Food Chemistry 112 (2009) 329–338

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Influence of simulated industrial thermal treatments on the volatile fractions of different varieties of honey

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ARTICLE INFO

Article history:
Received 17 March 2008
Received in revised form 20 May 2008
Accepted 21 May 2008

Keywords:
Honey
Thermal treatments
Volatile compounds
Physicochemical parameters
Pollen

ABSTRACT

The aim of this study was to determine if the volatile fraction of honey is affected by the application of standard industrial thermal treatment processes. Four types of Spanish honey were studied: three of floral origin (citrus, rosemary and polyfloral) and the fourth from honeydew. Each sample of honey was divided into three parts: one was left untreated, one was liquefied (at 45 °C for 48 h) and the other was both liquefied and pasteurized (at 80 °C for 4 min). All the samples analyzed were characterized to determine their melissopalynological, physicochemical (pH, moisture, total acidity, conductivity, hydroxymethylfurfural, and diastase activity), and volatile profiles. Type of honey had a greater impact on volatile fraction variations than did heat treatment. The overall volatile profile of each kind of honey permitted the classification of the honeys by botanical origin, revealing that there were practically no differences between the raw, liquefied, and pasteurized samples of each honey. These findings suggest that industrial processes conducted under controlled conditions should not significantly alter the intrinsic aroma of honey.

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1. Introduction

Appearance plays a key role in the commercial success of honey, as consumers demand a fluid, non-crystallized product. Recently harvested raw honey is in a liquid state, but it crystallizes with greater or lesser speed depending on numerous factors such as origin (botanical and geographical), temperature, moisture content, and sugar content. To slow down the natural crystallization process and ensure stability during its commercial life, raw honey is normally pasteurized prior to being packaged in order to dissolve sugar crystals and destroy yeasts. Before it can be pasteurized, however, the honey must be heated at a moderate temperature between 45 °C and 50 °C. This treatment is applied to the honey in the drums received from beekeepers. The heat liquefies the honey to facilitate the emptying of the drums and favour the subsequent filtration and blending stages required to produce a particular production batch.

Although some questions still remain about reactions that take place in food as a result of heat treatment (mainly Maillard reactions), it is known that they are related to transformations in flavour, aroma, taste and colour, and that they are closely associated with temperature, time, pH, the nature of reactants, etc. (i.e. the type of sugar and amino acid, or protein) (Martins, Jongen, & Van Boeckel, 2001).

All honeys have a characteristic basic flavour and aroma, determined largely by the composition of its volatile fraction. This fraction, in turn, has specific components which could be considered to be true flavour/aroma fingerprints. These volatile compounds may provide information about the botanical origin of honey, whether this has been produced by honeybees from the nectar of flowers or from exudates secreted by plants or insects (Radovic et al., 2001; Serra-Bonvehí & Ventura-Coll, 2003). Given that both monofloral and honeydew honey from certain plants tend to have a higher commercial value than other varieties, establishing botanical origin is an important part of the quality control process in these honeys.

The large number of studies published in the past three decades on the volatile profile of honey with different botanical and geographical origins illustrates the importance of determining a honey's aroma and flavour. Very few studies, however, have analyzed the effect of heat treatment on the volatile fraction of honey, and none of them has analyzed the effect of temperatures used in industrial processes. Many authors agree that certain volatile compounds such as furan derivatives (i.e. furfural, methylfurfural, and furfuryl alcohol) are normally good indicators of heat treatment and storage conditions (Castro-Vazquez, Diaz-Maroto, & Pérez-Coello, 2006). Wootton, Edwards, and Faraji-Haremi (1978), for example, used gas chromatography–mass spectrophotometry to study the effect of heating Australian honey at a temperature of 50 °C. They found that many high-point components of the honeys were decomposed during storage or processing at 50 °C and that other components, such as furfural, furanaldialdehyde, 2-acetylfuran, cetol, and other

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0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.05.068

unidentified compounds, increased in level. Visser, Allen, & Shaw, 1988, reported that when monofloral manuka honey was heated at 50 and 80 °C, there were significant variations in the peak areas (obtained by GC–MS) of many compounds. Maximum and minimum levels of these compounds, which indicate formation/decomposition reactions, were also seen at intermediate temperatures of 60 and 70 °C. More recently, Serra-Bonvehí & Ventura-Coll, 2003 investigated the importance of flavour profiling in identifying quality control indicators for fresh and heated monofloral honeys (*Lavandula stoechas, Castanea sativa, Dorycnium pentaphyllum, Rosmarinus officinalis, Eucalyptus spp.,* and *Robinia pseudoacacia*). These authors, on evaluating the effect of time and temperature on flavour by calculating a flavour index determined by spectrophotometry, observed that this index increased progressively by a factor of 10–40 during heat treatment. Although the volatile fraction of the above honeys was characterized in the study, the authors found no significant correlation between the flavour index and the variations in the different volatile compounds identified.

Increasing knowledge about the influence of heat on the volatile composition of honey is key to gaining a better understanding and control of variations in quality (Wootton et al., 1978; Anklam, 1998). One particularly interesting aspect is the effect that industrial processing has on flavour and aroma. To this end, the aim of this study was to analyze the influence of heat treatment on the volatile profile of four varieties of honey subjected to conditions similar to those used in industrial liquefaction and pasteurization processes. All the samples analyzed were previously characterized to determine their melissopalynological, physicochemical, and volatile profiles.

2. Materials and methods

2.1. Honey samples

Four types of Spanish honey; three of floral origin (citrus, rosemary and polyfloral) and one from honeydew (forest origin) were used in this study. The botanical origin of the samples was ascertained by melissopalynological analysis. These four varieties were chosen as they represent the most popular types of honey consumed in Spain. The samples were collected in 2005. For each type of honey, ten different raw batches (15 kg each) were obtained directly (to ensure freshness) from ten local beekeepers. Each sample was divided into three parts: one was analyzed in its raw state (unheated) and the other two were analyzed following heat treatment (liquefaction and liquefaction plus pasteurization). The raw samples were characterized on arrival at the laboratory and the others were preserved at 20 °C until processed. Volatile fractions were analyzed immediately following processing.

2.2. Heat treatment

The heat treatment temperatures were chosen considering the standard temperatures to which honey is exposed in industrial liquefaction and pasteurization processes. Accordingly, the liquefaction samples were placed in a temperature-controlled oven (Selecta model 20000207 80L, Barcelona, Spain) at 45 °C (±1 °C) for 48 h, and the commercial pasteurization of honey was simulated by pumping all honeys at 7 mL/s flow through silicone tubing (6 mm bore and 1 mm wall thickness by 1500 cm length) immersed in a temperature-controlled oil bath (Digiterm 200, Selecta, Barcelona, Spain) at 80 °C (±0.05 °C) corresponding to 4 min retention in the oil bath, using a variable speed peristaltic pump (Heidolph model Pumpdrive 5001, Schwabach, Germany). Given that not all the honeys had the same viscosity, the pump speed was adjusted in each case to achieve the desired pasteurization time. After thermal treatments, all the samples were quickly cooled approximately to 30 °C. Immediately after pasteurization, honey was placed in cylindrical flasks (4 cm diameter × 8 cm height) introduced in a water recirculation bath at 20 °C. Honey was kept in the bath until it reached the desired temperature (measured with PT100 sensor).

2.3. Melissopalynological analysis

Melissopalynological analysis was performed using the methods recommended by the International Commission for Bee Botany (Louveaux, Mauricio, & Vorwohl, 1978). Microscope slides were prepared without acetolysis solution to preserve all the components in the extracted sediments. A light microscope (Zeiss Axio Imager, Göttingen, Germany) at a magnification power of x400 with DpxView LE image analysis software attached to a DeltaPix digital camera was used in this analysis. 400 grains of pollen from each honey preparation were classified according to pollen grains in the literature (Sainz-Laín & Gómez-Ferreras, 1999).

2.4. Physicochemical analysis

5-Hydroxymethylfurfural content (HMF), diastase activity, pH, total acidity, electrical conductivity, and moisture content were analyzed in accordance with the harmonized methods of the European Honey Commission (Bogdanov, 2002). Colour was measured by reflectance spectroscopy using a spectrocolorimeter Minolta CM-3600d (Osaka, Japan), the samples were placed in 20 mm thick holders and measured against a black and white background. Translucency was determined by applying the Kubelka–Munk theory for multiple scattering to the reflection spectra (Hutchings, 1999). Colour coordinates CIEL* a* b* were obtained from R_∞ between 400 and 700 nm for D65 illuminant and from 2° observer (Talens, Martinez-Navarrete, Fito, & Chiralt, 2001).

All the tests were performed in triplicate.

2.5. Volatile compound analysis

Aromatic compounds were extracted by purge and trap thermal desorption; 30 g samples of each variety of honey spiked with 40 µg camphor as an internal standard were placed in a purging vessel flask and left in a water bath at 45 °C for 45 min. During this time, purified nitrogen (100 mL min^{-1}) was forced through a porous frit placed at the bottom of the vessel, producing a stream of bubbles which passed through the sample and the volatile compounds were collected. These were trapped in a 100-mg porous polymer (Tenax TA, 20–35 mesh) packed into a glass tube placed at the end of the system. The volatile compounds were subsequently thermally desorbed using a direct thermal desorber (TurboMatrix TD, Perkin ElmerTM, CT-USA). Desorption was performed under a 10-mL min^{-1} helium flow at 220 °C for 16 min. The volatiles were then cryofocused in a cold trap at −30 °C and transferred directly onto the head of the capillary column by heating the cold trap to 250 °C (at a rate of 99 °C/s).

GC–MS analyses were performed using a Finnigan TRACETM MS (TermoQuest, Austin, USA). Volatile compounds were separated using a BP-20 capillary column (SGE, Australia) (60 m length, 0.32 mm i.d., 1.0 µm film thickness). Helium at a constant flow rate of 1 mL min^{-1} was used as a carrier gas. The temperature was programmed to increase from 40 °C (2-min hold time) to 190 °C at 4 °C min^{-1} (11-min hold time) and finally to 220 °C at 8 °C min^{-1} (8-min hold time). The MS interface and source temperatures were 250 °C and 200 °C, respectively. Electron impact mass spectra were recorded in impact ionization mode at 70 eV and with a mass range of m/z 33–433. A total of three extracts were obtained for each sample.

Volatile compounds were identified by comparing their GC retention indices and mass spectra against authentic standards (Sigma–Aldrich, San Louis, Missouri and Acros Organics, Geel, Belgium). Compounds for which it was not possible to find reference volatiles were tentatively identified by comparing their mass spectra with spectral data from the National Institute of Standards and Technology 2002 library, from retention indices (Kondjoyan & Berdague, 1996) and from the literature (Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2005; Alissandrakis, Tarantilis, Harizanis, & Polissiou, 2007; Bouseta & Collin, 1995; Campos, Nappi, Raslan, & Augusti, 2000; Castro-Vazquez et al., 2006; Castro-Vazquez, Diaz-Maroto, & Pérez-Coello, 2007; Fisher & Scott, 1997; Rowe, 2004; Serra-Bonvehí & Ventura-Coll, 2003; Soria, Gonzalez, de Lorenzo, Martínez-Castro, & Sanz, 2004; Tananaki, Thrasyvoulou, Giraudel, & Montury, 2007; Visser et al., 1988; Wootton et al., 1978; Overton & Manura, 1994; Radovic et al., 2001).

The values were calculated as the ratios between the peak areas of each compound and the peak area of the internal standard. These ratios were the variables used in the statistical analysis.

2.6. Statistical analysis

Statistical analysis was performed using version 5.1 of the Statgraphics Plus software system. The data corresponding to each variable (volatile compound) were analyzed by multifactor analysis of variance (ANOVA) with examination of interactions between factors (heat treatment and type of honey). Multiple comparisons were performed using the least significant difference test (LSD), and statistical significance was set at $\alpha = 0.05$. Principal component analysis (Esbensen, 2000) was performed (Unscrambler version 9.7; CAMO Process AS, Oslo, Norway) on the means of the volatile compounds identified in the four types of honey (ten batches of each) in the different states analyzed (raw, liquefied, and pasteurized).

3. Results and discussion

3.1. Melissopalynological and physicochemical characterization of raw honey

A melissopalynological characterization of the 40 raw honey samples used in this study was carried out. Although this is a laborious task that requires skilled personnel, it is currently the most reliable and useful way of determining the botanical origin of honey (Von Der Ohe, Persano, Piana, Morlot, & Martin, 2004). It is well known that this identification is based on the relative frequencies of the types of pollen from nectariferous species. The first row in Table 1 shows the percentage of the most abundant pollen (ranges between minimum and maximum values) together with the other pollen types identified in every type of honey studied. As can be seen, the results varied considerably according to the origin of the honey. For example, the percentage of citrus pollen found in the ten batches of citrus honey ranged between 21% and 37.9%.; these percentages being high enough for this honey to be classified as citrus honey. This affirmation agrees with different authors (Sainz-Laín & Gómez-Ferreras, 1999; Von Der Ohe et al., 2004) as well as with the Valencian Community Regulations. (1998). It is important to note that in this work, the polyfloral honey had up to 7.4% citrus pollen; however, this level is not enough to consider this honey as citrus honey. For honeydew, low or very low dew elements (HDE: honey dew elements) were observed. This means that this kind of honey could be from a dry area (with low rainfall) as is the case of the Spanish Mediterranean, where HDE are very rare or inexistent (Gomez-Pajuelo, 2004). In this study, the confirmation of the classification of this honey as honeydew was complemented by the physicochemical characterization.

Table 1 also shows the minimum and maximum values for the different physicochemical parameters analyzed in the untreated samples. The values correspond to the results of the three tests performed on each of the ten samples of the four types of honey.

Total acidity, pH, and electrical conductivity were analyzed as they are considered by many authors to be useful indicators for differentiating floral honey from honeydew honey (Campos, della Modesta, da Silva, & Raslan, 2001). Moisture content is an important quality parameter for companies when buying fresh honey, as high moisture content can trigger yeast fermentation, limiting the shelf-life of honey during storage.

In relation to moisture, all the samples complied with the European Commission Directive relating to honey (2001), which stipulates that honey should have a maximum moisture content of 20%. This limit is used to control maturity and quality as moisture content not only depends on the season in which the honey is harvested and the climate conditions, but also on the quality of beekeeping practices (i.e. if the honey has been allowed to mature properly in the hive) (White & Bryant, 1996; Bogdanov, 1999). The pH values for all the samples analyzed are acceptable according to Bogdanov, 1999 (3.0–4.3).

As it could be expected, honeydew honey had the highest levels of conductivity, pH, total acidity, and diastase activity. In a characterization study of non-industrially produced honeys in Madrid, Spain, (Soria et al., 2004; Soria, Gonzalez, de Lorenzo, Martínez-Castro, & Sanz, 2005), the authors showed that the mean values for electrical conductivity, total acidity and pH, as well as for ash content and net absorbance, were higher in honeydew honey than in honey classified as nectar honey.

Rosemary and citrus honeys had the lowest total acidity and diastase activity levels, considerably lower than those obtained

Table 1
Melissopalynological (pollen) and physicochemical results (range between minimum and maximum values) of the untreated honeys

Pollen and physicochemical parameters	Citrus	Rosemary	Polyfloral	Honeydew
Pollen	-*Citrus* sp. *21–37.9% -others: Oxalis* sp., *Olea europaea* sp., *Quercus*, *Helianthus annus* L., *Carduus type*	-*Rosmarinus officinalis: 26–37%; -others: Prunus dulcis; Cistaceae; Leguminosae*	-*Citrus* sp.: *5–7.4% -others: Rosaceae, Brassicaceae, Quercus*	- *HDE/P: 1–2.3% -others: Erica* sp. pl., *Helianthus annus* L., *Rubus* sp. pl.
pH	3.88–4.00	3.74–3.99	3.67–3.89	4.02–4.25
Moisture (g/100g)	15.9–16.2	16.5–18.1	17.2–18.7	15.2–16.3
Total acidity (meq kg^{-1})	18.2–21.4	17.2–20.5	26.3–30.2	45.6–53.5
Conductivity (μS cm^{-1})	254–356	113–296	464–684	913–1085
HMF (mg kg^{-1})	4.31–5.27	3.10–3.79	1.89–2.31	4.36–5.35
Diastase activity (ID)	16.89–20.64	18.57–22.70	39.24–47.96	34.41–42.05

Ten different batches were analyzed for every type of honey (three replicates per sample).

for the honeydew and polyfloral. Other previous studies have proven that among floral honeys, citrus honey has low acidity and conductivity levels (Corbella & Cozzolino, 2006; Terrab, Gonzalez, Diez, & Heredia, 2003; Serrano, Villarejo, Espejo, & Jodral,2004). Finally, the results for the polyfloral honeys were in the intermediate range, which is logical given that they come from a variety of different nectars (Corbella & Cozzolino, 2006).

In this study, HMF content, which is widely recognized as an indicator of freshness, was always lower than 5.5 mg/kg. These low values demonstrate that the honey was indeed fresh and had not been heat treated (Bogdanov, 1999). This finding is corroborated by the fact that diastase activity was high in all the honeys analyzed, particularly so in polyfloral and honeydew honey. Diastase activity results were well over eight, the minimum level established by European Commission Directive relating to honey (2001).

To characterize the raw honey samples by colour, they were plotted in their corresponding positions on the a^*-b^* and a^*-L^* colour spaces (Fig. 1A and B). On the a^*-b^* colour space, the nearer a honey is to the origin, the less purity of colour it has, and the further away it is from the origin, the greater the purity. As can be seen, the honeys with the greatest purity of colour were the citrus and rosemary honeys; the honeydew honey, followed by the polyfloral honey, exhibiting the least purity of colour. Rosemary honey, and citrus honey in particular, had the greatest yellow component (highest b^* values), and polyfloral honey had the greatest red component (highest a^* value). Fig. 1B shows that rosemary and citrus honey were clearer (higher L^* value) than the other varieties. Honey dew not only showed the less purity of colour of all the analysed honeys, but it also showed to be the darkest (lowest L^* value).

The colour values obtained were within the expected ranges for each of the honeys studied. Most colour values reported in the literature generally correspond to measurements taken on the Pfund scale (mm) (Corbella & Cozzolino, 2006; Persano-Oddo, Gioia-Piazza, & Zellini, 1995). Although only a few studies have used CIELAB (L^*, a^*, b^*) to measure colour in nectar and honeydew honey (Soria et al., 2004; Terrab et al., 2003), their results were similar to this study.

3.2. Volatile composition of the four types of honeys

The volatile fraction of the four types of honey both before and after heat treatment (liquefaction and pasteurization) was analyzed. A total of 74 compounds were identified: 38 in citrus honey, 32 in rosemary honey, 29 in polyfloral honey, and 47 in honeydew honey. Table 2 shows the relative areas mean values of the identified compounds. Most of them had been previously identified in honey.

Of the 74 compounds identified, nine were found in all the samples analyzed, albeit in considerably different concentrations. These nine compounds were acetic acid; 2-methyl-2-propanol; 3-methyl-3-buten-1-ol; ethanol; phenyl ethyl alcohol; octane; dimethyl sulphide; D-limonene and linalool oxide, all compounds that had been previously reported by other authors as being present, albeit to considerably varying degrees, in a range of honeys, including the four varieties studied.

The most abundant compounds found in the batches of honeydew analyzed were 2-methy-1-butanol; 3-hydroxy-2-butanone (acetoin); 3 methyl-3-buten-1-ol and ethanol, although none of these compounds were exclusive to honeydew honey. Acetic acid and 3 methyl-3-buten-1-ol were found in all the honeys analyzed in this work. 2-methy-1-butanol was also isolated in polyfloral honey, although in much smaller quantities than in honeydew honey. Although 3-hydroxy-2-butanone was present in citrus and polyfloral honey, it was considerably more abundant in honeydew honey. Acetic acid and 3-hydroxy-2-butanone (acetoin) have also been found in abundant quantities in honeydew honey in previous studies (Campos et al., 2000; Soria et al., 2005). Of the 74 compounds identified, 20 were exclusive to honeydew honey. These were 2-methyl-propanoic acid; propanoic acid; 1,3-butanediol; 1-hexanol; 2,3-butanediol; 3-hexen-1-ol; 3-methyl-2-butanol; 3-pentanol; 1-cyclohexene-1-carboxaldehyde-5,5-dimethyl-3-oxo; 1-hydroxy-2-butanone; 2-hydroxy-3-pentanone; 2-methyl-4-hexyne-3-one; 3,5,5-trimethyl-2-ciclohexen-1-one; 1,1-bicyclopropyl-2-octanoic acid-2-hexyl-methyl ester; ethyl acetate; 2-furanmethanol; 2-furanmethanol-5-ethenyltetrahydro-α,α,5-trimethyl-trans; dihydro-2(3H)-furanone; dihydro-2-methyl-3(2H)-furanone and 2,4,5-trimethyl-1,3-dioxolane. Of note was the large number of furan compounds found in honeydew honey; of the seven furan compounds identified, five were found in this type of honey, four of them exclusively so.

Although some authors have suggested that benzene and phenolic compounds might be characteristic of honeydew honeys (Castro-Vazquez et al., 2007), in this study the benzene compounds identified (e.g. benzaldehyde, benzeneacetaldehyde, and phenyl-ethyl-alcohol) were not found in considerable quantities in honeydew honey and, in addition, they were not exclusive to this type of honey. Moreover, certain authors have suggested that benzene compounds and derivatives, together with terpenes and norisoprenoids, might be characteristic components of floral honeys, so much so in fact that in certain cases they have been classified as "floral markers" (Fisher & Scott, 1997; Serra-Bonvehí & Ventura-Coll, 2003). In this study, three of the six terpenes identified (D-limonene, hotrienol, and linalool oxide) were found in honeydew honey, linalool oxide being the most abundant in this type of honey.

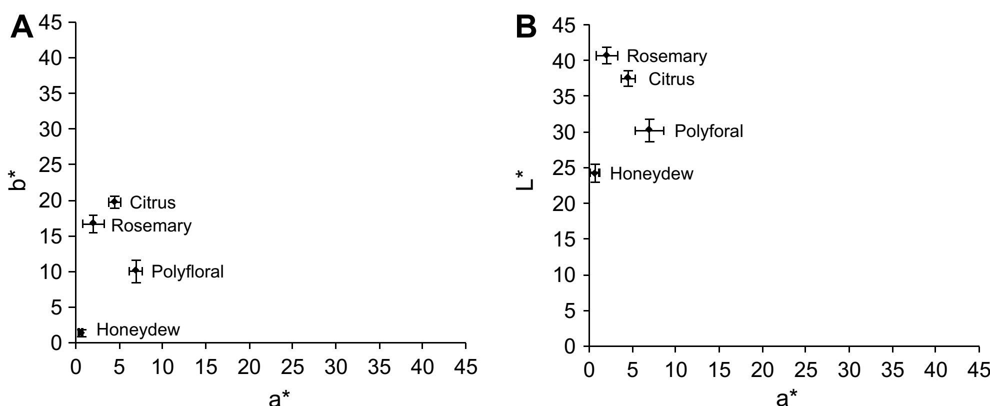

Fig. 1. Colour spaces (A: a^*-b^* and B: a^*-L^*) showing position of raw honeys.

Table 2
Relative areas mean values (with respect to internal standard) of the volatile compounds identified in samples before (*R*, raw) and after heat treatment (*L*, liquefaction and *P*, pasteurization) of honeys (citrus, rosemary, polyfloral, and honeydew); and ANOVA *F*-ratio for each of the two factors (treatment and type of honey) and their respective interactions for each of the variables analyzed

Volatile compounds	Citrus			Rosemary			Polyfloral			Honeydew			ANOVA *F*-ratio		
	R	*L*	*P*	*R*	*L*	*P*	*R*	*L*	*P*	*R*	*L*	*P*	*T*	*H*	$T \times H$
Acids															
2-Ethyl-hexanoic acid	–	–	–	0.08	0.09	0.08	–	–	–	–	–	–	0.24ns	400.46***	0.24ns
2-Methyl-hexanoic acid	–	–	–	0.27	–	–	–	–	–	1.18	1.74	1.68	0.21ns	63.08***	1.41ns
2-Methyl-propanoic acid	–	–	–	–	–	–	–	–	–	0.55	0.89	0.47	1.12ns	28.03***	1.12ns
Acetic acid	2.00	0.99	2.16	1.10	1.10	0.88	0.18	1.15	0.08	6.42	9.40	13.94	1.78ns	31.79***	2.11ns
Hexanoic acid	–	–	–	0.05	0.75	0.06	–	–	–	0.49	0.47	0.76	2.15ns	16.76***	3.90**
Propanoic acid	–	–	–	–	–	–	–	–	–	0.35	0.30	0.37	2.35ns	46.54***	1.58ns
Alcohols															
1,3-Butanediol	–	–	–	–	–	–	–	–	–	0.20	0.25	0.25	1.65ns	791.13***	1.84ns
1-Hexanol	–	–	–	–	–	–	–	–	–	0.90	1.12	1.00	1.48ns	256.83***	1.48ns
2,3-Butanediol	–	–	–	–	–	–	–	–	–	0.33	0.26	0.44	0.10ns	4.16*	0.1ns
2-Butanol	–	–	–	0.23	0.36	0.35	–	–	–	0.16	0.28	0.14	0.62ns	11.56***	0.37ns
2-Butoxy-ethanol	–	–	–	0.31	0.66	0.60	–	–	–	–	–	–	8.94**	216.62***	8.94***
2-Methyl-1-butanol	–	–	–	–	–	–	0.28	0.47	0.74	3.71	4.28	5.51	3.24ns	140.07***	1.86ns
2-Methyl-1-propanol	4.35	3.70	6.30	–	–	–	0.39	0.68	0.60	1.93	2.24	2.99	11.15***	189.49***	5.55***
2-Methyl-2-buten-1-ol	0.40	0.73	0.70	–	–	–	–	–	–	1.70	0.47	1.99	8.11**	74.04***	10.28***
2-Methyl-2-propanol	0.96	0.55	1.27	1.66	1.06	2.81	1.49	1.04	1.37	0.92	1.82	1.06	1.89ns	2.54ns	0.08ns
2-Methyl-3-buten-2-ol	0.43	0.44	0.59	1.05	0.94	0.93	–	–	–	0.58	0.60	0.85	0.56ns	27.36***	0.45ns
3-Hexen-1-ol	–	–	–	–	–	–	–	–	–	0.53	0.18	0.67	18.09***	175.09***	18.09***
3-Methyl-1-butanol	–	–	–	0.08	–	–	0.31	0.41	0.48	–	–	–	8.28**	133.65***	7.68***
3-Methyl-2-butanol	–	–	–	–	–	–	–	–	–	0.90	1.00	1.10	0.73ns	638.17***	0.97ns
3-Methyl-3-buten-1-ol	0.91	1.03	1.21	0.20	0.54	0.43	0.88	1.30	0.90	2.45	2.43	3.06	3.07ns	94.33***	1.66ns
3-Pentanol	–	–	–	–	–	–	–	–	–	0.14	0.20	0.26	1.64ns	222.39***	1.83ns
Benzyl alcohol	–	–	–	0.05	0.76	0.04	–	–	–	0.36	0.50	0.55	2.88ns	4.18***	2.47ns
Ethanol	0.21	0.26	1.08	0.39	0.27	0.26	0.98	1.22	1.32	2.36	3.00	3.50	2.35ns	39.26***	1.31ns
Nonanol	–	–	–	0.03	–	–	0.11	0.14	0.11	0.41	0.45	0.11	5.57**	34.9***	4.38*
Phenyl ethyl alcohol	0.32	0.45	0.49	0.05	0.77	0.70	0.08	0.14	0.12	0.36	0.53	0.53	3.98*	3.14*	1.58ns
Aldehydes															
1-Cyclohexene-1-carboxaldehyde-5,5-dimethyl-3-oxo	–	–	–	–	–	–	–	–	–	0.63	1.51	1.00	5.88*	1.64.84***	6.65***
2-Methyl-2-butenal	–	–	–	0.26	0.59	0.39	0.86	1.52	1.61	–	–	–	5.14*	82.54***	2.82*
3-Methyl-2-butenal	0.71	0.51	0.77	0.13	0.49	0.53	0.39	–	0.51	–	–	–	5.34*	27.37***	3.85**
3-Methyl-butanal	–	–	–	–	–	–	0.87	2.01	1.68	–	–	–	3.04ns	60.79***	3.04*
Benzaldehyde	1.38	1.20	1.77	0.37	0.87	0.77	–	–	–	0.12	0.25	0.20	16.68***	378.89***	11.39***
Benzeneacetaldehyde	0.82	0.99	1.15	0.24	0.85	0.51	–	–	–	–	–	–	7.53**	140***	4.41**
Decanal	0.29	0.27	0.44	0.07	0.24	0.26	–	–	–	0.15	0.18	0.12	6.20**	48.11***	3.77*
Hexanal	0.08	0.11	0.16	0.16	0.28	–	–	–	–	–	–	–	8.02**	41.81***	15.19***
Lilac aldehyde A	1.89	1.64	2.79	–	–	–	–	–	–	–	–	–	48.69***	1727.3***	48.69***
Lilac aldehyde C	1.81	1.51	2.67	–	–	–	0.33	0.49	0.44	–	–	–	38.81***	1115.61***	39.00***
Lilac aldehyde B	1.16	1.01	1.76	–	–	–	0.36	0.30	0.38	–	–	–	25.21***	593.27***	20.57***
Lilac aldehyde D	1.47	1.36	2.49	–	–	–	0.35	0.61	0.52	–	–	–	38.58***	743.54***	36.62***
Nonanal	0.74	0.85	1.00	0.74	0.39	1.05	0.31	0.50	0.38	–	–	–	7.87**	95.66***	7.07***
α-4-Dimethyl-3-cyclohexe-1-acetaldehyde	0.92	1.48	1.18	–	–	–	–	–	–	–	–	–	16.31***	879.07***	16.35***
Hydrocarbons															
Octane	0.46	0.39	0.52	1.19	0.64	1.06	0.54	0.77	0.56	0.18	0.16	0.16	1.03ns	26.61***	2.46ns

(*continued on next page*)

Table 2 (*continued*)

Volatile compounds	Citrus			Rosemary			Polyfloral			Honeydew			ANOVA *F*-ratio		
	R	*L*	*P*	*R*	*L*	*P*	*R*	*L*	*P*	*R*	*L*	*P*	*T*	*H*	*T* × *H*
Dodecane	–	–	–	0.10	0.22	0.08	–	–	–	–	–	–	2.77ns	23.44***	2.77*
Toluene	0.21	0.12	0.18	–	–	–	0.62	0.84	0.61	–	0.05	0.14	0.33ns	49.51***	1.24ns
m-Xylene o p-Xylene	–	0.08	0.08	0.27	–	–	–	–	–	–	–	–	23.34***	15.40***	15.24***
Ketones															
1-Hydroxy-2-butanone	–	–	–	–	–	–	–	–	–	1.00	1.66	1.54	0.94ns	43.86***	0.94ns
1-Hydroxy-2-propanone	0.32	0.21	0.27	0.07	–	–	–	–	–	0.80	2.39	1.92	1.22ns	18.05***	1.64ns
2,3-Butanedione	0.52	0.45	0.75	0.14	0.24	0.24	0.64	0.69	0.58	–	–	–	1.21ns	79.17***	2.57*
2-Hydroxy-3-pentanone	–	–	–	–	–	–	–	–	–	0.15	0.33	0.15	0.67ns	8.28***	0.67ns
2-Methyl-4-hexyne-3-one	–	–	–	–	–	–	–	–	–	2.44	3.01	2.39	0.25ns	42.29***	0.25ns
3,5,5-Trimethyl-2-ciclohexen-1-one	–	–	–	–	–	–	–	–	–	0.35	0.29	0.53	1.57ns	44.52***	1.57ns
3-Hydroxy-2-butanone	1.06	1.34	1.38	–	–	–	0.80	1.22	1.21	3.32	5.24	6.98	1.88ns	23.45***	1,12ns
6-Methyl-5-hepten-2-one	0.09	0.09	0.12	–	–	–	–	–	–	–	–	–	3.77*	366.83***	3.77**
Acetone	0.21	0.28	0.36	–	–	–	1.42	1.82	2.17	0.47	0.66	0.64	2.11ns	56.73***	0.85ns
Esters															
(1,1-Bicyclopropyl)-2-octanoic acid, 2-hexyl-methyl ester	–	–	–	–	–	–	–	–	–	0.19	0.53	–	1.80ns	20.60***	1.80ns
1-Metoxy-2-propyl acetate	–	–	–	0.08	0.38	0.18	–	–	–	–	–	–	5.9**	32.52***	5.9***
Antranilic acid methyl ester	0.71	0.66	0.85	–	–	–	–	–	–	–	–	–	1.43ns	45.83***	4.43**
Ethyl acetate	–	–	–	–	–	–	–	–	–	0.81	1.64	1.24	19.74***	527.11***	19.74***
Isopropyl mirystate	0.30	0.29	0.30	–	–	–	–	–	–	–	–	–	0.84ns	414***	1.00ns
Methyl salicylate	–	–	–	0.49	1.02	0.61	–	–	–	–	–	–	6.68**	128.70***	6.68***
Furanes															
1-(2-furanyl)-ethanone	0.13	0.16	0.18	–	–	–	–	–	–	0.27	0.41	0.38	2.62ns	78.51***	1.39ns
2-Furanmethanol	–	–	–	–	–	–	–	–	–	1.16	2.33	1.96	2.67ns	20.83***	2.67*
2-Furanmethanol-5-ethenyltetrahydro-αα,5-trimethyl-trans	–	–	–	–	–	–	–	–	–	1.52	2.09	1.95	1.49ns	173.91***	1.49
4,5,7a-Hexahydrobenzofuran-3,6-dimethyl-2,3,3a	0.20	0.45	0.48	–	–	–	–	–	–	–	–	–	42.76***	1003.77***	54.93***
Dihydro-2(3H)-furanone	–	–	–	–	–	–	–	–	–	0.39	0.40	0.50	0.77ns	123.77***	0.77ns
Dihydro-2-methyl-3(2H)-furanone	–	–	–	–	–	–	–	–	–	0.34	0.46	0.54	2.54ns	143.07***	2.54ns
Furfural	1.48	1.98	2.15	0.25	1.04	1.02	2.72	5.23	4.31	–	–	–	8.97**	82.27***	2.66*
Sulphur compounds															
Dimethyldisulphide	–	0.11	0.10	–	–	–	0.22	0.41	0.30	0.38	0.45	0.48	3.71ns	50.18***	0.86ns
Dimethylsulphide	0.09	0.12	0.15	0.20	0.17	0.11	0.42	0.65	0.71	0.70	0.47	0.69	0.42ns	20.43***	11.9ns
Terpenes															
β-Damascenone							0.22	0.34	0.33				3.23ns	192.57***	b-3.23*
β-Linalool	–	–	–	–	–	–	1.84	2.00	2.23	–	–	–	0.88ns	280.24***	0.88ns
D-Limonene	0.57	0.39	0.37	0.11	0.03	0.07	0.40	0.36	0.36	0.12	0.25	0.21	0.33ns	5.82**	2.18ns
Hotrienol	0.97	1.33	1.30	–	–	–	2.18	3.82	2.93	0.10	1.86	–	5.23*	27.20***	1.58ns
8-Hydroxylinalool	0.26	0.32	0.33										3.10ns	480.6***	
Linalooloxide	1.01	1.20	1.35	0.04	0.50	0.48	0.30	0.53	0.47	1.11	1.30	1.48	10.05***	106.32***	2.38ns
Miscellaneous															
2,4,5-Trimethyl-1,3-dioxolane	–	–	–	–	–	–	–	–	–	0.94	0.91	1.85	3.0ns	48.45***	3.0*

T: treatment; H: honey; ns: non significant.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

Soria et al. (2004) reported that in addition to the following physicochemical parameters: colour, electrical conductivity, acidity, ash content, and pH, certain volatile compounds, 3-hydroxy-2-butanone; 2,3-butanediol; 1-hydroxy-2-propanone and 1-(2-furanyl)-ethanone could be used to distinguish honeydew honey from nectar honey. The authors found that 3-hydroxy-2-butanone; 2,3-butanediol and 1-hydroxy-2-propanone were most positively correlated to honeydew honey and that 1-(2-furanyl)-ethanone was most positively correlated to nectar honey. In this work, the four compounds were more abundant in honeydew honey than in nectar honey, and 2,3-butanediol, for example, was present only in the former.

Of the 38 compounds identified in citrus honey, seven were exclusive to this variety. These were lilac aldehyde A; α-4-dimethyl-3-cyclohexe-1-acetaldehyde; 6-methyl-5-hepten-2-one; anthranilic acid methyl ester; isopropyl myristate; 4,5,7a-hexahydrobenzofuran-3,6-dimethyl-2,3,3a and 8-hydroxylinalool. As expected, anthranilic acid methyl ester was only found in citrus honey. As this compound is specific to this variety of honey, it is considered a reliable marker. Concentrations, however, have varied from one study to the next, and certain authors have found no traces of it in citrus honey (Overton & Manura, 1994; Pérez, Sanchez-Brunete, Calvo, & Tadeo, 2002). Concentrations reported by the literature range from approximately 500 to 3500 μg/kg (Ferreres, Giner, & Tomas-Barberán, 1994; Castro-Vazquez et al., 2007). In other studies where levels referred to the internal standard, these ranged from 0.1 to 2.7 (Alissandrakis et al., 2007). Similar values were found in this study for anthranilic acid methyl ester, as relative areas with respect to the internal standard for this compound were between 0.66 and 0.85. Some authors have even identified anthranilic acid methyl ester in honey other than citrus honey (Verzera, Campisi, Zappala, & Bonaccorsi, 2001). The considerable variations in the quantities of anthranilic acid methyl ester identified in different citrus honeys can be attributed to the fact that this compound undergoes significant changes in different environmental and storage conditions (White & Bryant, 1996). Serra-Bonvehí (1995), for example, suggested that the level of anthranilic acid methyl ester in citrus honey depended on the variety of fruit, moisture content, level of freshness, and beekeeping practices. Castro-Vazquez et al., 2006 suggested that the best marker of citrus honey was 2,6-dimethyl-10-methylene-2,6,11-dodecatrienal or β-sinensal, while Ferreres, Garcia-Viguera, Tomás-Lorente, and Tomás-Barberán (1993) proposed the hesperetin. None of these compounds were found in this study but this could perhaps be because the above authors used different methods (simultaneous distillation and extraction "SDE" followed by GC–MS and high performance liquid chromatography, respectively) to this study. Castro-Vazquez et al. (2006) believed that linalool derivatives and sinensal were primarily responsible for the floral, fresh, and orange-like aroma of citrus honey. Linalool is a component of essential oils from citrus flowers, and large quantities of linalool derivatives, such as 2,6-dimethyl-2,7-octadiene-1,6 (8-hydroxylinalool) and lilac aldehydes A, B, C and D have been found in citrus honey (Alissandrakis et al., 2007). These authors actually found that lilac aldehydes accounted for approximately 20% of the volatile compounds isolated in raw citrus honey, coinciding with reports in other papers that citrus honey contains large amounts of lilac aldehydes in comparison with other compounds.

Some authors have found α-4-dimethyl-3-cyclohexe-1-acetaldehyde; hotrienol and linalooloxides all to be the characteristic of citrus honey (Alissandrakis et al., 2005; Castro-Vazquez et al., 2007). In this work, α-4-dimethyl-3-cyclohexe-1-acetaldehyde was only found in citrus honey, but both hotrienol and linalool oxide were found in the other honeys studied. The findings obtained in this study coincide with those of other studies, in which hotrienol and linalool oxide were identified in a variety of honeys, including rosemary, heather, and eucalyptus honey (Castro-Vazquez, Pérez-Coello, & Cabezudo, 2003).

Benzaldehyde, associated with citric aroma (Alissandrakis et al., 2007; Fisher & Scott, 1997), was isolated in greater quantities in citrus honey than in the other varieties in this work.

In this study, the largest amount of limonene, an important component of citrus essential oil (Fisher & Scott, 1997), was found in citrus honey, followed by polyfloral honey. This is logical, as mentioned earlier, as some batches of polyfloral honey analyzed contained up to 7.4% citrus pollen, indicating that this honey was produced by bees that occasionally had fed on nectar from citrus flowers. This is why polyfloral honey may contain certain compounds that are typically found in citrus honey.

Thirty-two different volatile compounds in rosemary honey were identified, although only five were exclusive ones. These were 2-ethyl-hexanoic acid; 2-butoxy-ethanol; dodecane; 1-metoxy-2-propyl acetate and methyl salicylate. Castro-Vazquez et al. (2003) also found traces of 2-ethyl-hexanoic acid in this honey. The most abundant compounds in rosemary honey were: 2-methyl-2-propanol; acetic acid; 2-methyl-3-buten-2-ol and octane. The first three have been previously reported as being present in rosemary honey (Pérez et al., 2002; Serra-Bonvehí & Ventura-Coll, 2003). Other authors have found large amounts of 2,3-butanediol in rosemary honey (Castro-Vazquez et al., 2003; Pérez et al., 2002), although only using the liquid–liquid extraction method (the compound was not identified by either solid-phase or simultaneous distillation extraction methods using the same samples). Using solid-phase microextraction, De la Fuente, Martinez-Castro, and Sanz (2005) found that 2,3-butanediol was present in much smaller concentrations than the other alcohols identified (3-methyl-1-butanol; 3-methyl-3-buten-1-ol; 2-methyl-2-buten-1-ol and 3-methyl-2-buten-1-ol). In this study, 3-methyl-1-butanol and 3-methyl-3-buten-1-ol were also found in the analysis of rosemary honey. Although some authors believe that 3-hydroxy-2-butanone (acetoin) is characteristic of rosemary honey (Pérez et al., 2002; Castro-Vazquez et al., 2003), no evidence of it was found in this work.

Polyfloral honey had the smallest number of unique compounds, namely 3-methyl-butanal, β-damascenone (known for its characteristic honey aroma) (Belitz & Grosch, 1997) and β-linalool, cited by many authors as a volatile compound of honey with floral origin (De la Fuente et al., 2005; Alissandrakis et al., 2005; Castro-Vazquez et al., 2007). It should be noted that three of the lilac aldehydes identified in citrus honey, namely B, C, and D, were also found in polyfloral honey, although in much smaller quantities. This coincides with the previous findings in this work for D-limonene, and is again explained by the fact that polyfloral honey contains citrus pollen, providing further evidence that polyfloral honey contains compounds typically found in citrus honey, albeit to a lesser extent.

3.3. Changes in volatile profile induced by thermal treatments

To study the influence of the thermal treatments on the volatile fraction of honey, a multifactor analysis of variance (ANOVA) was carried out taking into consideration two factors: the thermal treatment applied (liquefaction/pasteurization) and the type of honey. In addition, the interaction between these factors was also considered. Table 2 shows the F-ratio obtained in this analysis for the analyzed volatile components. The F-ratio represents the quotient between variability due to the considered effect and the residual variance; the higher the F-ratio, the greater the effect that a factor has on a variable. Accordingly, type of honey had the greatest impact on variables (compounds) as it was significant for almost all the compounds identified. Treatment, in contrast, was only significant for 29 of the 74 compounds identified. Interaction between the two factors was significant in approximately half of

the compounds, which indicates that in these cases, the variation of volatile compounds with thermal treatments was different depending on the type of honey.

Of the 29 compounds that were significantly altered by heat treatment, 20 belonged to the family of alcohols and aldehydes. It is possible that the moderate conditions the honey samples were subjected to in this study (liquefaction for 48 h at 45 °C and pasteurization for 4 min at 80 °C), conditions similar to those found in many packaging plants, may have a greater effect on alcohols and aldehydes (leading to their formation, in most cases) than on other compounds. Visser et al. (1988), for example, found that certain aldehyde compounds, such as 4-methoxybenzaldehyde, increased significantly when honey was heated at 60 °C–70 °C (for periods ranging between 16 and 64 h). Levels of other aldehydes

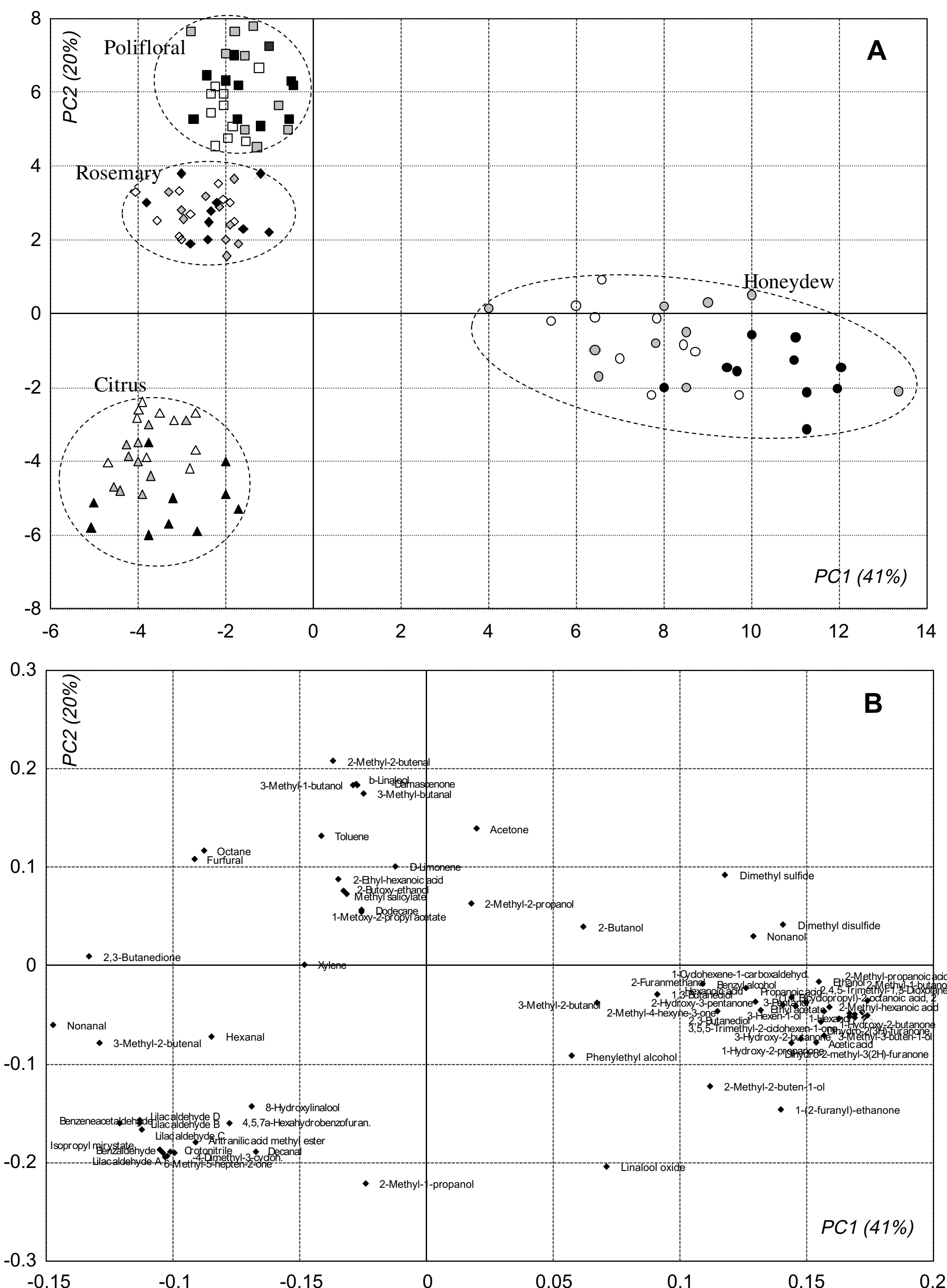

Fig. 2. Principal component analysis of volatile compounds. (A) Plot of the two principal component scores (white symbols: raw samples; grey symbols: liquefied samples and black symbols: pasteurized samples). (B) Plot of the two principal component loadings.

such as benzaldehyde, however, increased only when samples were heated at 80 °C. These authors also found increased levels of certain alcohols, including phenyl-ethyl-alcohol, on heating. The increase in levels of alcohols such as pentanol; 2-methyl-1-butanol; 3-methyl-1-butanol and propanol may originate from their corresponding amino acids (norleucine; isoleucine; leucine and α-aminobutyric acid (Wootton et al., 1978).

Furan compounds are of particular interest as they are usually formed by sugar degradation and Maillard reactions, meaning that they are good indicators of heat treatment processes and storage conditions. Compounds that can be formed in this way include furfural (from pentoses) and HMF (from hexoses). This study found that heat treatment had a significant effect on both furfural (found in the three honeys of floral origin) and 4,5,7a-hexahydrobenzofuran-3,6-dimethyl-2,3,3a (found only in citrus honey). The levels of both the compounds increased considerably following liquefaction, exhibiting little difference compared to pasteurization. The other five furan derivatives identified: 1-(2-furanyl)-ethanone; 2-furanmethanol; 2-furanmethanol, 5-ethenyltetrahydro-a,a,5-trimethyl-trans; dihydro-2(3H)-furanone and dihydro-2-methyl-3(2H)-furanone increased slightly (but not significantly) following liquefaction.

In this work, the HMF component, an important indicator of thermal treatment in honey, was quantified using the spectrophotometric method described in the Harmonized Methods of the European Honey Commission (Bogdanov, 2002) instead of being determined as a part of the volatile fraction. This is because this component is not easily volatizable unless present in large quantities. However, sometimes HMF has been identified as a volatile component (Bouseta & Collin, 1995) partly because the compounds were isolated by ebullition using a Lickens–Nickerson apparatus, which can contribute to the formation of important levels of this compound (Visser et al., 1988). In this work, the HMF content was measured in all the honey samples (raw, liquefied, and pasteurized). The mean values for liquefied (L) and pasteurized (P) honey, expressed in mg/kg were 6.0 (L) and 11.0 (P) for citrus honey, 5.1 (L) and 7.2 (P) for rosemary honey, 7.6 (L) and 9.9 (P) for polyfloral honey, and 6.2 (L) and 9.5(P) for honeydew honey. Although HMF content increased following liquefaction and pasteurization in all the samples analyzed, the mean values were well below the legally established maximum permissible limits (40 mg/kg) (European Commission Directive relating to honey, 2001). The ANOVA results showed that HMF content was affected by both heat treatment and honey type (Escriche, Visquert, Carot, Doménech, & Fito, 2008). The two factors and the interaction between them had a significant effect; the *F*-ratio values were as follows: 135.64^{***} for heat treatment, 10.37^{***} for type of honey, and 5.67^{***} for the interaction between both. These ratios clearly show that heat treatment had a much greater effect on HMF content than the type of honey did.

Once the individual behaviour of each compound was studied, a PCA was used to assess the overall effect of the type of honey and the thermal treatments on the volatile fraction. Fig. 2 shows the PCA results (a: scores of the samples, and b: loading) for the complete series of volatile compounds identified in the four types of honey (for raw, liquefied, and pasteurized samples). It was found that three of the principal components accounted for 76% of the variations in the data set. Specifically, 41% of the variability was explained by PC1, 20% by PC2, and 15% by PC3. The proximity of samples on the score plot indicates similar behaviour in terms of aromatic profiles and the proximity of compounds on the loading plot indicates that changes in concentration were correlated (similar change pattern). There are two clearly differentiated groups of samples on the plot; the one on the right corresponds to the honeydew honey and the one on the left to the floral honeys; within the floral honeys, citrus is at the bottom, rosemary, in the middle, and polyfloral, at the top. The first principal component differentiates between floral (nectar) and honeydew honey, and the second between the three types of floral honey. This indicates that differences between samples were most strongly influenced by the origin of the honey (honeydew or nectar). Liquefaction or pasteurization clearly did not exert a strong effect on volatile compounds as the samples were grouped according to the type of honey and not the type of heat treatment (raw, liquefaction, and pasteurization).

The loadings of each compound on the principal components clearly show that the grouping of the different types of honey is primarily influenced by certain compounds. For example, compounds that are only found in citrus honey (such as anthranilic acid, methyl ester, lilac aldehydes and hydroxylinalool) are largely responsible for the difference between this honey and the others. The same occurs with 1-methoxy-2-propyl-acetate; 2-ethyl-hexanoic acid and dodecane in the case of rosemary, and with furans (except furfural), sulphur compounds, and many acids and alcohols in the case of honeydew honey. The considerable difference between the aromatic components of honeydew and nectar honey corroborates the major physicochemical differences detected in this and other studies (Mateo & Bosch-Reig, 1998).

4. Conclusions

The profile of volatile compounds identified in honeys of different botanical origins made it possible to classify them by botanical origin and establish a clear differentiation between honeydew and nectar honey. Although it was found that heat treatment led to significant variations in levels of certain volatile compounds, the overall volatile fraction of each honey, determined by chemometric analysis, was only scarcely modified. It was found that honey type had a greater influence on the volatile fraction than did heat treatment (liquefaction and pasteurization) under moderate industrial-like conditions. It is therefore believed that industrial processes conducted under controlled conditions should not significantly alter the intrinsic flavour and aroma of honey. This finding is especially relevant for honey with greater commercial value as is the case of certain monofloral and honeydew honeys.

Acknowledgments

This study forms part of a project funded by the Consellería de Empresa, Educación y Ciencia de la Comunidad Valenciana (Valencian Regional Ministry of Business, Education, and Science) with project number CV2007-244), which the authors gratefully acknowledge. We thank the Foreign Language Co-ordination Office at the Polytechnic University of Valencia for their help in revising this paper.

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