Volatile profile in the accurate labelling of monofloral honey.

The case of lavender and thyme honey

Isabel Escriche*, Lara Sobrino*, Andrea Conchado*, Marisol Juan-Borrás*

*Institute of Food Engineering for Development (IUIAD). Food Technology Department (DTA).
Universitat Politècnica de València. P.O. Box 46022 Valencia, Spain.

bDepartamento de Estadística e Investigación Operativa Aplicada y Calidad, Centro de Gestión de la Calidad y del Cambio,
Universitat Politecnica de Valencia (UPV), Camino de Vera, s/n, 46022 Valencia, Spain

* Correspondence to: Isabel Escriche (iescrich@tal.upv.es)

ABSTRACT

The proliferation of hybrid plant varieties without pollen, such as lavender, has complicated the classification of specific types of honey. This study evaluated the correlation between the proclaimed type of monofloral honey (lavender or thyme) as appears on the label with the actual percentage of pollen. In addition, physicochemical parameters, colour, olfato-gustatory profile, and volatile compounds were tested. All the samples labelled as lavender were wrongly classified according to the usual commercial criteria (minimum 10% of pollen Lavandula spp.). In the case of lavender honey, there was significant agreement between commercial labelling and classification through organoleptic perception (81.8%), and above all between the commercial labelling and the volatile compounds (90.9%). For thyme honey, agreement for both parameters was 90.0%. These results offer compelling evidence that the volatile compounds are useful for the classification of lavender honey with low levels of pollen since this technique agrees well with the organoleptic analysis.
Keywords: accurate labelling; lavender honey classification; thyme honey; volatile compounds; GC-MS

1. Introduction

Thyme and lavender honey, although limited in production, are highly appreciated due to their organoleptic characteristics. Both, thyme (*Thymus* spp.) and lavender (*Lavandula* spp.) plants belong to the botanical family Labiatae and are part of the autochthonous Mediterranean vegetation, sharing environmental characteristics and botanical habitat and pollinating agents. These types of honey have a characteristic sweet flavour with sour notes, but may have salty notes when harvested late and/or contain some honeydew secretions. Thyme honey has an intense persistent aroma and its colour ranges from a very light amber to amber with red highlights. Lavender honey has a distinctive aroma, which is very characteristic of the lavender plant. Its colour varies from amber to very light amber, being clearer when purer, and darker due to a higher content of oak honeydew (Mateu, 2002).

The monoflorality of honey is determined by the botanical species that bees visit to obtain the nectar from flowers or the secretions of plants. Honey can be classified as belonging to a specific botanical origin when a certain percentage of pollen of this botanical species is present. The required percentage varies depending on the botanical species in question; for example, ranges between 10-20% for orange blossom honey or 70-90% for eucalyptus honey (Persano-Oddo & Piro, 2004; Juan-Borrás, Domenech, Conchado, & Escriche, 2015). However, pollen may be under represented, which causes problems in the botanical classification of honey (Persano-Oddo & Piro, 2004; Juan-Borrás et al., 2015, ANIPAM, 2008; The Apis Information Resource Center website, 2016). This problem has been observed in citrus honey because citrus trees sometimes
produce nectar before the anther produces pollen, or the nectar comes from sterile hybrid
varieties of citrus trees which are characterized by their small amounts of pollen (Juan-
Borrás, Domenech, & Escriche, 2015).

In the case of lavender honey, the problem is similar because the percentage of pollen
is usually very low or even non-existent. This is because in recent years the lavender crop
has consisted of hybrid varieties without pollen, which therefore is not present in the
nectar nor the honey (Persano-Oddo & Piro, 2004). This problem for the honey sector has
been noticed by the laboratories which carry out the botanical classification of honey.

For this reason, it is recommendable to complement the results of pollen analysis with
other determination techniques, such as physicochemical (Bogdanov, Ruoff, & Persano-
Oddo, 2004; Kádár, Juan-Borrás, Carot, Domenech, & Escriche, 2011), organoleptic
(González-Viñas, Moya, & Cabezudo, 2003; Castro-Vázquez, Díaz-Maroto, González-
Viñas, & Pérez-Coello, 2009), or chromatographic ones. Among the chromatographic
techniques, special attention should be paid to the identification of specific minority
components such as volatile compounds (Kádár et al., 2011; Juan-Borrás et al., 2014).

While some physicochemical parameters (colour, moisture, acidity, etc.) can vary
between types of honey, contributing to some extent to their organoleptic characteristics,
it is evident that in monofloral honey, flavour/aroma is the most distinctive pattern.
Although all types of honey have a common intrinsic flavour/aroma, every nectar from
blossom or secretions from plants give different specific aromas and flavours that strongly
influence their distinguishing features. It has been shown that different honeys have
certain specific compounds which some authors consider to be "fingerprints" or
"markers", which may be useful in their botanical classification (Kádár et al., 2011; Juan-
Borrás et al., 2014; Alissandrakis, Tarantilis, Harizanis, & Polissio, 2007; Kaskoniene &
Venskutonis, 2010; Castro-Vázquez, Leon-Ruiz, Alañon, Pérez-Coello, & González-
Porto, 2014). For instance, methylantranilate is important for the identification of orange blossom honey since this compound is only present in orange blossom nectar (Juan-Borrás, Domenech, & Escriche, 2015); likewise, formaldehyde and acetaldehyde in rape and clover honey, respectively; acetone in fir honey and diketones, sulphur compounds and alkanes in eucalyptus honey, among others (Bouseta, Collins, & Dufour, 1992; Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016).

Currently, the beekeeping sector is aware of the importance of increasing the offer of monofloral honey, especially from a particular geographical origin. For this reason, whenever it is possible, honey is marketed specifying its botanical origin on the label in order to inform consumers and to increase producers’ profit margins. Guarantying authenticity and differentiated quality, means that companies can increase the range of honey varieties on the market.

Although there are numerous works related to pollen, physicochemical parameters, and the volatile profile of different types of unifloral honey; as far as the authors know, there is no research focused on the relationship between this information and what appears on the label in terms of monoflorality. Therefore, the objective of this study was to investigate the correlation between the proclaimed type of monofloral honey (lavender or thyme) as appears on the label with the actual percentage of pollen, physicochemical parameters, colour, olfato-gustatory profile, as well as, volatile compounds. All this with the aim of evaluating the effectiveness of these techniques in the accurate classification of these monofloral honeys.

2. Material and methods

2.1. Honey samples

Honey samples, labelled as lavender or thyme and harvested in 2015 from the Valencian Region (Spain), were purchased locally from different retail outlets. Three batches of each
available brand on the market (11 lavender and 10 thyme) were acquired. Therefore, a
total of 66 honey samples were analyzed.

2.2. Melissopalynological analysis

The percentage of pollen from lavender and thyme present in each sample was quantified
following the criteria 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 ×400 with DpxView LE image analysis
software attached to a DeltaPix digital camera was used. Figure 1 shows micrographs of
grains of pollen of *Lavandula* spp. (A) and *Thymus* spp. (B) at 400× magnification. Light
micrographs are shown in the top row (A1 and B1) and DIC (differential interference
contrast optics) micrographs at the bottom (A2 and B2).

2.3. Physicochemical and colour analysis

Hydroxymethylfurfural content (HMF), moisture content, conductivity, °Brix and pH
were analyzed in accordance with the Harmonized Methods of the European Honey
Commission (Bogdanov, 2009). HMF was determined by HPLC-UV methodology using
a ZORBAX Eclipse Plus C18 column (4.6 x 150mm, 5 µm particle size, Agilent
Technologies, USA). Water-methanol (90:10, v:v), with a flow rate of 1 mL/min was
used as a mobile phase. The detector was set to 285 nm. EZChrom Elite system software
was used for HPLC data processing.

Water activity (a_w) was evaluated at 25 °C (± 0.2 °C) using an electronic dewpoint water
activity meter, Aqualab Series 4 model TE (Decagon Devices, Pullman, Washington,
USA), equipped with a temperature-control system.

Colour was measured using a millimetre Pfund scale (C 221 Honey Color Analyzer,
Hanna Instruments).
2.4. Volatile compound analysis: Extraction and GC–MS analysis

Volatile compounds were extracted by purge and trap at 45 °C for 20 min and trapped in a glass tube packed with Tenax TA (20–35 mesh), then purified nitrogen (100 mL min⁻¹) was bubbled through the sample (Escriche, Kadar, Juan-Borrás, & Domenech, 2011). Next, the compounds were thermally desorbed at 220 °C for 10 min (at 10 mL min⁻¹ helium flow) (TurboMatrix TD, Perkin ElmerTM, CT-USA), cryofocused in a cold trap at −30 °C, then the cold trap was heated to 250 °C (at a rate of 99 °C/s) which transferred them onto the capillary column.

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. Helium at a flow rate of 1 mL min⁻¹ was used as the carrier gas. The temperature programme was: from 40 °C (2-minute hold time) to 190 °C at 4 °C/min⁻¹ (11-minute hold time) and finally to 220 °C at 8 °C min⁻¹ (8-minute hold time). Electron impact mass spectra were logged in impact ionization mode at 70 eV (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 with those obtained from authentic standards from: Sigma-Aldrich (San Louis, Missouri and Acros Organics, Geel, Belgium) and Fluka (Buchs, Schwiez, Switzerland). The compounds for which it was not possible to find authentic standards were tentatively identified by comparing their mass spectra (m/z values of the most important ions) with spectral data from the National Institute of Standards and Technology 2002 library (always considering more than 80% percent probability value), as well as linear retention indices and spectral data published in the literature.
All the physicochemical, colour and volatile compound analysis were performed in triplicate.

2.5 Sensory analysis

The samples were evaluated organoleptically based on their monofloral olfactogustatory profile. This analysis was carried out by experienced staff in the honey quality control laboratory at the Universitat Politècnica of València (Spain). A scale from 0 to 3 was used to score the perceived intensity (ISO, 2003). Tasters placed a small amount of honey (approx 5 g) on their tongue, diluted it with saliva and projected it toward the back of their mouth to evaluate the flavour and aromas via the retronasal route. Then, the honey was swallowed slowly, and the taste persistence was evaluated. This procedure was followed for all samples.

2.6 Statistical analysis

An analysis of variance (ANOVA) (using Statgraphics Centurion for Windows) was applied to evaluate the physicochemical parameters, colour, volatile compounds, and phenolic and flavonoid compounds, according to the type of honey (lavender or thyme). The method used for multiple comparisons was the LSD test (least significant difference) with a significance level $\alpha=0.05$. In addition, data were analyzed using a principal component analysis (PCA) applying the software Unscrambler X.10. The number of components extracted was based on the Kaiser criterion (1960) that suggests keeping all principal components with eigenvalues higher than 1 (Kaiser, 1960). This solution was not rotated using orthogonal or oblique rotation. Subsequently, Stepwise Linear Discriminant Analysis (SLDA) was applied (using SPSS.18) to the loadings of these seven principal components with the aim of differentiating between varieties of honey.
This analysis permitted identification of the principal components with better predictive power, by means of a unique discriminant function.

3. Results and discussion

3.1. Physicochemical and colour characterization

Table 1 shows the average values of the quantified physicochemical parameters (HMF, moisture, electrical conductivity, °Brix, pH and aw) and the colour of the three batches of each brand commercially labelled as lavender or thyme honey.

The code for each brand refers to the average percentage of Lavender spp. (L) and Thyme spp. (T) pollen. The organoleptic scores of these samples were based on the monofloral (lavender or thyme) olfato-gustatory profile of the first named type of pollen, the perception intensity being measured from absence (0) to very intense (3). For example, the code “L1-T8 (2)” means that this brand has an average of 1% lavender pollen and 8% thyme pollen, and the (2) represents an intermediate olfato-gustatory intensity of lavender.

In the present work all the honey samples labelled as thyme satisfied the commercial criterion about the percentage of pollen (minimum 10% of pollen Thymus spp.), since they ranged from 11 to 16%. However, this was not the case for the group of samples labelled as lavender, as the pollen ranged between 0 and 7% when their commercial minimum criterion is also 10% Lavandula spp. pollen. The International Honey Commission in a study carried out with 84 European lavender and 253 thyme honey samples (Persano-Oddo & Piro, 2004), reported a slightly higher average percentage of Lavandula spp pollen (between 1% and 19%) than those observed in the present work. On the contrary, the average Thymus spp. pollen content was higher in Italian and Greek thyme honey (26%±18 and 40%±16.4, respectively). The different species of thyme
involved in each case may be the main reason for these differences (Persano-Oddo &
Piro, 2004). It seems evident that the “samples labelled as lavender” used in the present
study are sold only following the criterion of aroma/flavour reminiscent of this flower
(organoletic analysis) since this type of sample did not meet the pollinic criteria. This is
not surprising since, as noted above, currently lavender honey is underrepresented in
pollen due to the proliferation of hybrid varieties used in the perfume industry (Guyot-
Declerck, Renson, Bouseta, & Collin, 2002; Von Der Ohe et al., 2004; ANIPAM, 2008;
The Apis Information Resource Center website, 2016). Therefore, it is obvious that the
pollen percentage criterion is unrealistic at least referring to lavender honey

This table also illustrates the ANOVA results (F-ratio and significant differences)
obtained for the factor “type of labelled honey” carried out for the physicochemical
parameters. Significant differences were not found between the two types of labelled
honey (lavender and thyme) for any of the parameters analysed. This is because in both
groups of samples all the parameters analysed are in the same range of values: moisture
(13.73-19.60 and 15.60-17.60 g/100g); electrical conductivity (0.180-0.600 and 0.160-
0.640 S/cm), °Brix (78.73-84.00 and 80.60-82.50); pH (3.78-4.40 and 3.83-4.80); a_w
(0.49-0.60 and 0.48-0.58) and colour (40.00-89.00 and 47.00-84.00), respectively. These
values agree with the results obtained for European lavender and thyme honey in the
before mentioned study (Persano-Oddo & Piro, 2004), where the physicochemical
reported data were: moisture (15.20-18.10 and 14.00-17.00 g/100g); electrical
conductivity (0.120-0.310 and 0.250-0.540 S/cm); pH (3.50-4.00 and 3.50-4.10); and
colour (20.30-45.00 and 35.00-74.5), respectively. It is important to point out that in the
present work the colour Pfund for lavender honey was about double that in the European
study, probably due to the influence of the vegetation present in the surroundings.
In relation to HMF a large amount of variation was observed: from 4.51 to 48.19 mg/kg in the case of lavender honey, and from 0.93 to 58.88 mg/kg for thyme honey. In both types of honey, two brands clearly exceeded the maximum of 40 mg/kg (Council Directive 2001/110 relating to honey, 2002), with values of 44.74 and 48.19 mg/kg in lavender honey, and 48.75 and 58.88 in thyme honey. This shows that these samples were not properly handled, or the time between harvesting and retail sale was too long.

The information given by the physicochemical parameters and the colour shows that neither of them permits differentiation between honey from the same geographical habitat labelled as lavender or thyme, as is the case of the two types of honey studied here.

The volatile compounds that are liberated during the tasting and ingestion of honey decisively influences the aroma/flavour perceived. Therefore, it is logical to think that the volatile fraction of honey contains potentially usable information for the differentiation of lavender honey from other types of honey with which it might be confused.

3.2. Olfato-gustatory profile and volatile compound characterization

Around 30 major volatile compounds were identified and semiquantified in the volatile fractions of honey samples, including alcohols, aldehydes, ketones, acids, esters, terpenes and nitrogen compounds. The average values, standard deviation, and the ANOVA result of the volatile compounds analysed in both types of honey are shown in Table 2.

Alcohols, as in other types of honey, were abundant in the analysed samples. All of them had 2-propanol, 2-butanol and 1-butanol in similar amounts. Ethanol was more common in thyme honey samples, although without significant differences between both groups. The alcohol 1-hexanol was present in significantly greater amounts in the samples labelled as lavender; some authors propose 1-hexanol, among other compounds, as a typical marker of lavender honey. In fact, in the present study 1-hexanol was identified
in almost all the brands labelled as lavender (average value of 4.2 µg/kg) and only in one
brand labelled as thyme (T16-L3) with an average value in this brand of 1.4 µg/kg. The
existence of lavender pollen in this last sample shows that the honeybees visited lavender
flowers too, which could explain the occurrence of 1-hexanol in this sample. The large
amount of 1-hexanol found in lavender honey is in line with the results obtained in other
studies of Spanish lavender honey (Castro-Vázquez et al., 2009; Castro-Vázquez et al.,
2014).

In the present study, the levels of methyl alcohols in the two groups of honeys were
not found to be significantly different by the ANOVA. However, among them, 2-methyl-
3-buten-2-ol, 3-methyl-3-buten-1-ol, and 2-methyl-2-buten-1-ol, where more abundant in
samples labelled as lavender and 2-methyl-1-propanol and 2-methyl-1-butanol in thyme
samples. Apparently, the methyl alcohols contribute to the typically fresh aroma of this
type of honey (Castro-Vázquez et al., 2009; Bouseta, Collins, & Dufour, 1992; Da Silva
et al., 2016).

Aldehydes such as 3-methyl-1-butanal, 2-methyl-1-butanal, hexanal, heptanal furfural
and phenylacetaldehyde, were also identified. Phenylacetaldehyde, described as having a
honey-like aroma was present in most of the thyme honey samples and in very few of
those labelled as lavender. This agrees with the results obtained for Greek thyme honey
(Alişsandırakis et al., 2007; Karabagias, Badeka, Kontakos, Karabournioti, &
Kontominas, 2014), and for Spanish thyme honey (Castro-Vázquez et al., 2009).
However, other authors reported the importance of phenylacetaldehyde to characterize
lavender honey from different botanical species (Guyot-Declerck et al., 2002). In the
present study, only a few samples of lavender showed the presence of this compound.
Many authors considered that hexanal is one of the compounds most responsible for the characteristic flavour of lavender honey (Bouseta, Collins, & Dufour, 1992; Guyot-Declerck et al., 2002; Manyi-Loh, Roland, & Clarke, 2011). In fact, in the present work the importance of hexanal is of note as it appeared in almost all the samples labelled as lavender (reaching values of 5.0 µg/kg) and in T16-L3. Although this sample had a high enough percentage of pollen to be classified as thyme, it is noteworthy that it had the organoleptic characteristics and aromatic notes typical of lavender honey, as well as the presence of pollen from this plant.

The two groups of analysed samples showed opposing behaviour in terms of methyl aldehydes with significant differences between them; 3-methyl-1-butanal was almost exclusively found in the group of honey samples labelled as lavender, whereas 2-methyl-1-butanal in those of thyme.

Different ketones were present in almost all the samples analysed: acetone; 2-butanone, 2,3 butanedione 1-hydroxy-2-propanone, and 3-hydroxy-2-butanone. In general, ketones are very common in different types of European honey, acetone being one of the major volatile compounds detected (Da Silva et al., 2016). In the present work, all the ketones identified were more abundant in thyme honey than honey labelled as lavender, although only for acetone the differences were significant between the two groups.

Acetic acid and ethyl acetate were the only acid and ester identified, respectively. Acetic acid showed significant differences between groups, being present in all the samples labelled as lavender (average value of 2.3 µg/kg) and only in three of the thyme samples. However, not significant differences were found for ethyl acetate.
β-linalool and hotrienol (3,7-dimethyl-1,5,7 octatrien-3-ol) were the only honey terpenes identified in the present work. Different studies reported that the derivatives of β−linalool originated from flowers visited by honeybees are found only in specific types of honey (Da Silva et al., 2016). Several authors highlighted the importance of hotrienol in lavender honey, compared to other types of honey (Castro-Vázquez et al., 2009; Castro-Vázquez et al., 2014; Jerkovic & Kus, 2014). In the present work, β−linalool was identified in both honey samples, although was significantly higher in those of thyme, whereas hotrienol was almost exclusively found in the samples labelled as lavender (reaching values up to 4.8 µg/kg). Other volatile terpenes such as thymol or carvacrol, which were reported by other authors in thyme honey, were not found in this study. This was probably due to the different botanical species or the analytical extraction procedures applied for these compounds (Cacho, Campillo, Viñas, & Hernández-Córdoba, 2015).

Only four brands labelled as thyme showed small amounts of short-chain nitrogen compounds (2-methyl-propanenitrile, 2-methyl-butanenitrile). Unsurprisingly, nitrile derivatives could be present in honey samples, even becoming important compounds in the headspace fraction (Moreira & De Maria, 2005), in some cases reaching 21.7% of the total volatile compounds (Kaškoniene, Venskutonis, & Ceksteryte, 2008). For instance, phenylacetonitrile was reported as very abundant in thyme honey from Greece (Alissandrakis et al., 2007).

With the aim of transforming the initial set of volatile compound variables into a more reduced set of linearly uncorrelated variables, a principal component analysis was performed. This analysis was carried out using the average values from the three repetitions for each sample of honey. Seven components were extracted according to the Kaiser criterion (1960) (Kaiser, 1960), explaining 95.5% of the total variance. The first
component (PC1) explained 40% and was positively correlated with ethyl acetate, 2,3-
butanedione, 2-methyl-propanenitrile, 2-methyl-1-propanol, 3-methyl-butane
nitrile, 1-butanol, 2-methyl-1-butanol, among others. The second component (PC2 explained
22.0%) was positively correlated with 1-hexanol, hotrienol, hexanal, acetic acid and 2-
methyl-2-buten-1-ol, and others. Figure 2 shows the scores and loadings for the two
principal components. The score codes correspond to those explained in Table 1.
Proximity between samples labelled as lavender or thyme, indicates similar behavior in
terms of the volatile profile. The loading plot confirms that certain compounds are
responsible for differentiation between the two groups. Ethyl acetate, 2,3-butanedione, 2-
methyl-propanenitrile and 1-butanol, associated with PC1, as well as 1-hexanol,
hotrienol, hexanal, corresponding to PC2, are characteristic of thyme and lavender honey,
respectively.

Furthermore, the third component (11.5%) was positively correlated to octane and 3-
methyl-3-buten-1-ol, while the fourth (8.6%), fifth (5.8%), sixth (4.4%) and seventh
(3.2%) components were mainly correlated with ethanol, furfural, acetone and 2-methyl-
butanal, and 3-methyl-butanal, respectively.

Once the variability of the initial set of volatile compound variables was reduced to
seven principal components, a discriminant analysis was applied to examine the
predictive power of each principal component when distinguishing between groups. That
is to say, the previous seven principal components extracted using PCA were
subsequently used as predictors of honey type in the discriminant analysis. As a result of
this analysis, only one statistically significant canonical function was obtained. This
function explained 100% of the total variance (Wilks’ lambda=0.462, df=7, p=0.101;
Canonical correlation coefficient=0.734), and also discriminates correctly between honey
labelled as lavender or thyme in 85.7% of the samples. The discriminant function values at the group centroids were -1.186 for lavender and 0.890 for thyme.

The standardized coefficients of discriminant functions for each principal component used as a predictor were: PC1 (0.731); PC2 (-0.819); PC3 (0.323); PC4 (0.227); PC5 (-0.586); PC6 (0.363) and PC7 (-0.088). These data reveal that the second component showed the highest predictive power of the discriminant function, followed by the first component. This shows that the compounds most involved in the distinction between the two types of honey were: 1-hexanol, hotrienol, hexanal, acetic acid and 2-methyl-2-buten-1-ol.

The results of the discriminant analysis are shown in Figure 3. Next to the code for each sample appears the organoleptic score based on its monofloral (lavender or thyme) olfato-gustatory profile as was described in Table 1. Samples of thyme honey fell in the left region of the map, whereas the rest of the samples were placed on the right, though both varieties obtained negative scores for this discriminant function.

Table 3 summarizes the information concerning commercial labelling and possible classifications of samples according to several criteria: percentage of pollen, olfato-gustatory profile and volatile compound obtained from discriminant analysis. Additionally, chi–square tests are shown to assess the relationship between commercial labelling and the classification provided by each criterion.

None of the samples labelled as lavender should have been classified as such according to the pollen content (minimum 10% of *Lavandula* spp. pollen). Actually, they should be classified as polyfloral with the exception of two of them that could be classified as thyme honey. However, the organoleptic perception (in 81.8% of these samples) and the volatile profile (in 90.9%) conform to the information given on the samples labelled as lavender.
Regarding thyme honey, the information on the label is correct for all samples considering the pollen content (100%). With the exception of one sample, all of them showed the characteristic olfato-gustatory profile and volatile profile of thyme honey (90.0% in both cases). Chi–square tests confirm that there is a significant association between the commercial labelling and the classification given by the organoleptic perception ($\chi^2=10.83$, $p=0.001$) and overall by the volatile compounds ($\chi^2=13.74$, $p=0.000$).

4. Conclusion

This paper highlights the importance of a detailed review of the information that appears on commercial labels. Besides, the need to identify alternative analytic techniques to help organizations provide more accurate content in their labelling processes is underlined.

This work contributes to reinforcing the usefulness of the volatile fraction of honey to provide more accurate honey labelling. The results offer compelling evidence that the volatile compound profile can be used for the classification of lavender type honey since this technique agrees quite well with the organoleptic analysis. The volatile analysis has a clear advantage over the pollinic one in the case of lavender honey.

In general, when pollen content is under-represented, volatile analysis could be a complementary technique to the pollinic one. Obviously, this analysis would only be recommended when honey has specific identifiable organoleptic characteristics.

The present findings have important implications for solving problems in the honey sector regarding the correct classification of underrepresented monofloral honey. Organizations may benefit from this new approach to volatile information, and consumers may buy honey products with guaranteed botanical origin. Considering these results, there are still some undefined sources of unreliability that may influence the final classification of samples containing a mixture of lavender and thyme characteristics. We
are aware that the research needs to be expanded to include a greater number of samples from a wider time period.

Acknowledgment

The authors thank the Generalitat Valenciana (Spain) for funding the project AICO/2015/104.

References


Pollen viability and natural hybridization of Central European species


Figure captions

Figure 1. Pictures at 400× magnification of pollen of Lavandula spp. (A) and Thymus spp. (B). A1 and B1- Light micrographs. A2 and B2 - DIC (differential interference contrast optics) micrographs.

Figure 2. (2.a) Scores (brand samples) and (2.b) loading (volatile compounds) plots of the first two components. (L) and (T) = percentage of Lavender spp. and Thyme spp. pollen.

Figure 3. Score plots of the first two components of the PCA-DA model. (L) and (T) = percentage of Lavender spp. and Thyme spp. pollen, (0-3)=intensity of organoleptic perception of the first named honey.
Table 1. Olfato-gustatory profile and average values of the physicochemical parameters and colour of each of the brands commercially labelled as lavender or thyme honey. L= lavender; T= thyme. Brand codes refer to the average percentage of pollen of *Lavandula* spp. (L) and (*Thymus* spp.). The organoleptic score of the brands was based on their monofloral (lavender or thyme) olfato-gustatory profile, perception intensity from 0 (absence) to 3 (very intense).

<table>
<thead>
<tr>
<th>Brand Codes</th>
<th>Olfato-gustatory profile</th>
<th>Commercial information</th>
<th>Moisture (g/100g)</th>
<th>Electrical conductivity S/cm</th>
<th>°Brix</th>
<th>pH</th>
<th>a_w</th>
<th>Colour</th>
<th>HMF (mg/kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-T8</td>
<td>(2)lavander</td>
<td>lavender</td>
<td>15.60 (0.01)</td>
<td>0.250 (0.006)</td>
<td>82.65 (0.01)</td>
<td>3.95 (0.01)</td>
<td>0.53 (0.01)</td>
<td>47 (1)</td>
<td>4.51 (0.01)</td>
</tr>
<tr>
<td>L3-T12</td>
<td>(3)lavander</td>
<td>lavender</td>
<td>15.27 (0.12)</td>
<td>0.330 (0.001)</td>
<td>83.07 (0.06)</td>
<td>3.94 (0.01)</td>
<td>0.49 (0.01)</td>
<td>66 (1)</td>
<td>15.49 (1.9)</td>
</tr>
<tr>
<td>L3-T0</td>
<td>(1)lavander</td>
<td>lavender</td>
<td>18.07 (0.12)</td>
<td>0.330 (0.001)</td>
<td>80.40 (0.01)</td>
<td>3.85 (0.01)</td>
<td>0.60 (0.01)</td>
<td>89 (1)</td>
<td>44.74 (1.6)</td>
</tr>
<tr>
<td>L7-T0</td>
<td>(3)lavander</td>
<td>lavender</td>
<td>14.80 (0.01)</td>
<td>0.180 (0.001)</td>
<td>83.40 (0.01)</td>
<td>3.78 (0.01)</td>
<td>0.54 (0.01)</td>
<td>80 (1)</td>
<td>48.19 (1.6)</td>
</tr>
<tr>
<td>L1-T26</td>
<td>(0)lavander; (1)thyme</td>
<td>lavender</td>
<td>15.40 (0.01)</td>
<td>0.600 (0.001)</td>
<td>82.82 (0.08)</td>
<td>4.40 (0.01)</td>
<td>0.53 (0.01)</td>
<td>81 (1)</td>
<td>10.09 (0.8)</td>
</tr>
<tr>
<td>L3-T0</td>
<td>(1)lavander</td>
<td>lavender</td>
<td>15.60 (0.01)</td>
<td>0.500 (0.015)</td>
<td>82.62 (0.03)</td>
<td>4.23 (0.01)</td>
<td>0.52 (0.01)</td>
<td>77 (1)</td>
<td>8.48 (0.2)</td>
</tr>
<tr>
<td>L4-T0</td>
<td>(2)lavander</td>
<td>lavender</td>
<td>16.60 (0.01)</td>
<td>0.320 (0.010)</td>
<td>81.53 (0.06)</td>
<td>4.02 (0.01)</td>
<td>0.55 (0.01)</td>
<td>61 (1)</td>
<td>4.85 (0.2)</td>
</tr>
<tr>
<td>L5-T0</td>
<td>(3)lavander</td>
<td>lavender</td>
<td>19.60 (0.01)</td>
<td>0.480 (0.010)</td>
<td>78.73 (0.06)</td>
<td>4.05 (0.01)</td>
<td>0.59 (0.01)</td>
<td>51 (1)</td>
<td>4.75 (0.5)</td>
</tr>
<tr>
<td>L1-T8</td>
<td>(0)lavander; (1)thyme</td>
<td>lavender</td>
<td>15.80 (0.01)</td>
<td>0.490 (0.006)</td>
<td>82.43 (0.03)</td>
<td>3.98 (0.01)</td>
<td>0.50 (0.01)</td>
<td>70 (1)</td>
<td>8.81 (0.5)</td>
</tr>
<tr>
<td>L0-T0</td>
<td>(1)lavander</td>
<td>lavender</td>
<td>17.40 (0.01)</td>
<td>0.250 (0.010)</td>
<td>80.80 (0.01)</td>
<td>3.89 (0.01)</td>
<td>0.55 (0.01)</td>
<td>40 (1)</td>
<td>8.40 (1.3)</td>
</tr>
<tr>
<td>L0-T0</td>
<td>(1)lavander</td>
<td>lavender</td>
<td>13.73 (0.12)</td>
<td>0.350 (0.006)</td>
<td>84.00 (0.01)</td>
<td>4.23 (0.01)</td>
<td>0.49 (0.01)</td>
<td>68 (1)</td>
<td>16.48 (0.9)</td>
</tr>
<tr>
<td>T16-L3</td>
<td>(1)thyme; (1)lavander</td>
<td>thyme</td>
<td>16.60 (0.01)</td>
<td>0.330 (0.006)</td>
<td>81.60 (0.01)</td>
<td>3.84 (0.01)</td>
<td>0.58 (0.01)</td>
<td>60 (1)</td>
<td>4.29 (0.4)</td>
</tr>
<tr>
<td>T14-L0</td>
<td>(1)thyme</td>
<td>thyme</td>
<td>17.60 (0.01)</td>
<td>0.570 (0.006)</td>
<td>80.60 (0.01)</td>
<td>4.11 (0.01)</td>
<td>0.58 (0.01)</td>
<td>74 (1)</td>
<td>0.93 (0.3)</td>
</tr>
<tr>
<td>T14-L0</td>
<td>(2)thyme</td>
<td>thyme</td>
<td>15.60 (0.01)</td>
<td>0.400 (0.015)</td>
<td>82.50 (0.01)</td>
<td>4.80 (0.01)</td>
<td>0.55 (0.01)</td>
<td>78 (1)</td>
<td>1.63 (0.5)</td>
</tr>
<tr>
<td>T12-L0</td>
<td>(1)thyme</td>
<td>thyme</td>
<td>16.80 (0.01)</td>
<td>0.420 (0.017)</td>
<td>81.60 (0.01)</td>
<td>3.94 (0.01)</td>
<td>0.56 (0.01)</td>
<td>71 (1)</td>
<td>6.07 (0.7)</td>
</tr>
<tr>
<td>T11-L0</td>
<td>(1)thyme</td>
<td>thyme</td>
<td>15.60 (0.01)</td>
<td>0.420 (0.021)</td>
<td>82.50 (0.01)</td>
<td>4.18 (0.01)</td>
<td>0.49 (0.01)</td>
<td>78 (1)</td>
<td>10.18 (1.2)</td>
</tr>
<tr>
<td>Sample</td>
<td>Thyme</td>
<td>Thyme</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
<td>F-Ratio</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>T12-L0</td>
<td>1.00</td>
<td>0.166</td>
<td>16.60 (0.01)</td>
<td>0.160 (0.001)</td>
<td>81.60 (0.01)</td>
<td>3.83 (0.01)</td>
<td>0.49 (0.01)</td>
<td>47 (1)</td>
<td>58.88 (0.9)</td>
</tr>
<tr>
<td>T11-L0</td>
<td>1.00</td>
<td>0.340</td>
<td>17.60 (0.35)</td>
<td>0.340 (0.006)</td>
<td>80.67 (0.09)</td>
<td>3.85 (0.01)</td>
<td>0.53 (0.01)</td>
<td>84 (1)</td>
<td>48.75 (1.3)</td>
</tr>
<tr>
<td>T11-L0</td>
<td>1.00</td>
<td>0.500</td>
<td>15.80 (0.20)</td>
<td>0.500 (0.001)</td>
<td>82.40 (0.08)</td>
<td>3.90 (0.01)</td>
<td>0.48 (0.01)</td>
<td>72 (1)</td>
<td>8.52 (0.6)</td>
</tr>
<tr>
<td>T13-L0</td>
<td>1.00</td>
<td>0.340</td>
<td>16.20 (0.01)</td>
<td>0.340 (0.006)</td>
<td>82.03 (0.06)</td>
<td>3.91 (0.01)</td>
<td>0.56 (0.01)</td>
<td>81 (1)</td>
<td>12.44 (0.3)</td>
</tr>
<tr>
<td>T13-L0</td>
<td>1.00</td>
<td>0.640</td>
<td>16.40 (0.01)</td>
<td>0.640 (0.006)</td>
<td>81.70 (0.01)</td>
<td>4.31 (0.01)</td>
<td>0.58 (0.01)</td>
<td>69 (1)</td>
<td>6.64 (0.7)</td>
</tr>
</tbody>
</table>

**ANOVA**

<table>
<thead>
<tr>
<th>F-Ratio</th>
<th>F-Ratio</th>
<th>F-Ratio</th>
<th>F-Ratio</th>
<th>F-Ratio</th>
<th>F-Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.73ns</td>
<td>1.46ns</td>
<td>2.16ns</td>
<td>0.26ns</td>
<td>0.15ns</td>
<td></td>
</tr>
</tbody>
</table>

ns: Non significant
Table 2. Volatile compounds (average values and standard deviation) in samples labelled as lavender or thyme honey. The data were calculated (µg/kg of honey) assuming a response factor equal to one.

<table>
<thead>
<tr>
<th>VOLATILE COMPOUNDS</th>
<th>RI cal</th>
<th>ID</th>
<th>Samples labelled as lavender Mean (SD)</th>
<th>Samples labelled as thyme Mean (SD)</th>
<th>ANOVA F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Propanol</td>
<td>947</td>
<td>MS;RI</td>
<td>1.2 (1.1)</td>
<td>1.6 (1.5)</td>
<td>0.3**</td>
</tr>
<tr>
<td>Ethanol</td>
<td>957</td>
<td>MS;RI</td>
<td>20.3 (13.3)</td>
<td>38.1 (20.6)</td>
<td>3.6**</td>
</tr>
<tr>
<td>2 Butanol</td>
<td>1047</td>
<td>St;MS;RI</td>
<td>8.5 (6.1)</td>
<td>6.4 (9.0)</td>
<td>0.2**</td>
</tr>
<tr>
<td>2 Methyl-3-buten-2 ol</td>
<td>1065</td>
<td>MS;RI</td>
<td>6.1 (3.3)</td>
<td>5.2 (1.9)</td>
<td>0.5**</td>
</tr>
<tr>
<td>1 Butanol</td>
<td>1175</td>
<td>St;MS;RI</td>
<td>2.4 (3.1)</td>
<td>3.8 (6.5)</td>
<td>0.2**</td>
</tr>
<tr>
<td>2 Methyl-1-propanol</td>
<td>1119</td>
<td>St;MS;RI</td>
<td>6.7 (2.3)</td>
<td>9.5 (2.3)</td>
<td>0.69**</td>
</tr>
<tr>
<td>2 Methyl-1-butanol</td>
<td>1185</td>
<td>St;MS;RI</td>
<td>7.7 (4.4)</td>
<td>8.9 (5.9)</td>
<td>0.2**</td>
</tr>
<tr>
<td>3 Methyl-3-buten-1-ol</td>
<td>1277</td>
<td>St;MS;RI</td>
<td>6.3 (2.9)</td>
<td>5.2 (2.4)</td>
<td>0.78**</td>
</tr>
<tr>
<td>2 Methyl-2-buten-1-ol</td>
<td>1349</td>
<td>MS;RI</td>
<td>3.0 (2.1)</td>
<td>1.4 (1.3)</td>
<td>3.2**</td>
</tr>
<tr>
<td>1 Hexanol</td>
<td>1476</td>
<td>St;MS;RI</td>
<td>4.2 (2.2)</td>
<td>0.1 (0.5)</td>
<td>15.9**</td>
</tr>
<tr>
<td><strong>Aldehydes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methyl-1-butanal</td>
<td>912</td>
<td>St;MS;RI</td>
<td>1.6 (2.4)</td>
<td>&lt;0.001</td>
<td>2.37*</td>
</tr>
<tr>
<td>2-methyl-1-butanal</td>
<td>920</td>
<td>MS;RI</td>
<td>0.3 (0.5)</td>
<td>2.6 (2.5)</td>
<td>6.9*</td>
</tr>
<tr>
<td>Hexanal</td>
<td>1065</td>
<td>St;MS;RI</td>
<td>3.2 (2.4)</td>
<td>0.6 (1.2)</td>
<td>8.9**</td>
</tr>
<tr>
<td>Heptanal</td>
<td>1160</td>
<td>St;MS;RI</td>
<td>9.2 (6.9)</td>
<td>19.7 (2.8)</td>
<td>1.35**</td>
</tr>
<tr>
<td>Furfural</td>
<td>1460</td>
<td>St;MS;RI</td>
<td>3.2 (4.6)</td>
<td>2.9 (4.5)</td>
<td>0.02**</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>1609</td>
<td>MS;RI</td>
<td>1.0 (1.0)</td>
<td>2.8 (1.6)</td>
<td>1.8**</td>
</tr>
<tr>
<td><strong>Ketones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>814</td>
<td>MS;RI</td>
<td>1.0 (0.3)</td>
<td>2.0 (0.9)</td>
<td>4.7*</td>
</tr>
<tr>
<td>Compound</td>
<td>M.S.</td>
<td>ID</td>
<td>RI cal</td>
<td>Identification</td>
<td>First retention</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------</td>
<td>----</td>
<td>--------</td>
<td>------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>910</td>
<td>St;MS;RI</td>
<td>1.7 (0.7)</td>
<td>2.6 (1.9)</td>
<td>2.2&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3 Butanedione</td>
<td>970</td>
<td>MS;RI</td>
<td>2.8 (1.9)</td>
<td>7.4 (7.7)</td>
<td>3.5&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-Hydroxy-2-propanone</td>
<td>1268</td>
<td>MS;RI</td>
<td>2.2 (1.2)</td>
<td>2.9 (1.4)</td>
<td>1.2&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-Hydroxy-2-butanone</td>
<td>1322</td>
<td>St;MS;RI</td>
<td>2.6 (1.4)</td>
<td>4.8 (3.0)</td>
<td>1.9&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1486</td>
<td>St;MS;RI</td>
<td>2.3 (1.4)</td>
<td>0.9 (0.5)</td>
<td>5.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>909</td>
<td>St;MS;RI</td>
<td>1.1 (1.4)</td>
<td>1.4 (1.2)</td>
<td>0.6&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Terpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Linalool</td>
<td>1670</td>
<td>St;MS;RI</td>
<td>3.6 (0.15)</td>
<td>4.9 (0.15)</td>
<td>4.8&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hotrienol</td>
<td>1737</td>
<td>MS;RI</td>
<td>2.7 (1.1)</td>
<td>0.3 (0.2)</td>
<td>35.8&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Nitrogen compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Methyl-propanenitrile</td>
<td>1022</td>
<td>St;MS;RI</td>
<td>&lt;0.001</td>
<td>0.23 (0.35)</td>
<td>2.5&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Methyl-butanenitrile</td>
<td>1158</td>
<td>MS;RI</td>
<td>&lt;0.001</td>
<td>0.17 (0.27)</td>
<td>3.9&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ns: Non significant; *P<0.05; **P<0.01; ***P<0.001
RI cal: Linear retention indices calculated
ID: Method of identification, MS (comparison with mass spectrum stored in NIST library), St (comparison of retention time and mass spectrum with those of authentic standards), RI (comparison of linear retention indices with the literature)

Table 3. Comparison of commercial labelling and possible classifications of samples according to presence of pollen, organoleptic profile and volatile compounds.
<table>
<thead>
<tr>
<th>Brand Code</th>
<th>Commercial labelling</th>
<th>Pollen</th>
<th>Olfato-gustatory profile</th>
<th>Volatile compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-T8</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L3-T12</td>
<td>Lavender</td>
<td>Thyme</td>
<td>Lavender</td>
<td>Thyme</td>
</tr>
<tr>
<td>L1-T8</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>L3-T0</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L7-T0</td>
<td>Lavander</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L1-T26</td>
<td>Lavander</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Lavender</td>
</tr>
<tr>
<td>L3-T0</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L4-T0</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L5-T0</td>
<td>Lavander</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L0-T0</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>L0-T0</td>
<td>Lavender</td>
<td>Polyfloral</td>
<td>Lavender</td>
<td>Lavender</td>
</tr>
<tr>
<td>T14-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T14-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T12-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T11-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T12-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T11-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T11-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T13-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
<tr>
<td>T16-L3</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Lavender</td>
<td>Thyme</td>
</tr>
<tr>
<td>T13-L0</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
<td>Thyme</td>
</tr>
</tbody>
</table>

% of samples conform the information given on the Lavander label: - 0% 81.8% 90.9%

% of samples conform the information given on the thyme label: - 100% 90.0% 90.0%
<table>
<thead>
<tr>
<th>( \chi^2 ) (p-value)</th>
<th>-</th>
<th>-</th>
<th>10.83</th>
<th>13.74</th>
<th>(0.001)</th>
<th>(0.000)</th>
</tr>
</thead>
</table>

Figure 1
Figure 2
Figure 3