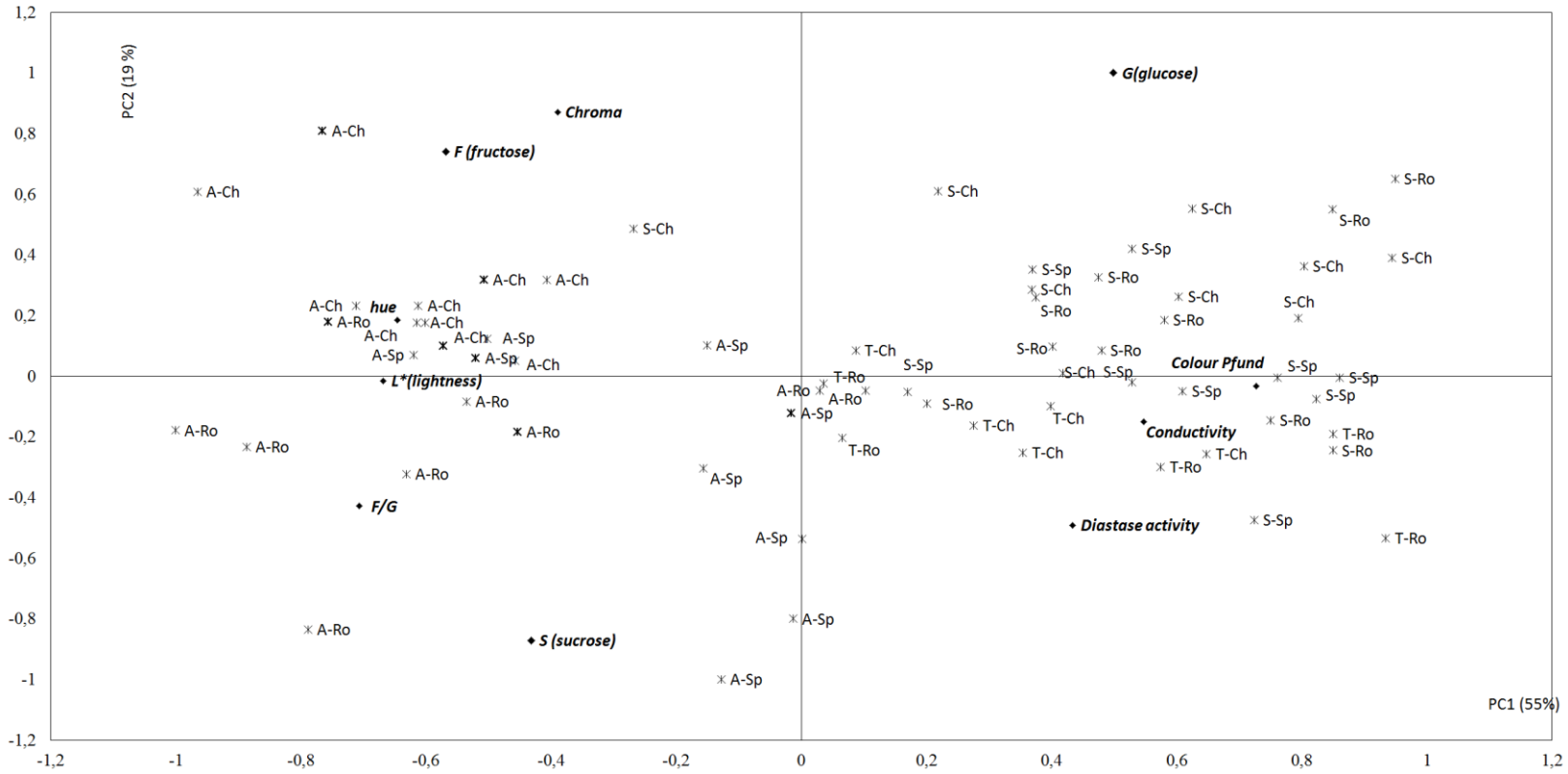

Figure 1. Biplot for the two principal components of the PCA model for the physicochemical parameters, sugars (fructose "F", glucose "G" sucrose "S" and F/G ratio) and colour (Pfund and CIEL*a*b) in acacia, sunflower and tilia honeys harvested in the different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz).

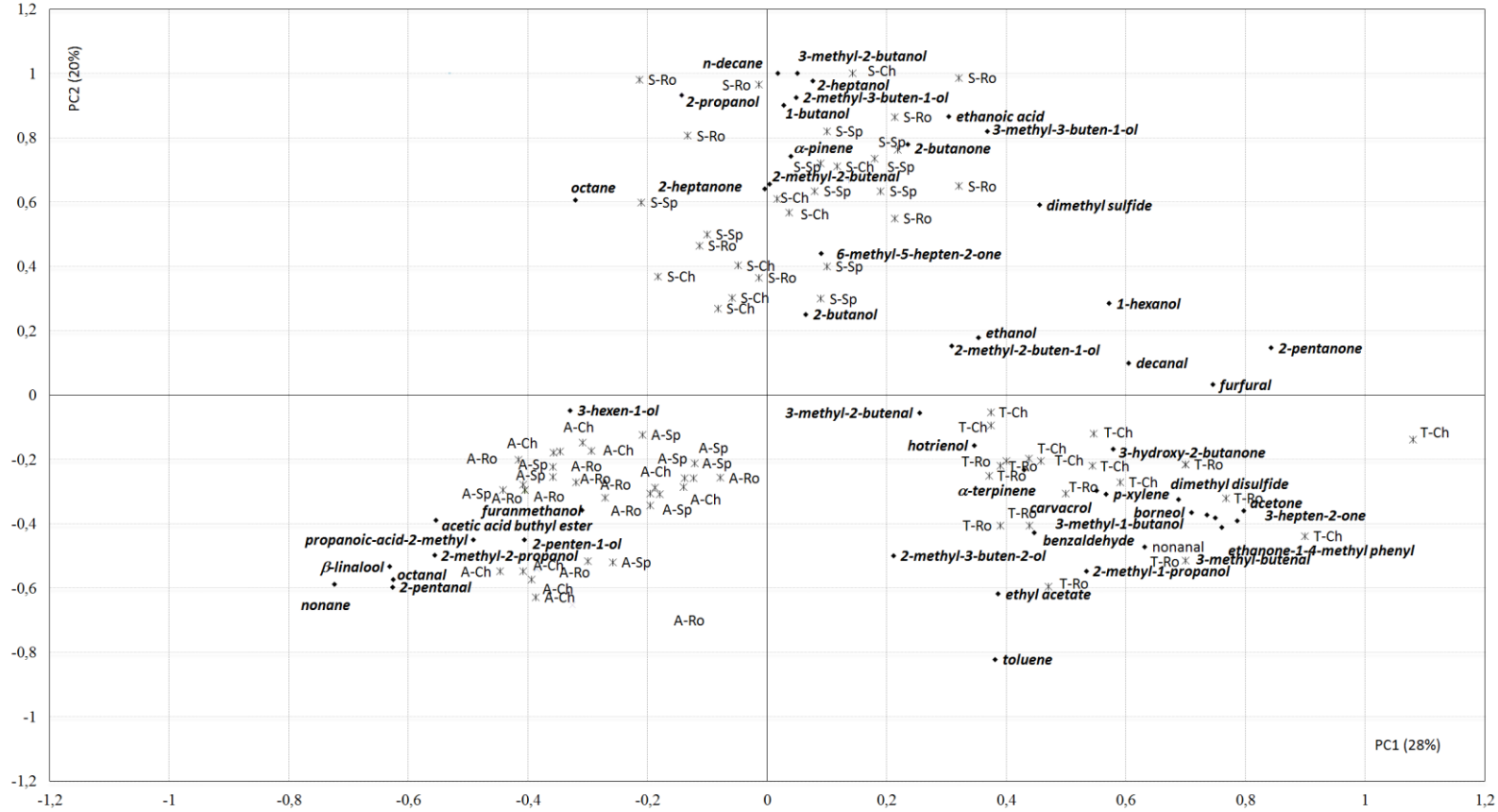

Figure 2. Biplot for the two principal components of the PCA model for the volatiles compounds identified in acacia, sunflower and tilia honeys harvested in the different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz).

Table.1 Physicochemical parameters, colour and principal sugars (average values and standard deviation) in acacia, sunflower and tilia honeys harvested in different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz). ANOVA results (F-ratio and significant differences) obtained for two factors: country and type of honey. For the country factor, each type of honey was considered separately.

	COUNTRY FACTOR											**TYPE OF HONEY FACTOR**			
	Acacia				**Sunflower**				**Tilia**						
Physico-chemical Parameters	**Sp**	**Ro**	**Cz**	**F-ratio**	**Sp**	**Ro**	**Cz**	**F-ratio**	**Ro**	**Cz**	**F-ratio**	**Acacia**	**Sunflower**	**Tilia**	**F-ratio**
HMF (mg kg^{-1})	3.3(1.8)	7.2(11.9)	3.3(2.2)	0.79ns	16.4(3.5)	23.4(0.4)	21.9(7.9)	0.57ns	7.1(6.5)	18.8(14.9)	2.22ns	4.8(7.6)	21.3(7.0)	15.5(13.9)	15.17***
Diastase activity (ºGoethe)	17.3(4.8)	10.4(4.6)	8.7(1.7)	9.93***	19.1(1.2)	10.1(0.8)	11.9(3.7)	4.33*	8.8(0.9)	14.6(5.2)	4.66ns	11.3(4.9)	12.7(4.2)	12.9(5.1)	0.66ns
Moisture (g/100g)	15.9 (0.2)	16.9(1.5)	17.0(1.0)	1.91ns	15.3(0)	17.3(0.14)	16.6(1.04)	2.33ns	17.5(0.12)	16.4(1.05)	3.92ns	16.7(1.16)	16.5(1.0)	16.7(1.1)	0.24ns
Conductivity (µS cm^{-1})	0.19(0.04)	0.17(0.03)	0.17(0.08)	0.32ns	0.44(0.0)	0.35(0.0)	0.43(0.12)	0.52ns	0.50(0.08)	0.80(0.12)	18.29**	0.17(0.05)	0.42(0.10)	0.71(0.17)	106.37***
Colour															
Pfund	9.1(2.5)	10.6 (2.2)	4.3 (1.3)	1.7ns	66.7 (0.5)	51.0 (3)	53.2 (14.6)	0.86ns	37.3(3.9)	42.2(17)	0.15ns	6.9(4.3)	56.3(12.6)	40.8(13.7)	24.8***
L*	50.5(4.3)	56.6(5.8)	54.6(1.8)	2.22ns	43.6(3.4)	48.1(0.1)	44.9(3.5)	0.53ns	49.6(1.2)	48.3(7.6)	0.05ns	54.1(4.7)	44.9(3.3)	48.6(6.2)	9.86***
Chroma (C^*_{ab})	19.6(3.1)	17.8(5.9)	17.8(2.6)	1.60ns	22.9(4.6)	27.4(2.5)	23.8(5.2)	0.27ns	27.2(1.3)	24.5(4.3)	0.68ns	17.3(4.4)	24.1(4.5)	25.4(3.7)	10.36***
Hue (h^*_{ab})	84.4(9.1)	93.2(7.2)	94.9(3.6)	2.96ns	70.4(2.8)	78.5(3.4)	74.6(8.3)	1.56ns	82.7(2.,2)	81.2(7.9)	0.06ns	91.3(7.7)	74.0(6.9)	81.6(6.2)	14.98***
Sugars (g/100g)															
Glucose	26.8(2.7)	26.9(2.8)	31.0(4.5)	7.86**	37.1(0.8)	33.9(1.62)	38.3(7.2)	1.33ns	29.7(0.8)	33.1(1.4)	18.8***	28.5(4.4)	36.3(6.6)	32.2(2.02)	24.49***
Fructose	40.2(3.6)	45.7(2.8)	49.2(6.6)	8.16**	39.3(0.6)	39.5(0.98)	43.0(6.9)	0.68ns	41.3(1.6)	41.9(1.4)	0.38ns	45.2(6.)	40.3(6.1)	41.7(1.5)	3.16ns
Sucrose	2.2(0.8)	1.7(0.6)	1.6 (0.3)	1.63ns	1.04(0.54)	0.60(0.37)	0.7(0.9)	4.92*	0.9(0.4)	1.5(0.1)	0.15ns	1.7(0.5)	0.8(0.5)	0.3(0.2)	7.80**
Fructose/Glucose ratio	1.5(0.1)	1.7(0.2)	1.5 (0.1)	4.49*	1.0(0.1)	1.16(0.02)	1.1(0.1)	3.48ns	1.3(0.1)	1.2(0.03)	38.68***	1.6(0.17)	1.06(0.07)	1.3(0.06)	68.28***

ns: Non significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Table.2. Volatile compounds (average values and standard deviation) in acacia, sunflower and tilia honeys harvested in different countries: Spain (Sp), Romania (Ro), and Czech Republic (Cz). ANOVA results (F-ratio and significant differences) obtained for two factors: country and type of honey. For the country factor, each type of honey was considered separately.

COMPOUNDS		COUNTRY FACTOR											TYPE OF HONEY FACTOR			
	RI	ACACIA				SUNFLOWER				TILIA						
		Sp	Ro	Cz	ANOVA F ratio	Sp	Ro	Cz	ANOVA F ratio	Ro	Cz	ANOVA F ratio	AC	SUN	TIL	ANOVA F ratio
ACIDS																
Ethanoic acid	1584	0.01(0.02)	<0.001	0.03(0.04)	3.80*	0.12(0.06)	0.19(0.13)	0.22(0.18)	0.5ns	0.02(0.02)	0.13(0.12)	3.17ns	0.01c	0.20a	0.10b	15.11***
Propanoic acid 2-methyl-	1697	0.06(0.02)	0.04(0.04)	0.01(0.01)	5.93***	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.03a	0.00b	0.00b	15.53***
ALDEHYDES																
3-Methyl-butenal	935	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.21(0.12)	0.19(0.08)	0.20ns	0.00b	0.00b	0.19a	84.27***
2-Pentanal	937	0.03(0.01)	0.04(0.02)	0.02(0.00)	2.60ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.03a	0.00b	0.00b	61.41***
2-Methyl-2-butenal	1129	0.06(0.03)	0.01(0.00)	0.02(0.01)	10.70***	0.13(0.01)	0.09(0.0)	0.14(0.18)	0.09ns	0.02(0.04)	0.13(0.06)	9.80**	0.02b	0.13a	0.02b	9.70***
3-Methyl-2-butenal	1236	0.07(0.02)	0.08(0.12)	0.06(0.01)	0.18ns	0.13(0.01)	0.05(0.00)	0.07(0.06)	1.23ns	0.02(0.00)	0.02(0.01)	0.24ns	0.07a	0.08a	0.09a	0.67ns
Octanal	1417	0.01(0.00)	0.01(0.00)	<0.001	2.69ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.01a	0.00b	0.00b	52.91***
Nonanal	1523	0.07(0.01)	0.06(0.04)	0.04(0.01)	2.27ns	0.02(0.01)	0.03(0.0)	0.03(0.02)	0.19ns	0.09(0.02)	0.18(0.13)	1.50ns	0.05b	0.03b	0.15a	14.73***
Decanal	1630	0.01(0.00)	0.03(0.01)	0.02(0.00)	8.11**	0.04(0.0)	0.04(0.0)	0.03(0.02)	0.03ns	0.04(0.00)	0.04(0.02)	0.48ns	0.02b	0.03a	0.04a	7.34**
Benzaldehyde	1675	0.13(0.06)	0.25(0.15)	0.20(0.05)	2.15ns	0.12(0.02)	0.14(0.01)	0.13(0.09)	0.02ns	0.14(0.05)	0.37(0.43)	1.04ns	0.2ab	0.13b	0.31a	2.42ns
ALCOHOLS																
2-Methyl-2-propanol	920	0.03(0.02)	0.04(0.04)	0.10(0.02)	8.07**	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.06a	0.00b	0.00b	25.74***
2-Propanol	947	0.03(0.01)	0.10(0.12)	0.02(0.01)	2.91ns	0.25(0.14)	0.19(0.01)	0.21(0.12)	0.11ns	<0.001	<0.001	-	0.05b	0.21a	0.00c	26.23***

Ethanol	956	0.40(0.20)	0.56(0.75)	0.38(0.33)	0.32ns	0.51(0.35)	0.38(0.06)	0.79(0.98)	0.23ns	1.25(0.24)	0.73(0.56)	3.01ns	0.45b	0.69ab	0.88a	2.25ns
2-Butanol	1047	0.20(0.14)	0.03(0.02)	0.05(0.13)	5.19*	0.73(0.90)	0.01(0.0)	0.12(0.21)	3.11ns	0.25(0.08)	0.06(0.02)	50.84***	0.08a	0.19a	0.11a	1.2ns
2-Methyl-3-buten-2-ol	1063	0.11(0.05)	0.11(0.12)	0.16(0.06)	0.89ns	<0.001	<0.001	<0.001	-	0.62(0.48)	0.14(0.06)	10.29**	0.13b	0.00c	0.28a	8.84***
2-Methyl-3-buten-1-ol	1062	<0.001	<0.001	<0.001	-	0.26(0.00)	0.20(0.06)	0.21(0.21)	0.08ns	0.31(0.07)	0.30(0.12)	0.01ns	0.00b	0.21a	0.00b	30.8***
2-Methyl-1-propanol	1119	0.05(0.02)	0.07(0.07)	0.05(0.02)	0.47ns	0.05(0.0)	0.01(0.0)	0.01(0.01)	5.60*	0.27(0.08)	0.16(0.10)	3.52ns	0.06b	0.01a	0.19a	28.99***
3-Methyl-2-butanol	1137	<0.001	<0.001	<0.001	-	0.42(0.04)	0.25(0.01)	0.36(0.04)	6.2*	0.01(0.00)	0.00(0.00)	1.16ns	0.00b	0.36a	0.00b	61.12***
1-Butanol	1175	0.10(0.03)	0.10(0.10)	0.08(0.02)	0.34ns	0.33(0.27)	0.15(0.01)	0.39(0.37)	0.41ns	0.11(0.02)	0.06(0.05)	2.55ns	0.09b	0.35a	0.08b	11.56***
3-Methyl-1-butanol	1233	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.61(0.04)	0.30(0.10)	32.03***	0.00b	0.00b	0.39a	114.95***
2-Penten-1-ol	1268	0.00(0.00)	0.14(0.10)	0.05(0.04)	8.18**	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.07a	0.00b	0.00b	10.72***
3-Methyl-3-buten-1-ol	1277	0.19(0.04)	0.11(0.08)	0.16(0.04)	1.59ns	0.44(0.02)	0.21(0.01)	0.50(0.21)	1.93ns	0.31(0.02)	0.30(0.11)	0.01ns	0.15c	0.45a	0.31b	27.38***
2-Heptanol	1449	<0.001	<0.001	<0.001	-	0.03(0.03)	0.01(0.0)	0.04(0.05)	0.37ns	<0.001	<0.001	-	0.00b	0.04a	0.00b	16.78***
2-Methyl-2-buten-1-ol	1449	0.14(0.03)	0.06(0.05)	0.13(0.05)	7.15**	0.09(0.00)	0.16(0.0)	0.15(0.09)	0.42ns	0.16(0.01)	0.19(0.13)	0.09ns	0.11b	0.14ab	0.19a	3.96*
1-Hexanol	1476	<0.001	<0.001	<0.001	-	0.10(0.0)	0.01(0.0)	0.01(0.01)	36.67***	0.02(0.00)	0.04(0.04)	0.52ns	0.00b	0.03a	0.03a	12.69***
3-Hexen-1-ol	1511	0.01(0.00)	0.03(0.04)	0.00(0.00)	2.97ns	<0.001	0.02(0.0)	0.01(0.0)	13.07**	<0.001	<0.001	-	0.018a	0.012ab	0.00b	3.54*
KETONES																
Acetone	836	0.45(0.27)	0.28(0.16)	0.09(0.01)	9.35**	0.33(0.05)	0.45(0.12)	0.23(0.08)	5.32*	0.85(0.27)	1.05(0.51)	0.54ns	0.24b	0.27b	0.99a	35.41***
2-Butanone	921	<0.001	<0.001	<0.001	-	0.66(0.56)	0.17(0.03)	1.19(1.72)	0.40ns	0.10(0.03)	0.42(0.18)	10.73**	0.00b	0.97a	0.33b	7.38**
2-Pentanone	1003	<0.001	<0.001	<0.001	-	0.13(0.11)	0.00(0)	0.26(0.32)	0.74ns	0.37(0.05)	0.57(0.31)	1.65ns	0.00c	0.21b	0.51a	30.11***
3-Hepten-2-one	1020	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.03(0.00)	0.08(0.04)	4.65ns	0.00b	0.00b	0.07a	46.32***
2-Heptanone	1212	<0.001	<0.001	<0.001	-	0.02(0.00)	0.01(0.00)	0.00(0.0)	1.48ns	<0.001	<0.001	-	0.00b	0.01a	0.00b	20.64***
3-Hydroxy-2-butanone	1425	0.06(0.03)	0.10(0.18)	0.01(0.00)	1.69ns	0.25(0.02)	0.10(0.02)	0.05(0.05)	13.43**	0.19(0.05)	0.31(0.16)	2.26ns	0.05b	0.08b	0.28a	16.42***
6-Methyl-5-hepten-2-one	1469	0.00(0.00)	0.01(0.01)	0.00(0.01)	0.80ns	0.02(0.0)	0.0(0.0)	0.19(0.0)	24.88***	0.01(0.00)	0.01(0.00)	0.67ns	0.01b	0.018a	0.01b	3.96*

Ethanone-1-4-methyl phenyl	1869	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.06(0.02)	0.32(0.25)	4.07ns	0.001b	0.001b	0.25a	21.65***
HYDROCARBONS																
Octane	802	0.07(0.04)	0.03(0.01)	0.03(0.0)	7.14**	0.12(0.01)	0.06(0.01)	0.04(0.06)	1.51ns	<0.001	<0.001	-	0.04a	0.05a	0.00b	10.2***
Nonane	902	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.38ns	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.01a	0.00b	0.00b	125.97***
n-Decane	1004	0.17(0.05)	0.16(0.05)	0.10(0.03)	5.16*	1.88(0.85)	1.00(0.0)	1.69(1.62)	0.22ns	<0.001	<0.001	-	0.14b	1.62a	0.00b	24.58***
Toluene	1069	0.07(0.02)	0.06(0.05)	0.08(0.02)	0.63ns	<0.001	<0.001	<0.001	-	0.06(0.02)	0.12(0.13)	0.75ns	0.07a	0.00b	0.11a	10.86***
p-Xylene	1164	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	0.00	0.22(0.10)	0.25(0.34)	0.50ns	0.01a	0.00a	0.18a	2.11ns
ESTERS																
Ethyl acetate	909	0.01(0.00)	1.42(1.36)	0.01(0.00)	8.32*	<0.001	<0.001	<0.001	-	1.17(0.91)	1.63(0.92)	0.71ns	0.55b	0.00b	1.50a	10.51***
Acetic acid butyl-ester	1098	0.05(0.02)	0.02(0.03)	0.11(0.05)	11.91***	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.06a	0.001b	0.001b	21.53***
SULFUR COMPOUNDS																
Dimethyl sulphide	<800	0.09(0.06)	0.08(0.08)	0.16(0.06)	3.20ns	0.62(0.16)	0.54(0.17)	0.40(0.20)	1.25ns	0.35(0.08)	0.27(0.23)	0.43ns	0.11c	0.30b	0.45a	21.92***
Dimethyl disulfide	1104	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.08(0.03)	0.32(0.37)	1.54sn	0.00b	0.00b	0.25a	11.63***
FURANES																
Furanmethanol	1576	0.06(0.05)	0.43(0.52)	0.08(0.04)	3.57*	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.21a	0.00b	0.00b	4.84*
Furfural	1606	0.32(0.06)	0.56(0.47)	0.18(0.06)	4.04*	1.13(0.17)	1.38(0.18)	0.71(0.29)	5.93*	1.15(0.11)	1.33(0.94)	0.14ns	0.36c	0.86b	1.28a	16.07***
TERPENES																
Carvacrol	1803	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.45(0.16)	1.32(0.18)	5.2*	0.00b	0.00b	1.07a	20.68***
α-Terpinene	1267	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.45(0.34)	0.10(0.05)	10.94**	0.00b	0.00b	0.20a	14.8***
α-Pinene	1024	<0.001	<0.001	<0.001	-	0.05(0.02)	0.08(0.02)	0.33(0.12)	5.80*	<0.001	<0.001	-	0.00b	0.25a	0.00b	4.58*
Borneol	1822	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	0.11(0.07)	0.22(0.18)	1.41ns	0.00b	0.00b	0.19a	29.72***

β-Linalool	1670	0.06(0.03)	0.09(0.04)	0.04(0.01)	5.09*	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	0.07a	0.00b	0.00b	51.32***
Hotrienol	1737	0.71(0.27)	0.39(0.49)	0.07(0.02)	2.66**	0.15(0.04)	0.24(0.03)	0.39(0.40)	0.41ns	0.24(0.02)	0.91(0.48)	7.33*	0.34b	0.33ab	0.72a	2.84ns

ns: Non significant; * p<0.05; ** p<0.01; *** p<0.001

Table 3. Standardized canonical discriminant function coefficients

Acacia honey	**Function 1**	**Function 2**
Variables	**88.8%**	**11.2%**
Diastase activity	1.588	1.304
Octane	2.592	0.575
2-Methyl-2-propanol	-2.815	0.533
2-Butanol	0.693	0.443
Acetic acid butyl ester	2.380	-0.905
2-Methyl-2-butenal	4.148	2.179
2-Penten-1-ol	1.699	0.876
2-Methyl-2-buten-1-ol	-1.402	-2.546
Sunflower honey	**Function 1**	**Function 2**
Variables	**94.3%**	**5.7%**
Sucrose	17.416	9.760
α-Pinene	-16.045	-4.218
2-Methyl-1-propanol	-5.927	7.059
3-Hydroxy-2-butanone	4,744	-4.320
6-Methyl-5-hepten-2-one	11.603	4.685
1-Hexanol	22.218	0.474

3-Hexen-1-ol	5.139	14.851
Tilia honey	**Function 1**	**Function 2**
Variables	**100%**	
Conductivity	0.758	
2-Butanone	1.036	
3-Methyl-1-butanol	-2.120	
Hotrienol	1.827	

Table 4. Classification results of the discriminant analysis carried out by cross validated procedure. Percentage of samples well classified by the model**.** Spain (Sp), Romania (Ro), and Czech Republic (Cz).

	Predicted Group Membership							
Floral and Country origin								
	Acacia Sp	Acacia Ro	Acacia Cz	Sunflower Sp	Sunflower Ro	Sunflower Cz	Tilia Ro	Tilia Cz
Acacia Sp	100	0	0	-	-	-	-	-
Acacia Ro	0	100	0	-	-	-	-	-
Acacia Cz	0	0	100	-	-	-	-	-
Sunflower Sp	-	-	-	100	0	0	-	-
Sunflower Ro	-	-	-	0	100	0	-	-
Sunflower Cz	-	-	-	0	10	90	-	-
Tilia Ro	-	-	-	-	-	-	100	0
Tilia Cz	-	-	-	-	-	-	0	100