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ORIGINAL RESEARCH PAPER

HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION (HPAEC–PAD) AND PHYSICOCHEMICAL ANALYSIS OF HONEY: EVALUATION OF THE COMPOSITION AND THE QUALITY

Ines Boudiar[1], Mabrouka Bouacha[2*], Sana Besnaci[3], Mina Kouch[2], Akila Abdi[1], Paul Schweitzer[4]

[1]University of Badji Mokhtar, Faculty of Sciences, Department of Biochemistry, Laboratory of Biochemistry and Microbiology, BP 12 Sidi Amar, 23000 Annaba, Algeria
[2]University of Badji Mokhtar, Faculty of Sciences, Department of Biochemistry, Laboratory of Biochemistry and Environmental Toxicology, BP 12 Sidi Amar, 23000 Annaba, Algeria
[3]University of Badji Mokhtar, Faculty of Sciences, Department of Biology, Laboratory of Cellular Toxicology, BP 12 Sidi Amar, 23000 Annaba, Algeria
[4]CETAM Lorraine, Laboratory of Analysis and Beekeeping Ecology, 1B, Jean Baptiste de la Salle Street, 57310 Guenange, France

*Corresponding author: mabrouka.bouacha@univ-annaba.dz

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Abstract: This study aims to evaluate the quality of five types of Algerian honey in terms of sugar profile and physicochemical properties. The honey samples were collected in different locations and under different climatic conditions. The carbohydrates were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Their general physicochemical parameters were also evaluated. The HPAEC-PAD was used for the simultaneous qualitative and quantitative analysis of six mono- and disaccharides. The averages of physicochemical properties such as moisture, *p*H, EC, HMF, and color (Pfund) were 16.32 ± 0.04; 4.36 ± 0.00; 0.38 ± 0.37 $mS \cdot cm^{-1}$; 09.84 ± 0.19 $mg \cdot kg^{-1}$ and 75.4 ± 0.24 mm, respectively. The results showed that fructose is quantitatively the major sugar in honey samples, followed by glucose, turanose, isomaltose, maltose, and sucrose. The estimated reducing sugars are between 72.13 % for Eucalyptus honey and 75.20 % for Euphorbia honey. Thus, the results showed that Algerian honey is natural, there is no sugar added to the nutrition of the bees, and it can be preserved for a long period.

Keywords: *carbohydrates, honey quality, HPAEC-PAD, physicochemical parameters, sugar profile*

INTRODUCTION

Traditionally, honey has always been consumed as a natural sweetener [1, 2]. It is the natural sweet substance produced by the honey bee (*Apis mellifera*) from the nectar of plants or secretions of living plant parts or excretions of plant-sucking insects on living plant parts [3, 4]. Honey is described as a supersaturated sugar solution, the nutritional values of honey are due to its complex and rich composition, especially in sugars [5]. This substance is composed of carbohydrates, water, and several minor components and about 200 reported substances, including amino acids, vitamins, minerals, enzymes, etc., are also present in honey [6, 7]. Beyond the energy value, the physicochemical properties are also provided by the carbohydrates. Bees collect nectar from a single type of flowers or different types of flowers. The honey produced is then monofloral or multi-floral, respectively [6].

The chemical composition of honey is diversified mainly according to its botanical source, geographical location, season, collection area, environment, processing, and storage conditions [2, 8]. These factors control the concentration and properties of sugar content in honey. Different types of disaccharides and trisaccharides have been identified in honey depending on the analytical technique used [9]. Moisture or water content is a significant indicator of honey shelf life, quality, and durability [10, 11]. It can lead to unintentional fermentation of honey caused by osmosis-tolerant yeasts, producing carbon dioxide and ethyl alcohol [12].

Electrical Conductivity (EC) is a criterion for distinguishing between flower honey and honeydew honey. EC is a useful indicator of botanical origin. It plays an important role in the authentication of monofloral honey [13] and in evaluating the physical characteristics of honey [14]. The quality of honey is evaluated by measuring 5-hydroxy methylfurfuraldehyde (HMF) in newly collected honey is usually absent. During storage, packaging, or exposure to heat, its level increases [15]. Honey sugars (hexoses) are converted to hydroxymethylfurfural by an acid-catalyzed dehydration mechanism [15]. The determination of HMF content is a crucial parameter to ensure the purity of honey [12]. Many factors make it very difficult to standardize the composition of honey and assign specific quality attributes. Honey quality is usually based on chemical, sensory, physical, and microbiological characteristics [16, 17]. Carbohydrate analysis plays a major role in food surveys on composition, adulteration, estimation of nutritional values, identification, origin control studies, etc. [16, 18].

Different analytical techniques can be applied to specify the concentration of sugars in honey, including the high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The technique is characterized by its rapid analysis and simple automation; the samples are injected directly (without or with minor pretreatment). It has high resolution; all classes of mono-, oligo-, and polysaccharides can be separated according to their structural characteristics (size, composition, etc.), making it a powerful analytical tool for carbohydrate separation. The HPAEC-PAD provides a sensitive and selective means of identifying simple and complex sugars in honey [19 – 21].

Algeria is classified as the largest country in Africa in terms of surface area; it is bordered to the north by the Mediterranean Sea, which results in all the Mediterranean bioclimates in the country (humid, subhumid, semi-arid, arid, and Saharan). These mountains characterize Algeria and divide the country into three types of environments

distinguished by their relief and morphology, resulting in significant biological diversity [22]. Algeria has significant plant genetic resources due to its geographical location between two floral empires: Holarctis and Paleotropis. This gives it a very diverse flora describing 3139 Algerian plant species, many of which are visited by honey bees.
Consequently, the production capacity of Algerian honey is extremely high [22, 23]. Algerian honey is very diverse from one plant to another within the same flora and from one flora to another as well as from one geographical area to another, however, few studies are conducted on the chemical composition and sugar content of Algerian honey types. Therefore, this study aims to contribute to the knowledge of Algerian honey and to evaluate its quality. It also aims to determine and quantify the sugar composition; monosaccharides and disaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and the physicochemical parameters of Algerian honey from five regions with different climates and botanical origins. The determination of these parameters and properties allows to evaluate its quality and ensure that it is maintained within the international standards and legislation values.

MATERIAL AND METHODS

Honey samples

Five natural honey samples were obtained from different floral sources (Table 1) and produced in different Algerian regions, where the climate differs from one region to another. The honey samples were collected from beekeepers during the summer of 2019 in hermetically-sealed sterile glass containers kept in a refrigerator at 4 °C until analysis. All the analyses were performed in the Laboratory of Analysis and Bee Ecology CETAM-LORRAINE in France.

Table 1. *Geographical and botanical origins of honey samples*

Honey sample	Region	Harvesting sites	Climate	Botanical source	Geographical origin
1	Laghouat	Aflou	Arid	*Hedysarum coronarium*	Field
2	Annaba	Berrahal	Humid	*Polyfloral*	Field
3	Djelfa	Guernini	Semi-arid	*Ziziphus lotus*	Field
4	Algiers	Baba Ahcen	Humid	*Eucalyptus sp*	Mountain
5	El Bayadh	El Mehara	Semi-arid	*Euphorbia sp*	Field

Physico-chemical analysis

Moisture content

An Abbé refractometer (RF 490, Euromex, Holland) was used at 20 °C using the refractometry method [24] to determine the water content. After converting the refractive index (RI) according to the Chataway table, the water content (g/100 g) was obtained according to the harmonized honey method by the International Honey Commission [25].

pH measurement

The measurement was carried out using a *p*H meter (CRISON 2000, Spain) in a 10 % honey solution diluted in distilled water [25].

Electrical conductivity

A knick conductivity meter (Consort C931, Turnhout, Belgium) was used according to previous method [25]. The results of the EC values (20 % honey solution) are expressed in milliSiemens per centimeter ($mS \cdot cm^{-1}$) at 25 °C according to international reference measurements.

Color intensity

The color intensity of the honey samples was determined by the Pfund scale method using a Cary 50 UV-visible spectrophotometer (Varian Inc., Agilent Technologies, USA) at wavelength $\lambda = 635$ nm. The results are expressed in millimeters (mm) Pfund's scale.

The Pfund was calculated using equation 1:

$$Pfund = -38.7 + 371.39 * OD \quad (1)$$

where *Pfund* is the value of the honey color in the Pfund scale, and *OD* is the optical density at the wavelength of 635 nm [26, 27].

Hydroxymethylfurfural (HMF)

The measurement of HMF content was carried out using the Winkler method, also known as the spectrophotometric method, based on absorption at 550 nm. This method involves measuring the UV absorbance of honey solutions with barbituric acid and *p*-toluidine [28]. The results obtained are expressed in $mg \cdot kg^{-1}$. A volume of 2 mL of honey solution (20 %) and 0.5 mL of the *p*-toluidine solution was put in two test tubes; 1 mL of distilled water [28] was added to the first tube and 1 mL of 0.5 % barbituric acid solution (sample) was added to the second tube.

Carbohydrate analysis using (HPAEC-PAD)

A quantity of 200 mg of each honey sample was weighed and dissolved in a 25 mL beaker with a few milliliters of highly purified water (HPW), then transferred to a 100 mL volumetric flask and made up to 100 mL with water.

After insertion of a 0.02 µm filter, the solution was injected into the chromatograph loop under the same conditions as for the lard solution (Dionex GP50 system, USA), with a Carbopac PA1 column (4×250), a PA1 guard column (4×50) and pulsed amperometric detection. The elution gradient composition contains two mobile phases [29]: phase A with highly purified water (HPW) and phase B with 0.2 M NaOH.

The flow rate was adjusted to 0.5 $mL \cdot min^{-1}$, 0.1 $mL \cdot min^{-1}$, 1 $mL \cdot min^{-1}$ for 16 min, 5 min, and another 16 min, respectively. For the detection conditions, the working electrode was maintained at the following potentials and times: Oxidation potential $E_1 = +0.2$ V ($t_1 = 500$ ms); Cleaning potential $E_2 = +0.7$ V ($t_2 = 100$ ms); Reduction potential (desorption of oxidized products) $E_3 = -0.9$ V ($t_3 = 100$ ms). Finally, the separation time lasted about 40 min. The crystallization of honey is usually indicated by the Fructose/Glucose ratio (F/G); when this ratio is high, the honey keeps its liquid texture [30]. To identify and quantify the carbohydrates present in the honey samples, six standards were used: fructose, sucrose, maltose, turanose, and isomaltose.

Statistical analysis

The physico-chemical parameters were compared to international standards such as Codex Alimentarius Commission, the International Honey Commission, and the European Union. Data were expressed as mean ± standard deviation (SD), and were calculated using SPSS Statistics for Windows, version 20 (SPSS Inc., Chicago, Ill., USA). Kruskal-Wallis one-way analysis of variance (ANOVA) was used to test the difference in statistical significance between samples concerning fructose, glucose, sucrose, maltose, isomaltose, and turanose content. A p value ≤ 0.05 was considered significant (approximately 95 % confidence level).

RESULTS AND DISCUSSION

Physico-chemical parameters

The results of the physico-chemical analyses of five samples of Algerian honey are summarised in Table 2.

Table 2. *Physicochemical characteristics of honey*
(all results in the table show the average of triplicates ± SD)

Honey type	Moisture [g/100 g]	*p*H	HMF [mg·kg^{-1}]	Electrical Conductivity [mS·cm^{-1}]	Color [Pfund]
Sulla	17.10 ± 0.00	3.78 ± 0.05	14.40 ± 0.15	0.17± 0.02	51.33 ± 0.57
Polyfloral	17.60 ± 0.17	4.20 ± 0.01	08.60 ± 0.00	0.57± 0.00	78.33 ± 0.00
Jujube	15.30 ± 0.01	4.63 ± 0.10	09.50 ± 0.05	0.39± 0.01	67.67 ± 0.29
Eucalyptus	16.60 ± 0.05	4.89 ± 0.05	03.10 ± 0.46	0.36± 0.02	92.00 ± 0.00
Euphorbia	15.00 ± 0.10	4.29 ± 0.05	13.60 ± 0.00	0.37± 0.00	87.83 ± 0.28

These results showed that the moisture content is below 20 % for all samples. High moisture increases the probability of yeast fermentation during storage. On the other hand, low moisture extends the shelf life of the honey. The moisture content varies between 15 and 17.6 %, with an average of 16.2 ± 0.07. Moisture is an important indicator of good quality(longest shelf life), stability, and ripening of honey according to the International Honey Commission [25]. In addition, international quality regulations specify that honey with a moisture content of 20 % or less is recommended [31]. The moisture content of sulla honey is lower than in previous reports [32 – 35]. Moisture is affected by the variation of the harvesting season, climatic conditions (air temperature, and relative humidity), the degree of maturity of the hive, the geographical origin and even the moisture content of the botanical origin [6]. The honey from polyfloral has the highest moisture content due to the Mediterranean climate (a humid climate) of this region (Annaba). These results are in line with the findings of Chefrour *et al.* (2009) [36]. Euphorbia honey has the lowest water content. The water content observed in jujube, eucalyptus, and euphorbia honey is between 15.3 % and 17.1 %. These results are consistent with some Algerian reports [22, 34, 36].

All the honey samples are acidic; the *p*H is varying between 3.78 and 4.89 with an average of 4.36 ± 0.00. Honey with a low *p*H (3.5) deteriorates quickly and requires important precautions for storage [37]. The *p*H values are affected by the floral source and the geographical area of harvest [38]. Like water content, *p*H influences the stability, shelf life, and even the texture of honey. Honey contains organic acids (gluconic acid) and inorganic ions (phosphate, chloride), which are responsible for the acidity of the honey. According to Bogdanov (2008) [5], the high acidity allows honey to resist microbial spoilage. This is due to the transformation of sugars into organic acids. Honey with a *p*H value between 3.5 and 4.5 is flower honey according to the standards [39]. The sulla, polyfloral, and euphrobia honey are flower honey, whereas jujube and eucalyptus honey are honeydew honeys according to their *p*H value between 4.5 and 5.5 corresponds to honeydew honey [11]. The *p*H results of sulla, polyfloral, jujube, and euphorbia honey confirm the data reported by several authors [32, 36, 40], whereas Benaziza-Bouchema and Schweitzer (2010) [34] found that eucalyptus honey from Algiers is more acidic than ours. The variation between *p*H results is influenced by the collection season and year depending on the collection localities in the same region [34].

All the Electrical Conductivity values of the honey are less than 0.8 $mS \cdot cm^{-1}$, ranging from 0.17 to 0.39 ($mS \cdot cm^{-1}$), with an average of 0.38 ± 0.37. The highest EC value was detected in polyfloral honey, while the lowest value was detected in sulla honey. Electrical conductivity (EC) is used to determine the physical characteristics of honey [14]. Likewise, it is a good criterion to identify the botanical and geographical origin [41]. It is also used to differentiate flower honey and honeydew honey [13]. Indeed, the EC of honey depends on mineral salts, organic acids, complex sugars, and protein concentration [42]. According to Persano Oddo *et al*. (2015) [43], the EC value is negatively correlated with the amount of pollen in the plant, which affects the physicochemical and sensory properties depending on the botanical origin. The EC value is less than 0.8 ($mS \cdot cm^{-1}$) for flower honey and mixed honey and greater than 0.8 ($mS \cdot cm^{-1}$) for honeydew and chestnut honey [43, 44]. The samples examined are typical of flower honey because of their EC values ranging from 0.17 to 0.57 ($mS \cdot cm^{-1}$). The results of EC are in agreement with previous reports on jujube and euphorbia honey [22, 45]. Some authors reported higher EC results for sulla and eucalyptus honey [33, 34]. In contrast, Chefrour *et al.* (2009) [36] found lower EC values in polyfloral honey.

The color of the studied samples varies from light amber (51 mm Pfund) to amber (92 mm Pfund), with an average of 75.40 ± 0.24. The honey samples of polyfloral, eucalyptus, jujube and euphorbia were dark amber and had the highest Pfund values, while only the honey of sulla was light amber. The color of natural honey ranges from light yellow to dark amber and even black [46]. Honey is graded by color, especially monofloral honey, according to the United States Standards for Grades of Extracted Honey. Its color depends on its botanical origins, and honey is usually marketed based on the Pfund color scale [46, 47]. According to Gonnet *et al*. (1986) classification [11], four honey samples are dark amber, and have the highest Pfund values according to the Pfund scale: polyfloral, eucalyptus, jujube, and euphorbia honey [11, 40].

In contrast, only sulla honey was light amber. Rebiai and Lanez (2014) [22] found a lighter color in eucalyptus honey but reported a dark color in euphorbia honey, although they come from the same botanical and geographical origin. This difference in color intensity is due to various factors: the storage period where the honey becomes darker

with time, the mineral content, and pollen color, which influences honey color, and pigments such as phenolics and flavonoids dotted with antioxidant activity [22, 47].
The concentrations of HMF ranged from 3.10 to 14.40 $mg \cdot kg^{-1}$, with an average of 9.84 ± 0.19 $mg \cdot kg^{-1}$, all HMF levels are below the maximum allowed (Table 2). According to the European Union, the HMF content in honey is set at 40 $mg \cdot kg^{-1}$ as a maximum limit [47]. In addition, the Codex Alimentarius has specified a value of 80 $mg \cdot kg^{-1}$ after packaging and 60 $mg \cdot kg^{-1}$ when the honey is freshly harvested and bottled. HMF is an essential parameter for the freshness and authenticity of honey [48, 49]. The results obtained prove that all honey samples are fresh and have not been exposed to any heat treatment during collection and storage. The HMF values from sulla are comparable to the results reported by Benaziza-Bouchema and Schweitzer (2010) [34]. Polyfloral, jujube, and eucalyptus honey revealed a minute amount of HMF compared to the results previously published by Zerrouk *et al.* (2018) [37] and Makhloufi *et al.* (2010) [35]. All samples have a lower HMF content than the Algerian honey reports. Only the HMF value of euphorbia honey is higher than the data of Haderbache *et al.* (2013) [40]. The HMF values are different depending on the storage period, which leads to its increase over time [37, 40].

Sugar analysis

The determination of the sugar composition of honey was carried out by HPAEC-PAD in monosaccharides and disaccharides (Table 3). Figure 1 shows that eucalyptus honey has the highest glucose content.

Table 3. *Sugar analysis of honey samples*

Sugar analysis	Sulla honey	Polyfloral honey	Jujube honey	Eucalyptus honey	Euphorbia honey
Glucose [g/100 g]**	31.20 ± 0.10	30.50 ± 0.10	31.00 ± 0.10	29.63 ± 0.10	32.20 ± 0.02
Fructose [g/100 g]	42.10 ± 0.10	42.97 ± 0.06	42.97 ± 0.10	42.13 ± 0.49	43.97 ± 0.10
Estimated reducing sugars [%]	73.30 ± 0.00	73.47 ± 0.15	73.90 ± 0.20	72.13 ± 0.57	75.20 ± 0.11
Estimated fructose/glucose ratio	1.34 ± 0.00	1.40 ± 0.00	1.38 ± 0.00	1.43 ± 0.01	1.33 ± 0.00
Estimated glucose/water ratio	1.82 ± 0.01	1.65 ± 0.00	2.02 ± 0.01	1.78 ± 0.01	2.14 ± 0.01
Sucrose [g/100 g]	1.00 ±0.03	0.43 ±0.11	0.99 ±0.00	0.20 ± 0.01	0.10 ± 0.01
Maltose [g/100 g]**	0.90 ±0.01	0.90 ±0.01	1.00 ± 0.05	0.99 ± 0.03	1.07 ± 0.03
Isomaltose [g/100 g]**	0.50 ±0.01	1.00 ± 0.01	1.20 ± 0.05	1.00 ± 0.03	1.14 ± 0.03
Turanose [g/100 g]**	1.10 ±0.04	0.90 ±0.00	1.09 ±0.17	1.00 ±0.01	1.00 ±0.00

**Strong significant difference in sugar content can be noticed between samples.

The results show a strongly significant difference in all sugars in all honey samples (P value ≤ 0.01) except for fructose and sucrose. Their distribution is the same in all samples, with a P-value of 0.09 and 0.07, respectively.

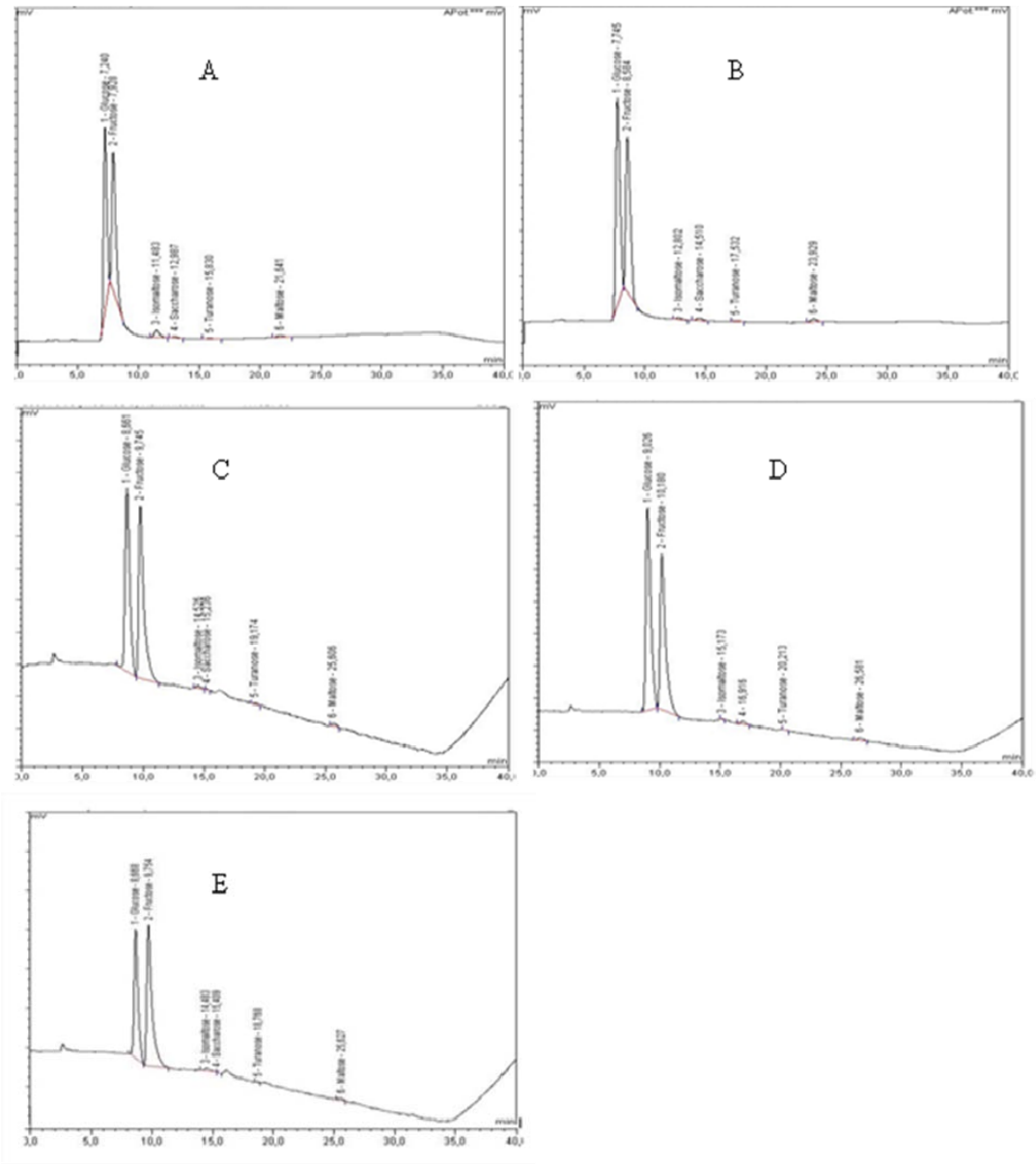

Figure 1. *Chromatographic profiles of sugars in honey samples*
A: Sulla honey, B: Polyfloral honey, C: Jujube honey, D: Eucalyptus honey,
E: Euphrobia honey

The profile of sugars differs from one type of honey to another, depending on the type of flower, climate, and geographical region [13]. Six sugars were identified and quantified in five honey samples (Tables 3). Fructose is quantitatively the major sugar in tested samples, followed by glucose, turanose, isomaltose, maltose, and sucrose. The values of fructose content vary from 42.10 % to 43.97 % with an average of 42.63 %. White *et al*. (1962) [7] reported that the fructose content of honey varies between 27.25 % and 44.26 % with an average of 28.19 %. All our samples are in agreement with this range of values [7]. The fructose content of polyfloral, jujube, and euphorbia

samples is in agreement with published results [32, 50]. However, Ouchemoukh *et al.* (2010) [51] reported higher fructose content in sulla honey, while our eucalyptus honey is rich in fructose compared to the results of Benaziza-Bouchema and Schweitzer (2010) [34]. It is clear that the fructose content is higher in some honey samples than in others, this depends on the botanical source precisely its nectar richness in fructose.

The glucose values vary between 29.60 % and 32.20 %, with an average of 30.90 %. The quality of the honey samples is proven as the glucose content is lower than fructose in all honey samples, this also proves that Algerian bees are naturally fed. Our results indicate that eucalyptus and polyfloral honey have slow crystallization; jujube, sulla, and euphorbia honey have fast crystallization. Honey with high glucose content increases the crystallization rate [51]. Regarding disaccharides, the sucrose content of the tested samples reported in Table 3 indicates high significant differences between the tested samples ($P < 0.05$). Sucrose is a crucial factor in the detection of honey quality.

According to the Codex International Standard for Honey, the sucrose content should not exceed 5 g/100 g [48]. The results have shown a very low sucrose content between 0.1 % and 1 %. The lowest sucrose content was detected in euphorbia honey. The sucrose content in all samples was lower than in previous results [36, 40, 51, 52]. The action of the invertase enzyme reduces the sucrose content. This occurs when honey is ripening in the cells, whereas harvesting it before ripening leads to high sucrose levels [53, 54].

Turanose is present in all samples. Its content varies from 0.90 % to 1.1 % with an average of 1.01 %. In this study, the turanose contents of sulla, polyfloral, and jujube honey are lower than the data of Haouam *et al.* (2016) [33] and Zerrouk *et al.* (2018) [37]. Although the values of eucalyptus honey are similar to the turanose levels of Ouchemoukh *et al.* (2010) [51]. The maltose content in our samples ranged from 0.9 to 1.1 %, these results are lower than the ratios of Haouam *et al.* (2016) [33], Zerrouk *et al.* (2018) [37], and Ouchemoukh *et al.* (2010) [51]. Reports on the concentration of isomaltose in Algerian honey are scarce, Ouchemoukh *et al.* (2010) [51] have quantified this sugar in various samples, and the results in sulla honey are very similar. However, a higher concentration of isomaltose is observed in polyfloral, jujube, and eucalyptus honey.

Among the samples studied, the Algerian sulla honey "*Hedysarum coronarium*" from the Saharan region "Laghouat" characterization reports in terms of physicochemical parameters and sugar profile is extremely rare. This honey is very unique and has many health benefits; it has a strong and pronounced taste and is known for its extraordinary medicinal properties [54, 55]. Many Mediterranean countries use euphorbia honey to treat asthma, sore throat, cardiovascular diseases, and high blood pressure, and to promote fertility in women [55]. The results of the physicochemical properties and sugar profile are in good agreement with international honey standards; the International Honey Commission (IHC), Codex Alimentarius Commission, and the European Union.

CONCLUSION

Reports on Algerian honey are rare concerning its enormous botanical and geographical diversity. Thus, this work consisted of evaluating the physicochemical characteristics

and determining the sugar profile. The quantification of sugars by HPAEC-PAD from different Algerian geographical origins allowed to assess its nutritional quality and detect its sugar composition variation from one region to another. All the samples contain a legal amount of sugar. The conductivity measurements showed that the honey samples are flower honey and mixed honeydew honey. This study shows that the results obtained are consistent with previous findings. Sugars are the main component of honey. Honey composition is affected by geographical location, botanical origin, climatic conditions, and season.

The results of the physicochemical characterization indicated that Algerian honey samples have a low moisture content, which allows good conservation for a long period. All tested samples comply with the requirements of the European Union, Codex Alimentarius Commission, and International Honey Commission standards, both for sugar composition and physicochemical parameters. Algerian honey is of good quality, which allows it to be marketed internationally and exported. The characterization of pollen and the quantification of other components such as polyphenol, glucose-oxidase enzyme, vitamins, and minerals, as well as the evaluation of the antibacterial, antifungal, and antioxidant activity of Algerian honey samples, provide additional information and contribute to the knowledge of the types of Algerian honey.

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