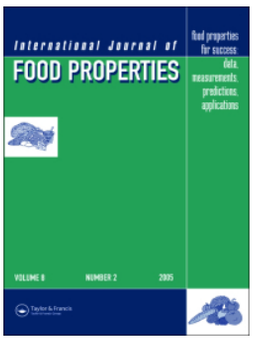

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Analysis of organic molecules, physicochemical parameters, and pollen as indicators for authenticity, botanical origin, type and quality of honey samples examined

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ABSTRACT

To contribute to Chilean honey's characterization, 12 honey samples were analyzed using comprehensive physicochemical and pollen analyses. Beekeepers donated samples from La Pintana, Linderos, Cajón del Maipo, and Chiloé. Physicochemical parameters required for honey authentication such as free acidity (in range of 9.5–46 meq/kg), hydroxymethylfurfural (0–8 mg/kg), humidity (14.4–16.9%), sugar profile, amino acid profile, organic acid profile, pH (3.8–4.7), electrical conductivity (0.25–1.47 mS/cm) and diastase activity (28.6–43.8 °G), were determined by conventional techniques and nuclear magnetic resonance (NMR) at an international quality control laboratory for honey analyses. The Chilean honey samples analyzed showed physicochemical properties in normal ranges and typical sugar profiles for natural honey, which confirmed their authenticity and high quality.

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Honey authentication; Apis mellifera; Chilean honey; physicochemical properties

Introduction

In Chile, apiculture involves more than 8,851 beekeepers, managing more than 779,000 Western honey beehives.[1] The apiaries are preferably located in the central-southern region, with higher populations in regions VI (O'Higgins), VII (Maule), and VIII (Biobio).[1] About 90% of the Chilean honey harvested is exported to the European Union and other countries. In 2018, honey export resulted in USD 29 million into the country.[1] The volume of produced honey fluctuates between 7,000 and 11,000 tons/year, with aproximately 93% exported to European Union.[1] Although the volumes mentioned representing around 0,6% of the world's honey market,[1] Chilean honey's excellent reputation provides the opportunity to enhance the national beekeeping practices.

Honey composition depends mainly on the quality, quantity, and diversity of plants that produce nectar in the same period of the year. Chile's tremendous floral resource provides numerous species for honeybees from which it is possible to obtain different types of monofloral honey .[2] The climate also causes honey to reach low humidity, allowing it to maintain its aroma and flavour.[2] However, the most considerable amount of honey harvested in the country is

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labelled or tagged as polyfloral.[3] Thus, foreign traders prefer to buy Chilean honey in bulk and mix it with honey from European countries to reduce the final product price. Since monofloral honey is in high demand and can be sold at a better price in the international market, it is essential to recognize Chilean honey's floral types and physicochemical properties. In this way, we can establish correct definitions and labeling standards for Chilean honey from different botanical and geographical origins. In addition, it is essential to check whether Chilean honey samples of different origins can meet the quality criteria demanded by food regulations, to avoid adulterations numerous species of foraging interest for honeybees from which it is possible to obtain different types of monofloral honey.[22]

Honey adulteration is the addition or fraudulent and intentional substitution of a substance to increase its apparent value and decrease its production cost. Types of adulteration comprise the addition of sweeteners (cane sugar or refined beet sugar), artificial feeding of honey bees during nectar flux, the early harvest of honey (indicating that sucrose was not completely transformed into glucose and fructose), use of filtrating resins by ionic exchange, and falsification of geographical or botanical origin.[4–6] Moreover, numerous chemical analysis methods have been implemented to detect evidence of adulteration in honey samples.[7–12] To the best of our knowledge, there are very limited studies in the literature that provide quantification data regarding honey organic molecules using ^{1}H-NMR methodology.[13–15] This study is the first attempt to test Chilean honey in a well-equipped European certified lab to fill in the gap of knowledge about Chilean honey quality.

Materials and methods

Collection and identification of samples

Twelve samples of raw beehoney, of approximately 250 g each, were requested as a donation from Chilean beekeepers (Table 1). In Chile, the capped frames are extracted from the hives. The frames are then uncapped without heat using a rake, a knife, or a chain traction machine. Honey obtained is centrifuged by using a manual or motorized centrifuge. Subsequently, the honey is decanted by weight for 2 days in a drum that contains a stainless steel strainer inside. In this way, wax remains are extracted in the recently harvested honey, and eventually, other foreign elements that it may contain, for example, limbs of bees and/or parts of them, among others. In Chile, most beekeepers store the honey in sealed drums. Also, there is the possibility of packaging the honey directly. To the extent that packaging is needed, it is also possible to melt it at 40°C. The honey samples were identified as multifloral (samples 1 to 10) or as monofloral (samples 11 and 12) by the supplier beekeepers. Later, samples were sealed, packaged, and sent, via airmail, to Quality Service

Table 1. List of analyzed honey samples.

No. samples	Production date	Honey type informed by the supplier	Production region
1	December, 2015.	Multifloral	Pichidegua
2	February, 2016.	Multifloral	Cajón del Maipo
3	February, 2016.	Multifloral	Chiloé.
4	March, 2016.	Multifloral	La Pintana.
5	March, 2016.	Multifloral	La Pintana.
6	May, 2016.	Multifloral	La Pintana.
7	January, 2016.	Multifloral	La Pintana.
8	March, 2016.	Multifloral	La Pintana.
9	Jannuary, 2016.	Multifloral	Puente Alto.
10	February, 2016.	Multifloral	Linderos.
11	January, 2016.	Monofloral	San José de Maipo.
12	February, 2016.	Monofloral	San José de Maipo.

International (QSI) GmbH based in Bremen (Germany) for conventional physicochemical and resonance nuclear magnetic (NMR) analyses.

Conventional physicochemical analysis

Free acidity	Free acidity was determined by potentiometric titration. For this, 10 g of honey were dissolved in 75 mL of Milli-Q water. By adding 0.1 N sodium hydroxide (NaOH), pH was left at 8,3.[,16,17]
Diastase activity (DA)	Diastase activity was evaluated by spectrophotometry. A buffered solution of soluble starch was used and the honey was kept in a thermoregulated bath at 40°C. The diastase value was calculated using the time it took for the absorbance to reach 0.235, and the results were expressed in Gothe degrees.[16,17]
Electrical conductivity (EC)	A 20% honey solution (w/v, dry matter base) was prepared in Milli-Q water for EC measurement at 20°C using a conventional conductivity meter.[16,17]
Hydroxymethylfurfural (HMF)	The absorbance of 5 g of honey dissolved in 25 mL of Milli-Q water was measured at 284 and 336 nm against a filtered solution treated with sodium bisulfite ($NaHSO_3$). The HMF was estimated using the following equation: HMF (mg/kg of honey) = $((Abs_{284} - Abs_{336}) \times 5 \times 149.7)/W$, in which Abs_{284}: Absorbance measured at 284, A_{336}: Absorbance measured at 336, 149.7: Factor = (126/16830) (10000/10) (100/5), W = weight of the honey sample (in grams). Factor 126: molecular weight of the HMF, 16830: molar absortivity of the HMF, 10000: mg/g, 10: centiliters/L,100: grams (g) of informed honey, and 5: mass of honey weighted in the analytical balance (g).[16,17]
Humidity	Honey water content was determined using a standard refractometer by placing drops of honey on its internal prism.[16,17]
pH	pH was measured in a solution of 4 g of honey dissolved in 30 mL of Milli-Q water using a conventional pH meter.[16,17]

Measurement proton nuclear magnetic resonance (^{1}H-NMR)

The sample preparation method was adapted from BruckerBiospin (BrukerBiospin, Rheinstetten, Germany). Homogenized honey samples (5 g) were diluted in 17.5 ml of NMR buffer (15.7 g of KH_2PO_4 and 0.05 g of NaN_3 in 1 L of deionized water adjusted to pH 3.1) 900 µl of this solution were taken and completed to 1 L with 100 µl of a standard Deuterium oxide solution containing 0.1% Na_3PO_4. The final solution was centrifuged at 14,000 rpm and 600 ul of the supernatant were transferred to NMR tubes for direct measurement.

Nuclear magnetic resonance is a recognized method for detecting exogenous sugars' addition to honey and determine its botanical and geographic origin. All measurements were made on a Bruker Ascend TM (400 MHz) food scanner, equipped with a probe PA BBI 400SI H-BB-D-05 Z (5 mm) and the Bruker SampleXpress device (BrukerBiospin, Rheinstetten, Germany) that allows automated sample loading. ^{1}H-NMR spectra were acquired at 300.1 K, using the pulse program *noesygppr1d* (spectrum 1D with water pre-saturation at 4.8 ppm) and *jresgpprqf* (2D spectrum J-resolved, displaying chemical drift and spin-spin coupling information). For the 1D spectrum, 32 scans and four simulations of 64 k points were acquired with a spectral width of 20.5524 ppm, receiver gain of 16 and acquisition time of 3.9846 s. The 2D spectra were made using four scans and 16 simulations of 8 k (F2-axis) and 40 k (F1-axis) points. The spectral width was 16.7057 ppm (F2) and 0.19 ppm (F1), receiver gain of 16, and acquisition time of 0.6127 s (F2) and 0.2564 s (F1). The NOESY spectra were used for quantification and the JRES spectra to verify the identity of the compounds. All spectra were standardized, corrected with a line basis, and calibrated using 2,2,3,3-D4-3-(Trimethylsilyl) Propionic Acid Sodium Salt as a reference at 0.0 ppm. The compounds were quantified using a routine profile for honey (release 1.0, BrukerBiospin, Rheinstetten, Germany) through automatic integration of the maximum point, calculated with an external standard.[18]

Study of pollen in honey samples

Ten grams of each honey sample were mixed with 20 ml of deionized water in conical tubes (50 ml) according to the method for Melissopalynology suggested by the International Honey Commission

(IHC). Solutions were centrifugated for 10 min at 1000 g to allow the decantation of the supernatant. Later, a second wash with deionized water was performed, and again the solution was centrifugated for 5 min at 1000 G.[19] Sediment was mixed with glycerol-gelatin and mounted on glass slides. Slides covered with cover slips were left for 10 min until the gelatin hardened. To make reference slides, after the parental plants were taxonomically identified in the University of Tehran (Iran) and the University of Urmia (Iran), the stamen of flowers were separated, washed with deionized water and sieved to remove large particles. After centrifuging during 5 min at 1000 G, sediment was mounted on glass slides as described above. At least 300 pollen grains were counted and identified *per* slide using light microscopy (magnification = 400x). For pollen identification, reference slides prepared for this study were compared to reference collections in QSI and University of Göttingen, and with PONET, an online pollen databank (http://ponetweb.ages.at).

Statistics

Infostat software version 2017 (2017, FCA, National University of Córdoba, Argentina) was used to perform descriptive statistical analyses of the results. Origin software was used to graph the results.

Results and discussion

Pollen in analyzed honey samples

Concerning the botanical origin, results showed that seven honey samples (samples 1, 4, 5, 8, 10, 11, and 12) corresponded to multifloral honeys, three samples were classified as honeydew (samples 6, 7, and 9), and two corresponded were monofloral (samples 2 and 3; belonging to Cajón del Maipo and Chiloé, respectively). In particular, the pollen abundance of sample 1 was: 33% *Brassicaceae* (Kreuzblütler, Crucifers), 34% *Rhamnaceae* (Kreuzdorngewächse, Buckthorn-Family), 16% *Salix* sp. (Weiden, Willow), 5% *Vicia* (Wicken, Vetch) -Type, 1% *Pirus*/*Prunus* (Obst, Fruit Blossom), and 11% of unidentified pollen. Pollen composition of sample 2 was: 60% *Quillaja saponaria* (Seifenbaum, Soap Bark Tree), 24% *Anacardiaceae* (Sumachgewächse), and 11% *Brassicaceae* (Kreuzblütler, Crucifers), *Azara* spec. (*Flacourtiaceae); Castanea sativa* (Edelkastanie, Chestnut) ü.r.; *Myrtaceae* (Myrtengewächse); *Rubus* (Himbeer, Rasp). In the case of sample 3, pollen was composed of: 97% *Eucryphia cordifolia* (Chilen. Scheinulme, Ulmo) ü.r., and 1% *Quillaja saponaria* (Seifenbaum, Soap Bark Tree), *Eucalyptus* spec.; *Azara* spec. (*Flacourtiaceae); Lotus* sp. (Hornklee, Trefoil) ü.r.; *Sapindaceae* (Seifenbaumgewächse); *Taraxacum* (Löwenzahn, Dande). For multifloral honey, taxa were identified by experts at the QSI laboratory (Table SI-1). The dominant pollen types for samples of multifloral honeys were similar to sample 1. The rest of the pollen types contributed with less than 1% of the total counted pollen. The determination of honey types was also carried out by experts from the QSI laboratory, which gave reliability and precision to our work. Under these results, we recommend beekeepers to perform palynological analyses of their harvested honey in each season. This should increase the commercial value of their honey and the possibility to access markets that pay a better price for a specific type of honey; for instance, honeydew is highly demanded in Austria, Germany, Greece, Slovenia, Switzerland, and Turkey. Samples 1 to 10 were previously identified as multifloral honeys by the supplier beekeepers, while samples 2 and 3 were classified as monofloral. Also, samples 6, 7, and 9 were classified as honeydew by the pollen study. In contrast, samples 11 and 12 were previously identified as monofloral honeys by the beekeeper, but they actually corresponded to multifloral honeys according to palynological analysis.

Sugars profile

The sugars profile of the honey samples analyzed was represented by monosaccharides (fructose, glucose, and mannose), disaccharides (gentiobiose, maltose, sucrose, and turanose), and a small

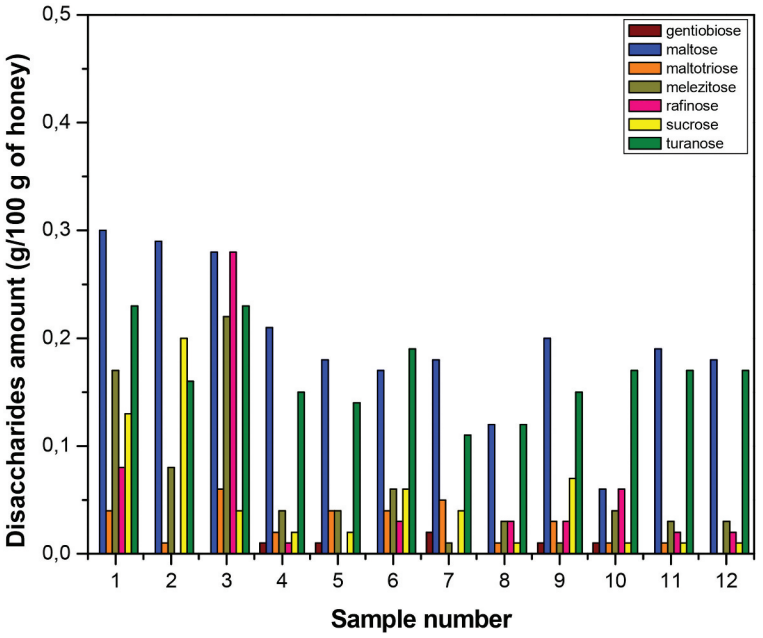

Figure 1. Amount of disaccharides in the analyzed honey samples.

number of other sugars (maltotriose, melizitose and raffinose) (Figure 1). This composition coincides with the values described by Cornejo[20] (Table SI-2) and Arvanitoyannis *et al.*[21] (Table SI-3). The most abundant monosaccharides in the analyzed honey samples were fructose (33.8 – 42.2 g/100 g) and glucose (28.5 – 34.1 g/100 g) (Table SI-4).

In our study, the dominant disaccharides corresponded to maltose (0.6–2.1 g/100 g), sucrose (0.03 –0.7 g/100 g), and turanose (1.1–1.9 g/100 g) (Figure 1). This result is partially in agreement with the information reported by Kaskoniene *et al.*[22] in monofloral (rapeseed and willow) and polyfloral honeys from Lithuania, except for turanose, whose values were lower compared to our results. According to literature, the differences observed between the two studies could be due to numerous factors, including the different botanical origins, geographical locations, climates, nectar flux, harvest conditions (date), processing, and storage of the analyzed honeys.[4,5,15,23] It should be noted that sample 4 had the highest maltose value (2.1 g/100 g), while the lowest value corresponded to sample 10 (0.6 g/100 g) (Figure 1). Regarding turanose, samples 2 and 6 presented the highest values, 1.8 g/100 g and 1.9 g/100 g, respectively, and the lowest values corresponded to samples 7 (1.1 g/100 g) and 8 (1.2 g/100 g). Also, the most abundant trisaccharide was maltotriose (0 – 0.5%) (Figure 1). Maltotriose values coincide with the study reported by De la Fuente *et al.*[24] in multifloral honeys from Spain. We could not detect this sugar in samples 3 and 12. On the other hand, there were considerable differences between blossom and honeydews, the latter containing a higher amount of oligosaccharides, mainly trisaccharides melezitose and raffinose, both absent in floral honeys.[25] In particular, sample 6 was high in melezitose (0.6 g/100 g), suggesting a honeydew origin.

Apart from the floral origin, several factors can determine the composition of sugars in honey. Among these, it is known that most of the disaccharides and trisaccharides present in honey are formed by the action of enzymes on the nectar carbohydrates. For example, acid reversion is one of these reactions, in which higher disaccharides and sugars are formed during storage. Additionally, these sugars modification processes may depend on factors other than pH and water content, which leads to an additional variability in their composition. Among the total sugars present in honey, it is possible to select the most characteristic sugars for a given honey type.[24] For example, the variables that most contributed to the geographical discrimination of honeys were: (1) maltose, moisture,

raffinose, fructose/moisture ratio, and phenylalanine in the case of pine honeys and (2) moisture, sum of fructose and glucose/moisture ratio, proline, glucose/moisture ratio, fructose/moisture ratio, sucrose, maltose, and turanose in the case of fir honeys.[15]

The sucrose amount did not exceed 5 g/100 g of honey in all the samples analyzed (range 0.03 – 0.7; Table SI-4), which is the limit value established for honey (Table SI-5). According to the quantification of sucrose carried out by conventional physicochemical analysis (data not shown) and by nuclear magnetic resonance (NMR), the Chilean honeys samples analyzed in this study would meet the standards established by the *Codex Alimentarius*[26] (Table SI-5). Moreover, it is possible to infer that the honey samples studied corresponded to natural honeys, showing no evidence of adulteration and inappropriate manipulation of the samples.[4,15,27] Fig SI-3 shows the ratio of fructose over glucose (F/G) of the analyzed honey samples, which was around 1 (1.1 – 1.38). This result suggests that fructose was the predominant sugar of the floral species that contributed as the nectar source, coinciding with results reported by Tornuk *et al.*[4] and De la Fuente *et al.*[24] for Turkish (1.08 – 1.27) and Spanish (1 – 1.6) multifloral honeys, respectively. It has been reported that honeys with high F/G remain liquid for more extended periods than honeys with lower ratios,[28,29] since a greater amount of fructose would modify the levels of saturated glucose, and could also impact on the taste of honey given that fructose is sweeter than glucose.[15,30] On the other hand, Fig SI-3 shows the sum of fructose and glucose (or F + G; value = 62.4 – 73.4), which coincides with the one described for honeys (≥60 g/100 g)(Codex; Table SI-6).[26]

Amino acid profile

The amino acid profile of the honey samples was as follows: proline (in a range of 474–4421 mg/kg of honey), glutamine (15–267 mg/kg of honey), phenylalanine (24–129 mg/kg of honey), tyrosine (0 –62 mg/kg of honey), alanine (10–55 mg/kg of honey), valine (4–29 mg/kg of honey), aspartic acid (0 –742 mg/kg of honey), leucine (0 – 38 mg/kg of honey), and isoleucine (0.0 mg/kg of honey) (Figure 2). Proline, which is usually the most abundant amino acid in honey and pollen,[31,32] was also the most

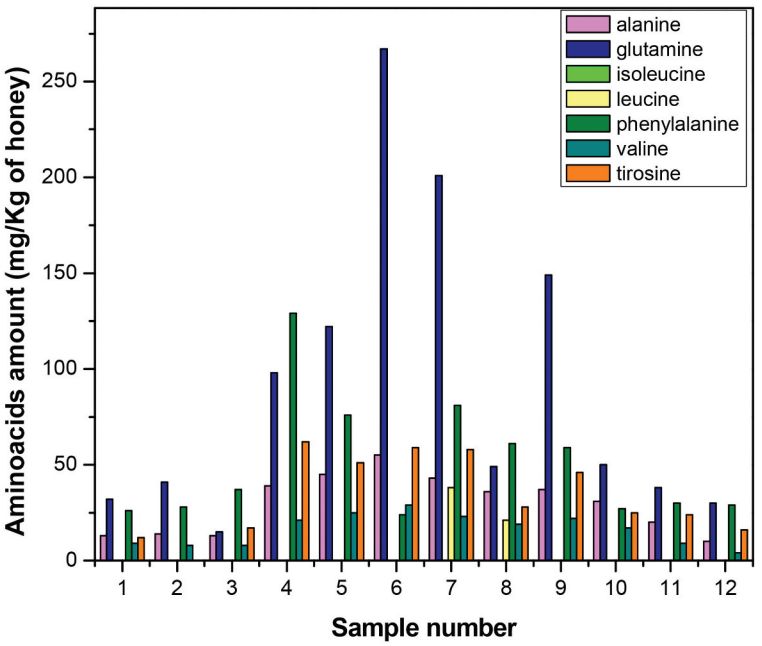

Figure 2. Amount of aminoacids in the analyzed honey samples.

abundant amino acid in our samples together with glutamine and phenylalanine. The amount of proline in our samples was ≥ 180 mg/kg, which allowed us to deduce that samples were not subjected to adulteration with common sugar or sucrose.[32–34] Intermediate aspartic acid values were detected in the analyzed honey samples except for samples 1, 2, 3, 8, 11 and 12 (Figure 3). Also, samples 6 and 7 presented ≥ 200 mg of glutamine/kg of honey. Samples 7 and 8 presented low average values of leucine (38 mg of leucine/kg of honey and 21 mg of leucine/kg of honey, respectively; Figure 2), which could be related to a similar pollen source (Table 1). The amino acids were found in low concentrations in the honey samples, which is consistent with the amino acid composition described for nectar honey.[31,32,35,36] All samples but sample 6 did not possess leucine (value = 0), and isoleucine was not detected in the analyzed honey samples (Figure 2). However, sample 6 had high amino acid levels, suggesting a honeydew origin (Figure 2 and Figure 3). Although the profile of amino acids can be an appropriate method to determine the botanical differentiation of honey,[33,37] it must be considered that during the storage and thermal treatment of honey, several compounds can be formed when the carbonyl group (COO^-) of a reducing sugar reacts with the free amino group (NH_2) of amino acids, peptides or proteins. Thus, since each amino acid has a different reactivity, their proportion in honey could be affected.[31]

Organic acids profile

Samples 6, 7, and 9 had the highest amounts of citric (1019, 324, and 515 g/kg of honey, respectively), formic (108, 76, and 65 g/kg of honey), and malic acid (1718, 1145, and 624 g/kg of honey), which confirmed their honeydew origin (Table 2). On the contrary, samples 2, 11, and 12 (Cajón del Maipo) had the lowest amounts of citric acid (52, 62, and 73 mg/kg of honey, respectively), suggesting a floral origin.[38,39] The existence of citric and malic acids in honey can contribute to their antioxidant capacity, since they can chelate metals, and therefore, synergistically improve the action of other antioxidants such as phenolic compounds.[40] Unlike the study by Cherchi *et al.*[41] and Mato *et al.*,[42] in this study, the following acids were not measured: α-ketoglutaric, galacturonic, glyoxylic, gluconic, glutamic, glutaric, 2-hydroxybutyric, α-hydroxyglutaric, isocitric, malonic, methylmalonic, oxalic, 2-oxopentanoic, propionic, and tartaric. However, three complementary acids: 3-phenyllactic (value = 0), kynurenic (value = 0) and pyroglutamic (value = 0–63 mg/g), not incorporated in previous

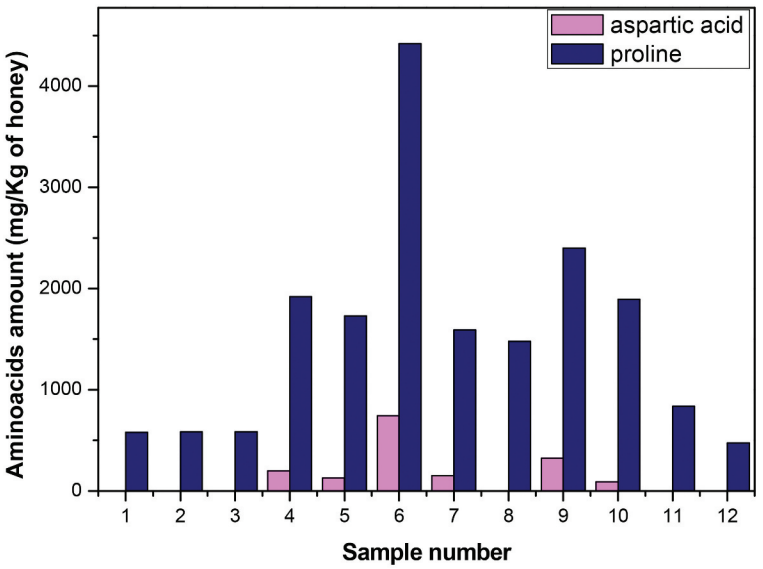

Figure 3. Amount of aspartic acid and proline in the analyzed honey samples.

Table 2. Composition of organic acids in the analyzed honey samples.

Sample number	acetic acid (g/kg)	citric acid (g/kg)	formic acid (g/kg)	fumaric acid (g/kg)	kynurenic acid (g/kg)	lactic acid (g/kg)	malic acid (g/kg)	3-phenyl lactic acid (g/kg)	pyro glutamic acid (g/kg)	quinic acid (g/kg)	shikimic acid (g/kg)
1	17	94	13	1	0	89	39	0	0	0	15
2	11	58	34	0	0	9	84	0	0	0	2
3	19	94	19	1	0	97	41	0	0	0	6
4	26	328	59	2	0	98	336	0	0	0	54
5	34	234	59	4	0	205	584	0	0	0	117
6	30	1019	108	4	0	77	1718	0	0	917	61
7	30	324	76	3	0	300	1145	0	0	0	127
8	23	133	45	2	0	182	41	0	63	0	30
9	30	515	65	2	0	209	624	0	0	0	48
10	25	75	58	3	0	24	310	0	0	0	18
11	18	73	34	2	0	21	58	0	0	0	19
12	25	62	37	0	0	7	88	0	0	0	24
Mean	24	250.8	50.6	2	0	109.8	422.3	0	5.2	76.4	43.4
Range	11 – 34	58 – 1019	13 – 108	0 – 4	-	7 – 300	39 – 1718	-	0–63	0 – 917	2 – 127
SD	6.7	281.2	26.2	1.4	-	94.4	529.6	-	18	264.7	41.1

studies were measured here. The presence of a variety of organic acids in honey can contribute to this product's antioxidant and antibacterial properties.[15] Moreover, organic acids can be useful indicators of fermentation (reflected in excessive concentrations of acetic acid),[39] and of the botanical and geographic origin of honey, if analyzed together with chemical, physical, organoleptic, and palynological analyses.[39,41,43]

Low molecular weight molecules

There is also a group of low molecular weight molecules (LMWM) that are less known but important to measure in honey. Acetoin ($C_4H_8O_2$), also known as 3-hydroxybutanone, is an organic compound produced during alcoholic fermentation by yeasts of the genus *Saccharomyces* (normal microbiota of honey). The decarboxylation of two pyruvic acids ($C_3H_4O_3$) leads to the formation of one molecule of acetoin. In turn, acetoin is a precursor molecule of 2,3-butanediol ($C_4H_{10}O_2$), an alcohol liquid, low volatile, soluble in water and organic solvents. Specifically, 2,3-butanediol is produced during lactic fermentation carried out by some bacteria naturally present in honey. Dihydroxyketone ($C_3H_6O_3$) or DHA, is a ketotriose commonly found in plants, whose levels are often high in freshly harvested honey. After some time, DHA is converted to methylglyoxal (MGO) through natural dehydration.[44] This chemical reaction is irreversible and occurs at a slow rate as honey ages. Thus, DHA is a good indicator of the potential levels of MGO that honey will develop in storage.[44] On the other hand, MGO is formed from sugars during heat treatment and/or prolonged storage of sugar-containing foods and beverages.[44] Finally, trigonelline ($C_7H_7NO_2$) is an alkaloid, zwitterion formed by the methylation of the nitrogen atom (N) of the niacin or vitamin B_3. In *in vitro* tests, trigonelline has inhibited the invasion of cancerous cells. Moreover, this molecule has regenerated axons and dendrites in nervous cells, suggesting its participation in biological processes related to the memory.[45] The results found in the analyzed honey samples were quite heterogeneous (Table 3). In this study, acetoin was not found, but the average of 2,3-butanediol was 8.5 ± 10. These results suggest that no alcoholic fermentation occurred during the honey storage, while lactic fermentation may have occurred in the samples. Also, all samples showed minimal quantifiable DHA and MGO (<10 mg/kg), similar results to other studies.[46–48] On the other hand, monofloral honeys obtained from *Leptospermum scoparium* (Manuka) in New Zealand, and from *Leptospermun polygalifolium* originated from Northern Rivers and Byfield (Queensland, Australia), exhibited high concentrations of DHA (>2000 mg/kg) with marked variability between samples.[49,50] Finally, trigonelline values resulted less uniform in the analyzed honey samples, ranging between 12 mg/kg (sample 12) and 172 mg/kg (sample 8). The regular consumption of honey with higher values of trigonelline could be beneficial to human health.

Table 3. Presence of other molecules in the analyzed honey samples.

Sample number	acetoine (mg/kg)	2,3-butanodiol (mg/kg)	dihidroxyacetone (mg/kg)	methylglioxal (mg/kg)	5-HMF (mg/kg)	trigonelline (mg/kg)
1	0	14	1	4	6	11
2	0	0	1	0	2	14
3	0	14	4	4	0	13
4	0	0	7	0	7	55
5	0	20	9	7	8	120
6	0	10	9	0	5	41
7	0	0	10	5	4	73
8	0	29	3	0	2	172
9	0	15	7	0	3	44
10	0	0	3	5	5	54
11	0	0	2	0	2	24
12	0	0	2	4	2	12
Mean	0	8.5	4.8	2.4	3.8	52.8
Range	-	0 – 29	1 – 10	0 – 7	0 – 8	11 – 172
SD	-	10	3.4	2.6	2.4	49.3

Table 4. Physicochemical parameters of the analyzed honey samples.

Sample number	Acidity (meq/kg)	Water (%)	EC (mS/cm)	Diastase (° G)	pH
4	31.4	16.2	0.76	37.3	4.2
5	33.4	15.9	0.79	43.8	4.1
6	46.0	16.6	1.47	42.6	4.7
7	41.0	16.3	1.11	41.6	4.6
8	29.5	16.9	0.51	42.4	3.8
9	34.4	14.4	0.92	35.7	4.4
10	21.5	15.9	0.62	35.9	4.4
11	15.1	14.6	0.36	39.1	4.3
12	9.5	15.1	0.25	28.6	4.6
Mean	29.1	15.8	0.75	38.6	4.3
Range	9.5 – 46	14.4 – 16.9	0.25 – 1.47	28.6 – 43.8	3.8 – 4.7
SD	11.8	0.9	0.4	4.8	0.3

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pH

The analyzed honey samples showed pH values between 3.8 and 4.7 (Table 4), similar to pH ranges reported for monofloral honey samples from Chile,[51] Australia,[30] Turkey,[4,52] Spain[34] and Greece.[53] These results indicate that none of the analyzed honey samples from this study were adulterated by the addition of artificial substances that elevate pH, like high fructose corn syrup (HFCS).[54–60]

Free acidity

The honey samples' free acidity values did not exceed 50 meq of acid per kg (9.5–46.0 meq/kg, Table 4). This indicates that the analyzed honeys were well conserved at the time of the analysis, showing no signs of sugar fermentation (which would have formed organic acids and increments of free acidity).[54–56,59–61] Therefore, all samples met the standards established by the *Codex Alimentarius* regarding free acidity (Table SI-6).[26]

Water content

The water content ranged between 14.4% and 16.9% in the analyzed honey samples (Table 4), coinciding with the values reported by Arvanitoyannis *et al.*[21] and Sabatini *et al.* (17%) (Table SI-3).[62] The sample with the highest moisture content corresponded to sample 8 (honey harvested in March 2016) and the less humid samples were 9 and 11 (honeys harvested in January 2016). This allowed us to confirm that the honeys analyzed in the present study were harvested, processed, and stored optimally.[4,5,15,23,27,52,53,63,64] The homogeneous moisture values may respond to related botanical origins, harvesting season, intensity of flux nectar, levels of maturation in the hive, similar manipulation by beekeepers during harvesting, as well as extraction, processing and storage conditions, and/or edaphic-geographical conditions (Metropolitan Region), and therefore, climatic similarity through the Region.[15,52,54–62,65] This allowed us to infer that there was no undesired fermentation of sugars in the samples during storage that could have altered their colour, crystallization, conservation, flavour, specific gravity, solubility, and viscosity.[26,27,53] The percentage of humidity in all honey samples analyzed in this work is in the optimum range, meeting the standards specified in both the Food Sanitary Regulation (FSR) (≤ 18%) (Table SI-5)[66] and the *Codex Alimentarius* (≤ 20%) (Table SI-6).[26] The low humidity percentage of Chilean honeys could be explained by favorable climatic conditions, which allows maintaining the original aroma and flavor.[2]

Electrical conductivity

The electrical conductivity (EC) of most of the analyzed honey samples was lower than 0.8 mS/cm (Table 4), comparable with most honeys and their mixtures(Codex; Table SI-6).[26] Samples 6, 7 and 9 exhibited values ≥ 0.8 mS/cm. Since EC is related to the acidity and ash content of honeys, higher EC values detected in the previous samples could be explained by a higher content of organic acids, ions, and proteins,[52] and by the origin of honeydew.[53,63] It is important to remember that samples 6, 7, and 9 were classified as honeydew, based on the results of citric and malic acid abundances, pollen identification, and NMR analysis. Although in the present study we did not quantify the ash content in the honey samples, it is possible to infer that it should be normal in most of the samples (maximum ash value of 0.8%) according to FSR,[66] due to the positive correlation between the presence of minerals and the EC.

Hydroxymethylfurfural

Chilean honey samples analyzed in this study presented values of HMF in the range 0 to 8 (Table 3). It is possible to deduce that our samples effectively corresponded to fresh, natural honeys,[63] which were not adultered, overheated, and stored for a long time.[4,67–69] A scheme of the formation of HMF from glucose and fructose was proposed by Zhao *et al.* (Figure SI-4).[70] The variability observed between samples could be explained by factors such as the floral source, moisture content, presence of organic acids (citric acid), pH, sugar profile (predominance of glucose or fructose), and water activity.[4,54–56,58,60,61,71,72] Given the low values of HMF in the analyzed honey samples, adulteration by adding inverted syrup is ruled out.[23,30,54–57,60,61,73] Regarding the amount of HMF, the analyzed honey samples met the standards established in the FSR (Table SI-5)[66] and *Codex Alimentarius* (≤ 40 mg/kg) (Table SI-6).[26]

Diastase

The diastase activity, an enzyme that is naturally present in honey, was in the range of 28.6 and 43.8 ºG in the studied samples. The differences detected could be due to numerous factors, which include the amount of nectar flow, the age of the Western honey bees, the period of nectar collection, the physiological period of the colony, the sugar content of the nectar, and pollen consumption.[54–56,60,61,74] Besides, it is possible to deduce that there was no overheating of the honeys and that their conservation degree was adequate.[75] About the diastase activity, all honeys analyzed met the standards established by the FSR (Table SI-5)[66] and *Codex Alimentarius* (≥ 8 Göthe units) (Table SI-6).[26]

Conclusion

Our study's principal aim was to contribute to the knowledge of the characterization of Chilean honeys with different origins. Also, we meant to determine through the utilization of conventional techniques and nuclear magnetic resonance (NMR), the physicochemical properties of the honeys to solve if they fulfilled the requirements of the Chilean and International regulation about authenticity and quality of multifloral honey. Our samples presented physicochemical properties in normal ranges, according to the parameters reported by the *Codex Alimentarius* (2001) and the Chilean Food Sanitary Regulation (1997), and typical sugar profiles for natural honeys, which confirms their authenticity. The mellisopalynological analysis allowed the classification of the honey's origin (floral or honeydew), the identification of the honey type (monofloral or multifloral), and the relative abundance of pollen in the samples. ^{1}H-NMR can be a useful technique to describe in detail the botanical and/or geographical origin of honey if they are examined together with conventional physicochemical, organoleptic and palynological analysis.[15] In Chile, it is necessary to deepen the study and implementation of these specific and sensitive techniques to analyze physicochemical parameters of monofloral and multifloral honeys from *A. mellifera* similar to

those proposed in the present study, in order to combine criteria for the final product characterization. This would allow to direct different kinds of honey to national and/or foreign markets and to establish specific quality control measures to ensure food safety and sovereignty.

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