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ORIGINAL RESEARCH ARTICLE

Spanish honeys with quality brand: a multivariate approach to physicochemical parameters, microbiological quality, and floral origin

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This study consisted of a palynological, microbiological, and physicochemical characterization of fifteen samples of Spanish honey sold under quality brands with different botanical and geographical origins from two consecutive harvest years (2010 and 2011). Eight of the fifteen honey samples were classified as monofloral honey from botanical origins *Persea americana, Castanea sativa*, *Rosmarinus officinalis*, *Eucalyptus* sp., and *Thymus* sp. With regard to microbiological analyses, mold, and yeast counting, *Staphylococcus aureus*, *Salmonella*, sulfite-reducing clostridia, and *Escherichia coli* were not detected in any of the samples. Aerobic mesophilic microorganisms were detected only in some samples and the counts in these cases were low. Despite the great variability between samples, the results obtained in the physicochemical analysis were consistent with the limits set by the Council Directive 2001/100. Honey samples showed high variability between two consecutive harvests, since, even if they had similar geographical origins they showed different nectar floral origins.

Mieles españolas con marca de calidad: un enfoque multivariado de parámetros fisicoquímicos, calidad microbiológica y origen floral

Este estudio consistió en la caracterización botánica, microbiológica y fisicoquímica de quince muestras de miel españolas acogidas a marcas de calidad diferenciada de diferentes orígenes botánicos y geográficos y procedentes de dos cosechas consecutivas (años 2010 y 2011). Ocho de las quince muestras de miel estudiadas fueron clasificadas como mieles monoflorales de Persea americana, Castanea sativa, Rosmarinus officinalis, Eucalyptus sp. y Thymus sp. En relación a los análisis microbiológicos los recuentos de mohos y levaduras, Staphylococcus aureus, Salmonella, clostridios sulfito reductores y Escherichia coli no fueron detectados en ninguna de las muestras. Se detectaron microorganismos aerobios mesófilos solo en algunas muestras y en este caso los recuentos fueron bajos. Aunque se detectaron coliformes, estos podrían estar asociados a un origen ambiental. A pesar de la gran variabilidad entre las muestras, los resultados obtenidos en el análisis fisicoquímico se encontraron dentro de los límites establecidos por la Directiva del Consejo 2001/100. Las muestras de miel mostraron una gran variabilidad entre cosechas porque aunque tuvieron un mismo origen geográfico, hubo diferencias en su origen floral.

Keywords: Honey; quality brands; microbiological analysis; pollen profile; physicochemical characteristics

Introduction

According to European legislation (Council Directive 2001/110. EU, 2001), honey is a natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which bees collect, transform by combining with specific substances, deposit, dehydrate, store, and leave in honey combs to ripen and mature.

Carbohydrates are the major components of honey, essentially reducing sugars such as fructose and glucose, as well as small amounts of disaccharides and polysaccharides. Honey also contains other minor compounds including minerals, protein, vitamins, organic acids, enzymes, flavonoids, phenolic acids, and volatile compounds, as well as other phytochemicals. Honey composition is inherently quite variable and depends mainly on the floral source. However, certain external factors such as seasonal, environmental, and processing conditions also play an important role (Juan-Borrás, Domenech, Hellebrandova, & Escriche, 2014; Moura Kadri, Zaluski, & De Oliveira Orsi, 2017).

Since ancient times, honey has been used not only as a food, but also for therapeutic purposes. More recently, some scientific studies have identified many of their bioactive properties (Escuredo, Silva, Valentão, Seijo, & Andrade, 2012; Ferreira, Aires, Barreira, & Estevinho, 2009; Tuberoso, Boban, Bifulco, Budimir, & Pirisi, 2013),

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Table 1. Floral origin according to label, year of harvest and geographical origin of honey samples studied.

Sample identification	Botanical origin according to label		Harvest year	Production area
H1	Avocado	(*Persea americana*)	2010	PDO *Miel de Granada*
H1a	Avocado	(*Persea americana*)	2011	PDO *Miel de Granada*
H2	Chesnut	(*Castanea sativa*)	2010	PDO *Miel de Granada*
H2a	Chesnut	(*Castanea sativa*)	2011	PDO *Miel de Granada*
H3	Lavender	(*Lavandula scoechas*)	2010	PDO *Miel de La Alcarria*
H4	Rosemary	(*Rosmarinus officinalis*)	2010	PDO *Miel de La Alcarria*
H4a	Rosemary	(*Rosmarinus officinalis*)	2011	PDO *Miel de La Alcarria*
H5	Blackberry	(*Rubus* sp.)	2010	PGI *Miel de Galicia*
H5a	Blackberry	(*Rubus* sp.)	2011	PGI *Miel de Galicia*
H6	Eucalyptus	(*Eucalyptus* sp.)	2010	PGI *Miel de Galicia*
H6a	Eucalyptus	(*Eucalyptus* sp.)	2011	PGI *Miel de Galicia*
H7	Thyme	(*Thymus* sp.)	2010	Province of León
H7a	Thyme	(*Thymus* sp.)	2011	Province of León
H8	Heather	(*Erica* sp.)	2010	Province of León
H8a	Heather	(*Erica* sp.)	2011	Province of León

which primarily depend on the botanical origin of honey (Giorgi, Madeo, Baumgartner, & Lozzia, 2011). Therefore, palynological analysis is essential in studies with honey to establish their floral origin and so, to understand their matrix variability (Escriche, Kadar, Domenech, & Gil-Sánchez, 2012; Tuberoso et al., 2014).

The European Council Directive relating to honey defines the requirements to physicochemical parameters which honey has to comply to ensure its authenticity. In addition, there is a complementary and more restrictive legislation for honey toward quality brands that define not only physicochemical parameters but also geographical origin together with palynological and sensory attributes to ensure products with top quality and higher economic added value. In Spain there are seven quality brands for this product: PDO (Protected Designation of Origin) *Miel de Granada*, PDO *Miel de La Alcarria*, PGI (Protected Geographical Indication) *Miel de Galicia,* PDO *Miel de Tenerife*, PDO *Miel Villuercas-Ibores*, PDO *Miel de Liébana*, and PDO *Miel de Campoo-Los Valles*.

Current legislation does not include specifications of hygiene or microbial contamination. Most of the microbiological analyses of honey are focused on the detection of *Clostridium botulinum*, since honey is the only spore reservoir of these bacteria that could cause infant botulism (Midura, 1996). However, the evaluation of their hygienic quality and microbiological safety is very important considering the possible sources of contamination such as: pollen grains, the digestive tracts of honey bees, dust, air, soil, and nectar, beekeepers equipment or buildings. The main aim of this work was, therefore, to carry out a palynological, microbiological, and physicochemical characterization of different types of Spanish honey sold under quality brands as well as two types of organic honey, assessing the variability that could occur in the product between two consecutive harvests.

Materials and methods

Sample collection

Two types of honey representative of each quality brands for this product existing in Spain to the date of the sample collection (PDO *Miel de Granada* (Orden APA/3209/2002, 2002), PDO *Miel de La Alcarria* (Orden de 3 noviembre, 1993), PGI *Miel de Galicia* (Orden APA/2186/2004, 2004)), and two types of organic honey from the province of León (Spain) were selected. Except for one sample from *La Alcarria*, all samples were collected in two consecutive harvests, providing two sets corresponding to the harvests of years 2010 and 2011 (Table 1). A total of 15 samples were studied. The samples were stored in dark conditions at room temperature until the moment of analysis being homogenized by agitation before each determination.

Botanical origin identification

Pollen analysis was carried out using the method recommended by the International Commission of Bee Botany (ICBB) (Louveaux, Maurizio, & Vorwohl, 1978). Quantitative analysis was conducted by examining each of the preparations under the optical microscope (Nikon Eclipse 80 i) at 400 and 1000 magnification. An average of 650 pollen grains in each honey sample were identified using various keys and literature (Hesse et al., 2009; Moore, Webb, & Collinson, 1991) and the pollen data base of Department of Biodiversity and Environmental Management of the University of León. The appearance frequency of the different pollen types was divided into the following five classes: predominant pollen (> 45% of the total pollen detected in honey); secondary pollen (16–45%); minor important pollen (3–15%); minor pollen (1–3%); and sporadic pollen (< 1%).

Table 2. Pollen types and their frequencies in the fifteen types of honey samples studied.

		Honey samples														
Family	Polen types	H1	H1a	H2	H2a	H3	H4	H4a	H5	H5a	H6	H6a	H7	H7a	H8	H8a
Apiaceae	*Bupleurum lancifolium*	R	R	–	–	–	–	–	–	–	–	–	–	–	–	–
	Conium maculatum	R	R	R	–	R	–	–	–	–	T	R	–	–	–	–
	Eryngium campestre	T	–	–	–	–	–	R	–	–	–	–	T	R	–	–
	Orlaya daucoides	T	–	–	–	–	–	–	–	R	–	–	T	–	–	R
	Other Apiaceae	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Scandix pecten-veneris	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–
Aquifoliaceae	*Ilex aquifolium*	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–
Anacardiaceae	*Pistacia terebinthus*	–	T	–	R	R	R	R	–	–	–	–	–	–	–	–
Asteraceae	*Anthemis arvensis*	T	R	R	R	R	R	–	–	–	–	–	–	R	–	R
	Artemisia campestris	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–
	Bellis annua	R	T	R	R	–	R	–	–	–	–	–	–	–	–	–
	Carlina corymbosa	R	R	–	–	R	–	–	–	–	–	–	–	–	–	–
	Centaurea calcitrapa	R	–	R	R	R	R	–	–	–	–	–	–	–	–	–
	Centaurea cyanus	–	–	–	–	–	–	–	–	–	–	–	–	R	–	–
	Cirsium vulgare	M	T	R	–	R	R	–	–	–	–	–	R	R	–	R
	Helianthus annuus	R	R	–	–	M	R	R	–	–	–	–	T	R	–	–
	Scorzonera laciniata	T	T	–	–	R	R	–	–	–	–	–	–	–	–	–
	Taraxacum sp	T	R	R	–	R	–	–	–	–	–	–	–	–	–	T
	Xanthium strumarium	–	–	–	–	R	R	R	–	–	–	–	–	–	–	–
Betulaceae	*Corylus avellana*	–	–	–	–	–	–	–	–	–	R	–	–	–	–	–
Boraginaceae	*Echium vulgare*	S	T	T	R	R	–	–	R	R	R	R	T	T	–	M
	Cynoglossum cheirifolium	–	–	–	–	–	–	–	R	R	T	–	–	–	–	–
	Lithodora fruticosa	–	–	–	–	T	R	–	–	–	–	–	R	R	–	–
	Myosotis discolor	–	–	–	R	–	–	–	R	–	–	–	–	–	–	R
Brassicaceae	*Capsella bursa-pastoris*	M	R	R	R	T	M	–	R	–	–	–	R	T	–	M
	Raphanus raphanistrum	T	–	–	–	R	T	–	–	–	R	–	T	–	–	–
	Sinapis arvensis	R	–	–	–	–	–	–	–	–	–	–	R	R	–	–
Campanulaceae	*Jasione montana*	R	–	R	T	–	–	–	–	–	–	–	–	R	–	T
Caprifoliaceae	*Viburnum tinus*	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–
Caryophyllaceae	*Arenaria serpyllifolia*	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–
Cistaceae	*Cistus ladanifer*	–	R	R	R	–	R	R	–	–	–	–	–	R	–	T
	Cistus psilosepalus	R	–	–	R	–	–	–	–	R	R	–	–	–	–	–
	Cistus salvifolius	–	–	–	–	–	–	–	–	–	–	–	–	–	–	R
	Halimium halimifolium	R	T	T	T	R	R	–	–	–	–	–	R	M	R	R
	Helianthemum salicifolium	R	S	R	R	M	S	M	–	–	–	–	T	M	–	T
	Other Cistaceae	–	–	–	–	–	R	–	–	–	–	–	–	R	–	R
Convolvulaceae	*Convolvulus arvensis*	–	R	–	–	R	–	–	–	–	–	–	R	–	–	–
Chenopodiaceae	*Chenopodium album*	–	–	–	–	–	R	R	–	–	–	–	–	–	–	–
	Beta vulgaris	–	M	–	–	–	–	R	–	–	–	–	–	–	–	–
Crassulaceae	*Sedum acre*	–	–	R	T	R	–	–	–	–	–	–	R	R	–	R
Cyperaceae	*Carex hallerana*	R	–	R	R	R	R	–	–	–	–	–	–	–	–	–
Dipsacaceae	*Scabiosa atropurpurea*	R	–	–	–	R	–	R	–	–	–	–	–	–	–	–
Ericaceae	*Erica* sp	–	–	–	–	R	–	R	–	–	–	–	–	–	–	R
	Erica arborea	–	R	–	–	–	R	R	–	T	M	–	R	R	R	T
	Erica australis	–	–	–	–	–	–	–	R	–	R	–	–	–	R	R
	Erica cinerea	–	–	–	–	–	–	–	–	–	–	–	R	–	R	T
	Erica umbellata	–	R	–	–	–	–	–	R	T	R	R	–	–	R	S
Fabaceae	*Acacia dealbata*	–	R	–	–	–	–	–	R	–	R	R	–	R	–	R
	Cytisus scoparius	S	S	M	M	R	M	S	T	R	T	M	M	M	R	M
	Lotus sp	R	T	–	–	–	–	–	R	R	–	–	S	–	–	R
	Onobrychis viciifolia	–	–	–	–	R	R	–	–	–	–	–	M	–	–	–
	Ononis spinosa	M	T	–	–	–	–	–	–	–	–	–	R	–	–	–
	Trifolium pratense	R	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Trifolium repens	–	–	–	R	R	–	–	R	–	–	–	–	R	–	R
	Vicia sativa	–	–	–	–	R	R	R	–	–	–	–	R	R	–	R
Fagaceae	*Castanea sativa*	R	T	**P**	**P**	–	R	R	P	P	T	M	S	–	**P**	S
	Quercus pirenaica	–	R	–	T	–	–	–	–	R	R	R	–	–	–	–
	Quercus rotundifolia	R	M	–	R	S	T	R	–	–	–	–	R	M	–	M
Lamiaceae	*Lamium amplexicaule*	–	–	–	–	–	–	–	–	–	–	–	T	R	–	–
	Lavandula stoechas	–	R	R	R	–	–	–	–	–	–	–	–	–	–	–
	Lavandula latifolia	R	R	–	–	M	–	–	–	–	–	–	–	–	–	R
	Thymus sp	R	R	R	R	M	T	R	–	–	–	–	R	**S**	–	R
	Rosmarinus officinalis	R	R	R	–	M	**S**	R	–	–	–	–	T	R	–	R

(*Continued*)

Table 2. (Continued).

Family	Polen types	Honey samples														
		H1	H1a	H2	H2a	H3	H4	H4a	H5	H5a	H6	H6a	H7	H7a	H8	H8a
	Teucrium scorodonia	–	–	R	–	R	R	–	–	–	–	–	T	–	–	–
Lauraceae	*Persea americana*	T	**S**	–	–	–	–	–	–	–	–	–	–	–	–	–
Liliaceae	*Asparagus acutifolius*	–	R	–	–	–	–	–	–	–	–	–	R	–	–	–
Myrtaceae	*Eucalyptus* sp	M	M	R	R	–	–	–	M	T	**P**	**P**	R	–	–	T
Oleaceae	*Fraxinus angustifolius*	–	R	–	–	–	R	–	–	–	–	–	–	–	–	–
	Olea europaea	M	T	T	R	R	–	–	–	–	–	–	–	R	–	M
	Other Oleaceae	–	–	R	–	–	R	–	–	–	–	–	R	–	–	–
Papaveraceae	*Hypecoum imberbe*	R	–	–	–	M	T	T	–	–	–	–	R	P	–	–
	Papaver argemone	–	–	–	–	–	–	–	–	–	–	–	–	R	–	–
	Papaver rhoeas	T	R	–	–	M	R	R	–	–	–	–	T	T	–	–
Plantaginaceae	*Plantago* sp	R	–	R	–	R	–	–	–	–	R	R	R	R	R	R
Pinaceae	*Pinus* sp	–	–	–	–	–	R	R	–	–	R	R	–	–	–	–
Poaceae	*Festuca arundinacea*	–	R	–	–	–	–	–	R	R	R	–	–	R	–	R
	Zea mays	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–
Polygonaceae	*Rumex* sp	R	R	–	R	R	–	–	–	–	–	–	R	–	–	–
Rafflessiaceae	*Cytinus hypocistis*	–	R	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnaceae	*Frangula alnus*	–	–	–	R	–	–	–	–	–	–	–	–	–	R	T
	Rhamnus alaternus	R	R	–	R	R	T	M	R	R	–	–	T	T	–	–
Resedaceae	*Reseda luteola*	R	–	–	R	R	R	R	R	–	R	–	M	–	T	R
Rosaceae	*Crataegus monogyna*	T	T	–	R	R	–	–	T	T	R	T	M	R	M	R
	Other Rosaceae	–	–	–	–	–	–	–	S	S	–	–	–	–	–	–
	Prunus spinosa	T	–	R	–	M	M	T	–	R	R	T	R	T	R	–
	Rubus ulmifolius	T	T	M	M	M	R	R	M	M	–	R	T	R	T	M
Rutaceae	*Citrus* sp	R	–	R	R	–	R	–	–	–	–	–	–	R	–	T
Salicaceae	*Populus alba*	R	–	–	R	R	T	T	–	–	–	–	R	–	–	–
	Salix sp	–	–	R	R	–	–	–	–	–	R	T	R	–	–	T
	Salix fragilis	M	T	–	–	T	S	P	–	–	–	–	–	–	–	–
	Salix triandra	–	–	–	–	M	–	–	–	–	–	–	–	–	–	–
Scrophulariaceae	*Scrophularia canina*	–	–	R	–	–	–	–	–	–	R	–	–	–	–	T

P = predominant pollen (> 45%); S = secondary pollen (16–45%); M = minor important pollen (3–15%); T = minor pollen (1–3%); R = sporadic pollen (< 1%). Sample in which % of a pollen type was enough to classify it as monofloral (highlighted in bold).

Microbiological analysis

Aerobic mesophilic bacteria count was performed on standard plate count agar (PCA) after incubation at 30 °C for 48 h. Molds and yeasts count was carried out following the protocol of ISO 21527-2:2008. For counts of sulfite-reducing clostridia spores aliquots of 10, 5, 1, and 0.1 ml of the initial suspension were taken, then were thermally treated in test tubes at 80 °C for 5 min and covered with agar SPS (sulfite-polymixin-sulfadiazine), being incubated at 37 °C for 5 days. For *S. aureus*, serial dilutions were inoculated on Baird-Parker agar with egg yolk tellurite and sulfadimidine solution and incubated at 37 °C for 24 h. Three to five characteristic colonies were then selected to verify the presence of coagulase and catalase activities. Microbial counts were expressed as colony forming units per gram of honey (cfu/g). Total coliforms and *E. coli* counts were estimated according to the Association of Official Analytical Chemists Official Method 2005.03 using SimPlate® -Coliforms/*E. coli* (Biocontrol) (AOAC International, 2010). *Salmonella* detection was accomplished following the AOAC Official Method 989.13 using 1-2 Test® (Biocontrol) (AOAC International, 1995). The determinations were performed in triplicate.

Physicochemical analysis

Water content (moisture %), electrical conductivity (mS/cm), hydroxymethylfurfural content (mg/kg), free acidity (meq/kg), diastase activity (Gothe degrees), and pH were analyzed using the AOAC Official Methods (AOAC International, 2006) and the Harmonized Methods of the European Honey Commission (Bogdanov et al., 1997). Water activity (a_w) was determined using an Aqualab CX-2 system (Decagon, Pullman, USA) at 20 °C. Viscosity of honey samples (Pa·s) was determined at 25 °C using a rheometer Bohlin CSR Rheometer-10. Color was measured by the tristimulus method CIE L^*a^*b using a Konica Minolta CM-700d spectrophotometer (Osaka, Japan) in terms of L (lightness), a (redness and greenness), and b (yellowness and blueness) and according to Pfund color scale as described by Ferreira et al. (2009).

The content of majority sugars was performed by high-performance liquid chromatography (HPLC) as described by Bogdanov et al. (1997). A Breeze-2 Waters chromatograph (Milford, USA) equipped with a differential refractive index (DRI) detector was used. The separation was carried out with an analytical column of stainless steel with modified silica gel amino groups and a particle size diameter of 5 μm. Identification was performed by

Table 3. Microbial counts of honey samples studied (mean ± standard deviation).

Sample	Aerobic mesophiles[a]	Molds and yeasts[a]	*S. aureus*[a]	Total coliforms[b]	*E. coli*[b]	Sulfite-reducing clostridia[c]	*Salmonella*[d]
H1	16.7 ± 5.8	< 10	< 10	< 1	< 1	ND	ND
H1a	339.4 ± 67.0	< 10	< 10	40.0 ± 20.0	< 1	ND	ND
H2	13.3 ± 5.8	< 10	< 10	< 1	< 1	ND	ND
H2a	< 10	< 10	< 10	33.3 ± 23.1	< 1	ND	ND
H3	< 10	< 10	< 10	< 1	< 1	ND	ND
H4	< 10	< 10	< 10	66.7 ± 23,1	< 1	ND	ND
H4a	< 10	< 10	< 10	106.7 ± 30,6	< 1	ND	ND
H5	< 10	< 10	< 10	< 1	< 1	ND	ND
H5a	< 10	< 10	< 10	86.7 ± 23.1	< 1	ND	ND
H6	< 10	< 10	< 10	< 1	< 1	ND	ND
H6a	< 10	< 10	< 10	126.7 ± 23.1	< 1	ND	ND
H7	13.3 ± 5.8	< 10	< 10	60.0 ± 34.6	< 1	ND	ND
H7a	16.7 ± 5.8	< 10	< 10	140.0 ± 20.0	< 1	ND	ND
H8	16.7 ± 5.8	< 10	< 10	33.3 ± 23.1	< 1	ND	ND
H8a	13.3 ± 5.8	< 10	< 10	93.3 ± 46.2	< 1	ND	ND

[a]Colony-forming units per gram of honey (cfu/g).
[b]Enumerated by the Most Probable Number (MPN).
[c](in 0.01 g).
[d](in 25 g).
ND = not detected.

comparing the retention times obtained with the standards solutions and quantification was accomplished by the external standard method. The determinations were carried out in triplicate.

Statistical analysis

Statistics were performed using different packages (gplots, ggplot2, reshape2, RColorBrewer, scales, grid, psych, and Hmisc) of the open source statistical program R (version 3.3.2) (R Core Team, 2014). The average values for the physicochemical variables studied were compared among the different honey samples to stablish significant differences between them ($p < .05$). One-way ANOVA (HDS test) was applied to variables which showed homogeneity of variances and a Welch's test (Duncan test) to variables without homogeneity of variances. In addition, an integrated study of the results obtained in palynological and physicochemical analysis was carried out to establish the relationship between the variables studied and the similarities or differences between honey samples. For this purpose, cluster analysis, degree of linear correlation (Pearson correlation coefficient), and principal components analysis (PCA) were used.

Results and discussion

Botanical origin identification

The botanical origin determination was based on the relative frequencies of occurrence of the pollen types of nectariferous plants. Ninety-two pollen types belonging to 36 botanical families (Table 2) were identified. About 73% of the identified pollen came from nectariferous plants while the remaining 27% derived from pollen or pollen and honeydew producers plants. The pollen type *Cytisus scoparius* was found in all samples. Other pollen types frequently detected were *Rubus ulmifolius* (found in 93% of samples), *Castanea sativa* (87%), *Echium vulgare* and *Crataegus monogyna* (80%), and *Prunus spinosa* (73%). The number of pollen types present in each sample ranged from 13 (sample H8) to 47 (sample H1). This variability was the result of the existing vegetation in the different geographical areas and the preference of bees for some plant species.

In general, a honey is considered monofloral if the relative frequency of occurrence of a certain type of nectariferous pollen exceeds 45%. However, since there are numerous pollen types that are over or underrepresented, this percentage varies significantly for the different types of flower honey (Von Der Ohe, PersanoOddo, Piana, Morlot, & Martin, 2004). European legislation does not establish requirements for minimal contents of pollen present in honey to label them taking into account their botanical origin. However, these specifications appear in Spanish regulations for quality brands. Based on these standards and what described by Von der Ohe et al. (2004), eight of the fifteen samples studied were monofloral honey, the rest did not reach the minimum content in a specific pollen type to be included in this group and were classified as multifloral honey.

Sample H1 was a multifloral honey, sample H1a corresponded to an avocado honey (*Persea americana*) and samples H2 and H2a were chestnut honey (*Castanea sativa*) as established by the regulations of the PDO *Miel de Granada* (Orden APA/3209/2002, 2002). Pollen types of the characteristic vegetation of Granada were also found as is described in the regulations for this quality brand. Samples H3 and H4a were multifloral honey and sample H4 corresponded to a rosemary honey (*Rosmarinus officinalis*), as established by the regulations of the PDO *Miel de La Alcarria* (Orden de 3 de noviembre, 1993). Pollen of crop plants was less than 10% and

Table 4. Physicochemical parameters studied in the different honey samples (mean value ± standard deviation).

Sample	*M*	a_w	FA	pH	*L*	*a*	*b*	mm Pfund	Color classification
H1	16.67 ± 0.12 ^{fg}	0.565 ± 0.005 ^{c}	41.29 ± 0.37 ^{a}	5.42 ± 0.06 ^{a}	26.27 ± 0.50 ^{i}	21.24 ± 0.99 ^{a}	21.71 ± 0.88 ^{f}	139.07 ± 0.22 ^{b}	Dark Amber
H1a	16.33 ± 0.12 ^{gh}	0.558 ± 0.003 ^{cd}	39.38 ± 1.02 ^{ab}	4.80 ± 0.04 ^{b}	34.71 ± 2.39 ^{fg}	22.81 ± 0.23 ^{a}	34.26 ± 3.25 ^{bcd}	112.45 ± 0.75 ^{e}	Amber
H2	15.47 ± 0.12 ^{j}	0.524 ± 0.006 ^{h}	27.71 ± 1.31 ^{de}	4.50 ± 0.05 ^{c}	28.62 ± 1.13 ^{hi}	8.53 ± 0.93 ^{c}	36.66 ± 1.79 ^{bc}	118.65 ± 0.93 ^{d}	Dark Amber
H2a	15.73 ± 0.31 ^{ij}	0.555 ± 0.003 ^{cde}	37.69 ± 0.51 ^{b}	4.52 ± 0.02 ^{c}	36.75 ± 1.22 ^{ef}	21.47 ± 0.62 ^{a}	39.15 ± 2.34 ^{b}	124.09 ± 0.57 ^{c}	Dark Amber
H3	16.93 ± 0.12 ^{ef}	0.542 ± 0.003 ^{fg}	29.19 ± 0.52 ^{d}	3.48 ± 0.06 ^{g}	40.52 ± 1.29 ^{de}	5.55 ± 0.45 ^{d}	32.15 ± 0.02 ^{cd}	61.45 ± 0.94 ^{h}	Light Amber
H4	17.87 ± 0.12 ^{bc}	0.564 ± 0.006 ^{c}	18.20 ± 0.92 ^{g}	3.58 ± 0.04 ^{fg}	52.92 ± 1.67 ^{b}	0.58 ± 0.09 ^{f}	16.03 ± 0.37 ^{g}	17.13 ± 0.93 ^{o}	Extra White
H4a	16.53 ± 0.12 ^{fg}	0.545 ± 0.001 ^{fg}	15.98 ± 0.76 ^{hi}	3.69 ± 0.06 ^{f}	41.67 ± 1.24 ^{d}	0.49 ± 0.06 ^{f}	14.16 ± 0.59 ^{g}	32.61 ± 0.75 ^{n}	White
H5	13.60 ± 0.20 ^{k}	0.555 ± 0.001 ^{cde}	23.64 ± 0.53 ^{f}	4.18 ± 0.07 ^{d}	46.59 ± 0.62 ^{c}	13.80 ± 0.74 ^{b}	49.75 ± 1.52 ^{a}	48.08 ± 0.57 ^{k}	Extra Light Amber
H5a	17.20 ± 0.20 ^{de}	0.574 ± 0.001 ^{b}	25.60 ± 0.27 ^{ef}	4.15 ± 0.05 ^{d}	35.72 ± 1.50 ^{fg}	7.99 ± 0.56 ^{c}	29.37 ± 3.38 ^{de}	57.74 ± 0.57 ^{i}	Light Amber
H6	16.87 ± 0.12 ^{ef}	0.549 ± 0.003 ^{def}	18.90 ± 0.59 ^{g}	3.95 ± 0.03 ^{e}	28.53 ± 1.80 ^{hi}	4.68 ± 0.21 ^{d}	33.18 ± 1.91 ^{cd}	38.92 ± 0.74 ^{m}	Extra Light Amber
H6a	16.07 ± 0.12 ^{hi}	0.545 ± 0.001 ^{efg}	15.31 ± 0.15 ^{i}	3.96 ± 0.03 ^{e}	58.51 ± 1.98 ^{a}	3.98 ± 0.48 ^{de}	46.72 ± 1.08 ^{a}	43.26 ± 0.21 ^{l}	Extra Light Amber
H7	17.47 ± 0.12 ^{cd}	0.538 ± 0.003 ^{g}	19.72 ± 0.77 ^{g}	3.93 ± 0.01 ^{e}	55.23 ± 0.85 ^{ab}	2.44 ± 0.13 ^{e}	23.99 ± 0.52 ^{ef}	51.55 ± 0.75 ^{j}	Light Amber
H7a	17.93 ± 0.12 ^{b}	0.603 ± 0.003 ^{a}	17.65 ± 0.79 ^{gh}	4.49 ± 0.04 ^{c}	41.37 ± 1.62 ^{d}	5.49 ± 0.30 ^{d}	36.64 ± 1.71 ^{bc}	74.45 ± 0.94 ^{g}	Light Amber
H8	18.20 ± 0.20 ^{b}	0.600 ± 0.002 ^{a}	38.47 ± 0.62 ^{b}	3.99 ± 0.06 ^{e}	47.52 ± 1.03 ^{c}	5.06 ± 0.27 ^{d}	26.10 ± 1.87 ^{ef}	110.35 ± 0.57 ^{f}	Amber
H8a	18.73 ± 0.12 ^{a}	0.609 ± 0.002 ^{a}	33.67 ± 0.45 ^{c}	4.20 ± 0.04 ^{d}	31.81 ± 0.14 ^{gh}	7.76 ± 0.54 ^{c}	22.62 ± 1.67 ^{f}	169.53 ± 0.21 ^{a}	Dark Amber

Sample	HMF	Da	EC	V25	Glu	Fru	Mal	Suc
H1	2.01 ± 0.09 ^{h}	38.53 ± 0.57 ^{d}	0.95 ± 0.02 ^{a}	8.17 ± 0.04 ^{f}	27.03 ± 0.97 ^{abc}	34.89 ± 0.99 ^{b}	6.53 ± 0.38 ^{ab}	0.72 ± 0.06 ^{c}
H1a	5.25 ± 0.22 ^{d}	44.70 ± 0.32 ^{c}	1.34 ± 0.01 ^{b}	11.75 ± 0.07 ^{e}	25.46 ± 1.02 ^{bcd}	35.81 ± 0.63 ^{ab}	5.08 ± 0.29 ^{cd}	ND ^{d}
H2	2.62 ± 0.05 ^{g}	62.77 ± 0.57 ^{b}	0.86 ± 0.01 ^{d}	11.42 ± 0.04 ^{e}	24.56 ± 1.58 ^{cd}	37.30 ± 0.76 ^{ab}	6.06 ± 0.19 ^{abc}	ND ^{d}
H2a	1.53 ± 0.06 ^{i}	65.93 ± 0.39 ^{a}	0.86 ± 0.01 ^{d}	20.11 ± 0.24 ^{c}	26.22 ± 1.18 ^{abc}	37.16 ± 1.12 ^{ab}	3.93 ± 0.21 ^{de}	ND ^{d}
H3	13.12 ± 0.20 ^{a}	35.24 ± 0.52 ^{e}	0.25 ± 0.01 ^{j}	11.82 ± 0.19 ^{e}	29.32 ± 1.33 ^{a}	37.24 ± 1.28 ^{ab}	5.82 ± 0.38 ^{abc}	ND ^{d}
H4	8.10 ± 0.12 ^{c}	11.61 ± 0.77 ^{m}	0.11 ± 0.01 ^{k}	15.31 ± 0.25 ^{d}	29.32 ± 1.28 ^{a}	34.74 ± 1.41 ^{b}	4.14 ± 0.24 ^{de}	ND ^{d}
H4a	2.97 ± 0.09 ^{f}	21.14 ± 0.53 ^{k}	0.13 ± 0.00 ^{k}	12.83 ± 0.08 ^{e}	28.64 ± 1.46 ^{ab}	35.72 ± 1.90 ^{ab}	3.50 ± 0.18 ^{e}	3.61 ± 0.20 ^{a}
H5	1.98 ± 0.09 ^{h}	15.78 ± 0.60 ^{l}	0.62 ± 0.01 ^{f}	7.26 ± 0.03 ^{f}	23.70 ± 0.74 ^{cd}	36.58 ± 1.31 ^{ab}	6.83 ± 0.34 ^{a}	ND ^{d}
H5a	3.03 ± 0.08 ^{f}	27.59 ± 0.53 ^{h}	0.51 ± 0.01 ^{g}	7.78 ± 0.16 ^{f}	26.30 ± 1.25 ^{abc}	37.28 ± 1.48 ^{ab}	3.75 ± 0.45 ^{e}	ND ^{d}
H6	2.01 ± 0.05 ^{h}	11.13 ± 0.45 ^{m}	0.42 ± 0.01 ^{h}	14.75 ± 0.17 ^{d}	24.65 ± 0.69 ^{cd}	36.51 ± 1.29 ^{ab}	5.59 ± 0.64 ^{bc}	0.50 ± 0.00 ^{d}
H6a	1.50 ± 0.08 ^{i}	23.27 ± 0.57 ^{j}	0.53 ± 0.01 ^{g}	11.62 ± 0.13 ^{e}	25.09 ± 0.86 ^{bcd}	38.57 ± 1.33 ^{ab}	4.94 ± 0.39 ^{cd}	ND ^{d}
H7	4.71 ± 0.03 ^{e}	25.05 ± 0.34 ^{i}	0.32 ± 0.01 ^{i}	50.63 ± 4.20 ^{a}	21.86 ± 1.33 ^{d}	39.63 ± 0.56 ^{a}	4.27 ± 0.42 ^{de}	1.19 ± 0.12 ^{b}
H7a	1.51 ± 0.04 ^{i}	32.93 ± 0.38 ^{f}	0.32 ± 0.01 ^{i}	7.30 ± 0.05 ^{f}	24.03 ± 1.39 ^{cd}	36.70 ± 0.96 ^{ab}	5.73 ± 0.52 ^{abc}	0.50 ± 0.00 ^{d}
H8	9.35 ± 0.09 ^{b}	27.65 ± 0.29 ^{h}	0.89 ± 0.02 ^{c}	8.27 ± 0.06 ^{f}	24.52 ± 0.85 ^{cd}	36.53 ± 2.10 ^{ab}	5.79 ± 0.61 ^{abc}	ND ^{d}
H8a	2.66 ± 0.06 ^{g}	31.30 ± 0.54 ^{g}	0.74 ± 0.01 ^{e}	25.85 ± 0.83 ^{b}	29.46 ± 2.12 ^{a}	38.97 ± 1.79 ^{a}	1.79 ± 0.07 ^{f}	0.57 ± 0.07 ^{cd}

M = Moisture (%); a_w = Water activity; FA = Free acidity (meq/kg); mm Pfund = Color obtained by UV-Vis spectrophotometry and calculated to Pfund scale; L = Lightness parameter obtained by tristimulus method CIE *L*a*b*; *a* = Parameter representing the redness and greenness color variable obtained by tristimulus method CIE *L*a*b*; *b* = Parameter representing the yellowness and blueness color variable obtained by tristimulus method CIE *L*a*b*; HMF = Hydroxymethylfurfural content (mg/kg); Da = Diastase activity (Gothe degrees); EC = electrical conductivity (mS/cm); V25 = Viscosity (Pa·s); Glu = Glucose (%); Fru = Fructose (%); Mal = Maltose (%); Suc = Sucrose (%). Different letters in the same variable denote significant differences according to HDS test ($p < .05$) between the different honey samples.

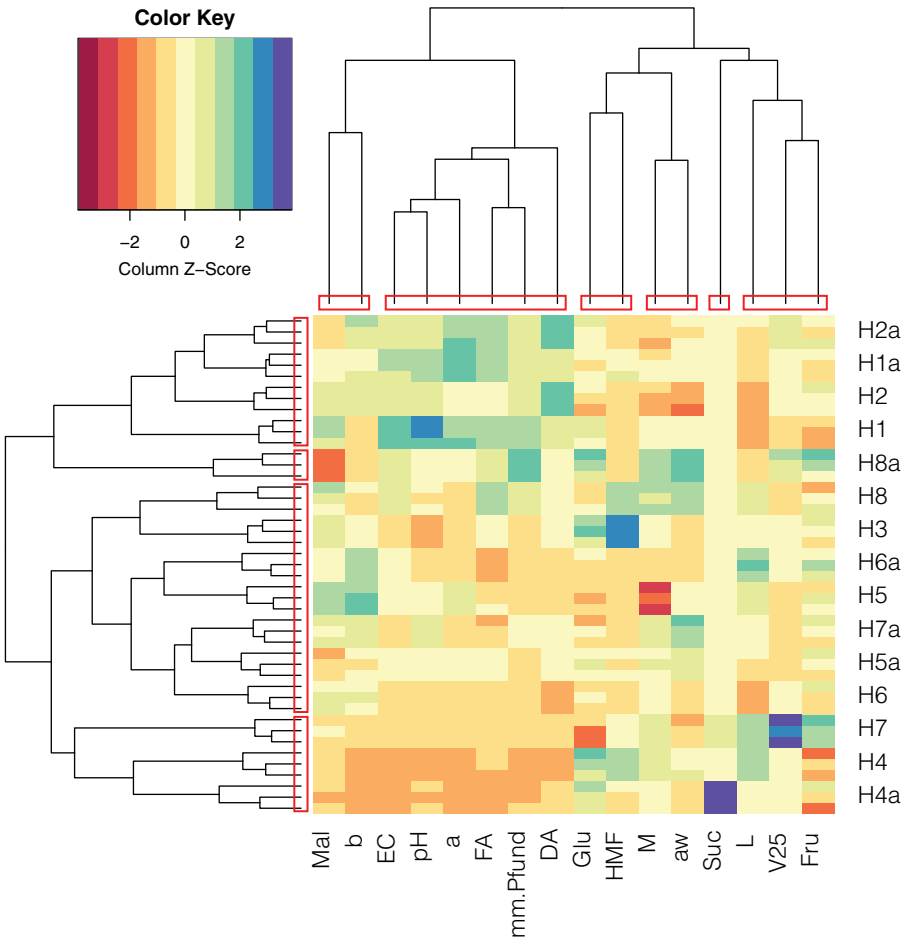

Figure 1. Heat map plot to visualize the physicochemical parameters' clustering of the honey samples analyzed. *M*—Moisture (%); a_w—Water activity; FA—Free acidity (meq/kg); EC—Electrical conductivity (mS/cm); HMF—Hydroxymethylfurfural content (mg/kg); DA—Diastase activity (Gothe degrees); mm Pfund—Color obtained by UV-Vis spectrophotometry and calculated to Pfund scale; *L*—Lightness parameter obtained by tristimulus method CIE *L*a*b*; a—Parameter representing the redness and greenness color variable obtained by tristimulus method CIE *L*a*b*; b—Parameter representing the yellowness and blueness color variable obtained by tristimulus method CIE *L*a*b*; V25—Viscosity (Pa·s).

pollen of *Cistus ladanifer* and *Lavandula stoechas* were less than 3% of the total pollen. Moreover, within the multifloral honey, the sample H3 showed a minimum rate of 5% of at least one of the pollen types of thyme, rosemary or lavender. Samples H5 and H5a were multifloral honey and samples H6 and H6a were eucalyptus honey (*Eucalyptus* sp.) as established by the regulations of the PGI *Mel de Galicia* (Orden APA/2186/2004, 2004). Pollen types belonging to the *Ericaceae* and *Rosaceae* families, that are characteristic of honey from this region, were also found. Samples H7 and H8a were multifloral honey and samples H7a and H8 were monofloral thyme honey (*Thymus* sp.) and chestnut honey (*Castanea sativa*), respectively, as described by Von der Ohe et al. (2004). Furthermore, pollen types of the characteristic vegetation of the province of León were found, mainly from *Ericaceae* and *Rosaceae* families and to other species such as *Capsella bursa-pastoris*, *Halimium halimifolium, Hypecoum imberbe* or *Lotus* sp. which were previously described by Valencia-Barrera, Herrero, and Molnar (2000).

The variability of the pollen types present in the different samples studied are the result of its diverse floral and geographical origin, as well as of climatic variations (Anklam, 1998), which explains why honey from similar production areas and two consecutive harvests showed different pollen spectra.

Microbiological analysis

The results of microbiological analysis are shown in Table 3. Aerobic mesophilic microorganisms were not detected in eight samples while the rest showed low counts. In general aerobic mesophilic microorganisms could reach 10^3 cfu/g depending on the degree of freshness, harvesting time, and handling conditions (Snowdon & Cliver, 1996). None of the samples reached the limit of detection for molds and yeasts, probably due to the low water activity observed which was indicative of an adequate beekeeping management and good extraction and processing practices (Gomes, Feás, Iglesias, & Estevinho, 2011). *S. aureus* was not detected in any

Table 5. Correlation factors of each physicochemical parameter with the five main principal components and the cumulative variance percentage explained.

Variable	PC 1	PC 2	PC 3	PC 4	PC 5
M	0.28	–0.81	0.08	–0.31	0.16
a_w	–0.13	–0.58	–0.03	–0.48	0.57
FA	–0.82	–0.38	–0.07	–0.14	–0.29
pH	–0.89	0.01	0.04	0.14	0.26
EC	–0.93	–0.07	0.04	–0.04	0.01
HMF	0.30	–0.28	–0.35	–0.46	–0.67
Da	–0.71	–0.05	0.22	0.18	–0.38
mm Pfund	–0.81	–0.47	0.23	–0.04	–0.03
L	0.65	0.25	0.20	–0.32	–0.09
a	–0.90	0.09	–0.02	0.10	–0.08
b	–0.31	0.76	0.20	–0.30	0.05
V25	0.29	–0.21	0.74	0.15	–0.26
Glu	0.12	–0.60	–0.42	0.18	–0.21
Fru	0.15	–0.01	0.83	–0.16	–0.12
Mal	–0.30	0.65	–0.40	–0.29	–0.09
Suc	0.42	–0.13	–0.12	0.74	0.03
VPC	33.58	51.94	63.87	73.51	81.31

M = Moisture; a_w = Water activity; FA = Free acidity; EC = Electrical conductivity; HMF = Hydroxymethylfurfural content; Da = Diastase activity; mm Pfund = Color obtained by UV-Vis spectrophotometry; *L* = Lightness; *a* = Parameter representing the redness and greenness color; *b* = Parameter representing the yellowness and blueness color; V25 = Viscosity at 25 °C; Glu = Glucose; Fru = Fructose; Mal = Maltose; Suc = Sucrose; VPC = Cumulative variance percentage explained by the principal components (%).

sample and all of them were negative for *Salmonella* and sulfite-reducing clostridia. The absence of spores of sulfite-reducing clostridia was also indicative of appropriate hygienic conditions during extraction and processing of honey. However, this does not completely guarantee the absence of *Clostridium botulinum* (International Commission on Microbiological Specifications for Foods, 2005). For this reason, consumption of honey in children less than one year and immunocompromised people is not recommended. Although in some honey samples coliforms were detected, *E. coli* was never found in any of them. Estimated counts of total coliforms were not significant considering that their presence in unprocessed foods does not necessarily indicate a low hygienic quality (Blackburn, 2006). Since *E. coli* and *Salmonella*, as indicators of fecal contamination, were not detected and other microorganisms indicators of hygiene were observed in low counts, it was possible to conclude that coliforms detected in the honey samples analyzed may be associated with microorganisms of genera such as *Citrobacter*, *Klebsiella*, *Serratia*, or *Enterobacter* which are mainly related to an environmental origin or are part of usual bees microbiota (Blackburn, 2006; Snowdon & Cliver, 1996).

Physicochemical analysis

Table 4 shows that for all physicochemical parameters all honey samples presented values within the legal limits established by the Council Directive 2001/110. EU (2001). Moisture contents below 20% are related to low values of water activity (a_w) which prevent the growth of osmophilic yeasts, capable of producing abnormal fermentations in honey (Snowdon & Cliver, 1996). Moisture content of honey depends primarily on environmental conditions and beekeeping practices and may vary according to season and year of harvest (Acquarone, Buera, & Elizalde, 2007), which would explain, at least in part, the significant differences observed in some samples between successive harvests. The results obtained indicate that ripening period and honey extraction conditions were proper. The values of a_w which limit the growth of osmotolerant yeasts found naturally in honey varies between 0.61 and 0.62 (Zamora & Chirife, 2006). Thus fermentative phenomena that could deteriorate honey during storage were not expected in these samples. Moisture content and a_w showed an intermediate correlation with high degree of significance ($r = 0.61$; $p < .001$). Even though, it should be noted that a_w provides information on the availability of water present in honey, which depends mainly on the fructose (Fru) and glucose (Glu) contents but also on the state in which these sugars are present. Indeed, if Glu crystallizes due to its lower solubility, honeys' a_w increases (Gleiter, Horn, & Isengard, 2006). This fact explains why higher moisture contents were not always associated to higher a_w values and vice versa.

Free acidity (FA) primarily comes from organic acids. Gluconic acid, originated from glucose, is the main compound but other organic acids from the nectar could be present. The FA values were below 50 meq/Kg as required by the Council Directive 2001/110 which indicates the absence of undesirable fermentations. Free acidity values can be affected by botanical or geographical origin as well as by the honey harvest season (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016; Mato, Huidobro, Simal-Lozano, & Sancho, 2006; Tornuk et al., 2013). All honey samples showed an acid character. pH values were similar to those described by other authors for different types of honey (Anjos et al., 2015; Giorgi et al., 2011; Habib, Al Meqbali, Kamal, Souka, & Ibrahim, 2014). pH has a great importance in the texture of honey, its stability and its shelf life (Bertoncelj, Golob, Kropf, & Korošec, 2011), therefore, although the legislation does not establish limit for pH values, these must be low.

Electrical conductivity (EC) is related to mineral content, organic acids and proteins which explains the high correlation observed between this parameter and the pH and FA values ($r = 0.84$, $p < .001$ in both cases). H1 and H1a samples showed higher EC values than that specified in the Council Directive 2001/110 but, they were in accordance to the specific regulations for this type of honey (Orden APA/3209/2002, 2002). The values of hydroxymethylfurfural content (HMF) and diastase activity (Da) were within the limits set out in the regulations. These two quality parameters in honey are indicators of freshness and processing conditions, and their values increase or decrease, respectively, with the ageing of the

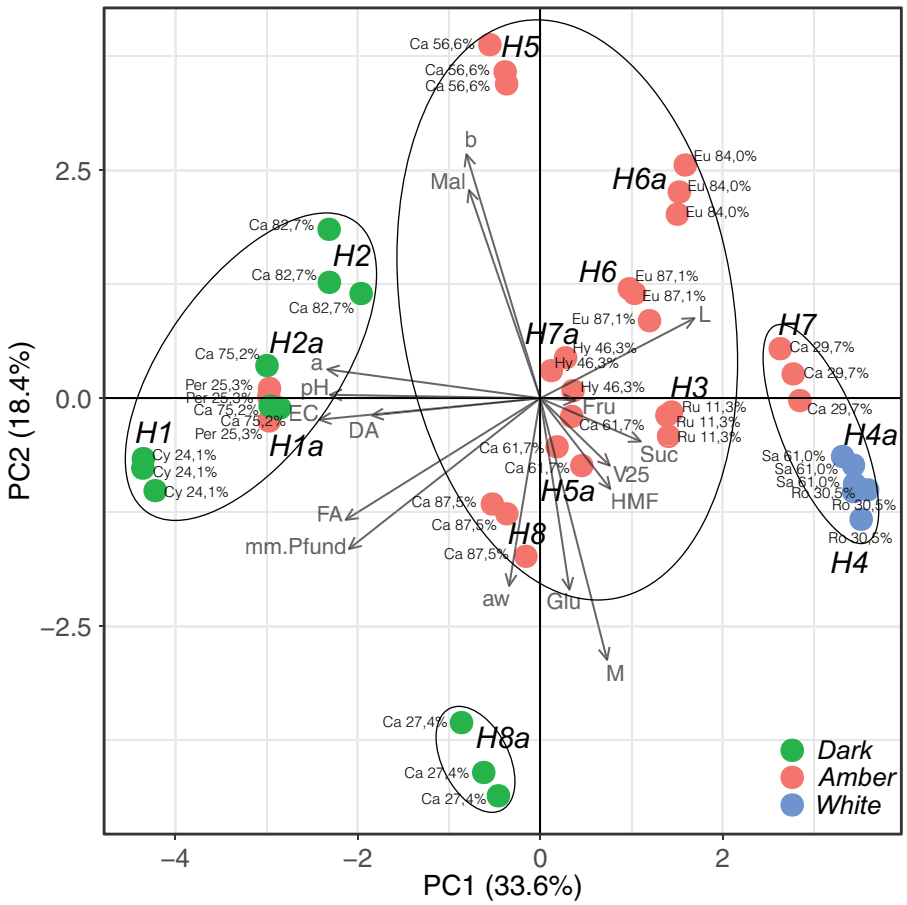

Figure 2. PCA biplot with honey samples plotted onto the first two principal components. *M*—Moisture (%); a_w—Water activity; FA—Free acidity (meq/kg); EC—Electrical conductivity (mS/cm); HMF—Hydroxymethylfurfural content (mg/kg); DA—Diastase activity (Gothe degrees); mm Pfund—Color obtained by UV-Vis spectrophotometry and calculated to Pfund scale; *L*—Lightness parameter obtained by tristimulus method CIE *L*a*b*; *a*—Parameter representing the redness and greenness color variable obtained by tristimulus method CIE *L*a*b*; *b*—Parameter representing the yellowness and blueness color variable obtained by tristimulus method CIE *L*a*b*; V25—Viscosity (Pa·s).

product and exposure to overheating. Carbohydrate composition of honey depends on botanical and geographical origin, on environmental and climatic factors, as well as processing and storage conditions (Ouchemoukh, Schweitzer, Bachir Bey, Djoudad-Kadji, & Louaileche, 2010). Fru and Glu were the major sugars in all samples and the sum of both sugars exceeded 60% set by the Council Directive 2001/110 but, were slightly lower than those reported by other authors (De la Fuente, Ruiz-Matute, Valencia-Barrera, Sanz, & Martínez Castro, 2011). Maltose (Mal) and sucrose (Suc) contents were similar to those described by other authors (Bentabol Manzanares, Hernández García, Rodríguez Galdón, Rodríguez Rodríguez, & Díaz Romero, 2014).

Honey color depends on many factors among which the content of minerals and pigments, such as carotenoids and flavonoids, play an important role. The CIE method is a three-dimensional method wherein the *L* dimensional axis indicates the brightness and *a, b* axes indicate chromaticity or color; *a* related to red (positive values) and green (negative values) and *b* to yellow (positive values), and blue (negative values) colors. Values of $L \leq 50$ have been associated with darker honey (Juan-Borrás et al., 2014) and this study showed that, in general, honey with higher values on Pfund scale presented lower values in *L* parameter and higher values in *a* parameter. These results were confirmed by an intermediate correlation with high degree of significance between the results obtained in Pfund scale and *L* ($r = -0.58$, $p < .001$) and *a* parameters ($r = 0.61$, $p < .001$). According to Pfund scale, honey samples showed color intensities ranging from extra- white (H4 sample) to dark amber (H8a sample).

Honey viscosity is influenced by temperature, moisture content, and botanical source (Yanniotis, Skaltsi, & Karaburn, 2006). Viscosity varied greatly between the different honey samples but within expected values being similar to those described by other authors (Dobre, Georgescu, Alexe, Escuredo, & Seijo, 2012). In most of the physicochemical parameters studied significant differences between the types of honey as between the two harvests were observed. Honey, which is a natural product, is unlikely to standardize. This variability

comes from the wide diversity of floral origins, type of soil in which the plants were located (Anklam, 1998) and changing climatic conditions, which are factors that change between harvest periods. These factors affect parameters such as mineral content and consequently EC, color or FA among others. Modifications during processing and handling of honey are also other variables that affects parameters such as M content, a_w, HMF content or DA (Acquarone et al., 2007; Habib et al., 2014; Moura Kadri et al., 2017). These facts explain that all samples showed significant differences between harvests for some of these parameters.

Some authors (Feás, Pires, Iglesias, & Estevinho, 2010) have proposed the designation "fresh," "raw," or "virgin" honey to indicate the virginal nature of honey (pure and natural) and to its wholeness referring to nothing was added, removed or altered. This appellation comprised physical and chemical requirements more restrictive than those under Community law: maximum humidity of 18% and maximum HMF content of 25 mg/kg. All honey samples could be labeled as "Virgin honey" except samples H8 and H8a that showed moisture contents higher than 18%.

Cluster analysis

Cluster analysis was performed with the aim of grouping the most similar or homogeneous samples and physicochemical variables. All variables were normalized (mean acquired the value 0 and standard deviation the value 1) to eliminate the differences associated with the units in which each parameter was expressed. Figure 1 shows the heat map plot (a two-dimensional representation of dendrograms) that allows to visualize the clustering of the physicochemical parameters data changes in relation to the samples analyzed. This analysis resulted in the formation of six groups (regarding variables) and four groups (for samples). Variables were grouped as follows: Group 1 included the variables Mal and parameter *b*. Group 2 included the variables EC, pH, parameter *a*, FA, color (mm Pfund) and DA. Group 3 comprised the variables Glu and HMF content. Group 4 included the variables moisture and a_w. Group 5 corresponded to the variable Suc. Group 6 comprised the variables *L*, viscosity, and Fru.

Regarding samples, four groups were formed: Group 1 included samples H2a, H1a, H2, and H1, which were characterized by relatively higher values of FA, pH, EC, DA, mm Pfund, parameter *a*, and Mal content, as well as, intermediate values in the remaining parameters. Group 2 corresponded to sample H8a that, in general, presented intermediate values for all parameters and higher values for the variable mm Pfund and low for Mal contents. Group 3 included samples H8, H3, H6a, H5, H7a H5a, and H6, characterized by relatively low values of pH, EC, DA, mm Pfund, and parameter *a*, high values for the parameter Mal, and intermediate values for the rest of the parameters. Group 4 formed by samples H7, H4, and H4a, characterized by relatively low values of FA, pH, EC, DA, mm Pfund, Mal content, and parameters *a* and *b*.

The cluster analysis revealed, once again, that a great variability existed among honey samples. On the contrary, it also showed that the overall variability found in the results of repeated analyses for the same parameter in each sample was not large enough to disperse them, and, therefore, the three results of each honey sample were found together in the dendrogram. Moreover, the formed clusters did not follow a pattern depending on the botanical origin or the color characteristics that could be related to physicochemical parameters.

Principal component analysis

As performed for the cluster analysis, all variables were normalized. A *scree* plot (a line plot of the variance of the principal components), was used to define the optimal number of main components that the model should have to explain a high percentage of data variability without introducing noise. It was verified that with five main components the model explains 81.31% of the total variability of the original data. Table 5 presents the degree of importance of each studied variable regarding principal component analysis and allowed to conclude that: principal component 1 (PC 1) was negatively correlated with the variables FA, pH, EC, Da, and with color variables mm Pfund and parameter *a*, and positively correlated with the parameter *L*; PC 2 was positively correlated with parameter *b* and Mal content and negatively correlated with moisture, a_w and Glu content; PC 3 was positively correlated with viscosity (V25) and Fru; PC 4 was positively correlated with Suc; and, the PC 5 correlated positively with a_w and negatively with HMF. Also, through Figure 2 visualization, the PCA biplot with samples plotted onto the first two PCs, it was possible to establish 4 groups of samples, which were in accordance to the clusters presented in the Figure 1.

Group I included samples characterized by higher values of FA, EC, pH, DA, mm Pfund, and parameter *a* and by low contents of Suc, Fru, HMF, viscosity, and parameter *L*. Group II corresponded to H8 sample, which possessed high values of the variable mm Pfund and a_w, high moisture and Glu content and low values for parameter *b* and Mal content. Group III was composed by a set of samples with very diverse characteristics. In general, it could be highlighted the high content in Mal, high values for the parameter *b* and low values of moisture, Glu, and a_w. Group IV included honey samples with high content of Suc, Fru, HMF, and those presenting higher viscosity and luminosity, as well as those with low values of pH, EC, FA, DA, mm Pfund, and parameter *a*.

In this study, in contrast with the described by other authors (Escriche, Kadar, Domenech, & Gil-Sánchez, 2012), was not possible to clearly differentiate samples

using the botanical origin and color characteristics as discriminating factors. In the central zone of PC 1, two groups were formed: one of them had several samples of amber color while the other group was formed by a single sample of dark color. In the left and right quadrants, two other groups were formed, which simultaneously include samples of dark and amber colors (left) and amber and white colors (right).

Conversely, considering the pollen spectrum it could be observed that samples with very similar primary pollen percentages, such as samples H8 and H2 or H8a and H7 belonged to different groups while, on the contrary, samples containing different primary pollen types (of four different plant species) were grouped together. This result was in agreement with the fact that the chemical composition of each honey sample was dependent on the pollen profile and not only on the most abundant pollen. This conclusion corroborates the need to perform pollen analysis in honey studies, since it allows to establish accurately honeys variability. Moreover, from the point of view of the consumer, a label should include at least the two or three most prevalent pollens to ensure correct honey's classification.

Conclusions

A palynological, microbiological, and physicochemical characterization of fifteen samples of Spanish honey from different geographical and botanical origins was accomplished. Eight samples were classified as monofloral honeys from avocado, chestnut, rosemary, eucalyptus, and thyme. The remaining were multifloral honeys. The microbiological counts obtained suggest that beekeeping practices were carried out hygienically and honey is safe for the consumers. All values obtained for the physicochemical parameters were within the maximum limits set down by European legislation. In addition, all samples except H8 and H8a could be labeled as "Virgin Honey." The samples varied widely regarding the botanical and geographical origin. Also, it was observed that honey harvested in consecutive years have significantly different chemical characteristics, explained by the honey's floral origin, which showed the pollen analysis relevance in honey studies. According to the results obtained in palynological analysis several honey samples were mislabeled. A greater control and a commitment from all involved parties would be necessary to ensure the authenticity of honey.

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No potential conflict of interest was reported by the authors.

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