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Relationships between selected physicochemical parameters, pollen quantity, microbiological characteristics and honey quality

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ABSTRACT

The aim of this work was to characterize selected physicochemical parameters, pollen quantity, and microbiological characteristics of six honey samples collected in three northeastern Algerian wilayas (Souk Ahras, Guelma and Annaba) and one commercial honey (Saint Clara brand). The samples were compared using the parameters actually measured: diastatic index, proline, hydroxymethylfurfural (HMF) content, pollen quantity, and yeast and mold loadings. Statistical analyses highlighted important variations between studied samples. The honeys from Souk Ahras generally showed higher pollen quantities, relatively high diastatic index and proline values, and lower HMF and microbial counts, based on the measured parameters. Some Guelma and Annaba honeys and the commercial honey presented higher HMF, yeast and mold counts, indicating less favorable profiles with respect to the measured indicators. Spearman correlation analyses found significant associations between several studied parameters. Positive correlations between HMF and yeasts and negative correlations between diastatic index and yeasts were demonstrated. The results of multivariate analyses (PCA and hierarchical clustering) divided the samples into three groups according to their measured analytical parameters and sampling location. The findings provide that honey quality is depending on several factors among which pollen, physicochemical and microbiological parameters seem to play an important role as quality markers.

Keywords: honey quality, Algerian honey, physicochemical parameters, pollen.

INTRODUCTION

Honey is produced by bees from plant nectar and dew. Some of its components (carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen, and wax) come from its maturation; others are added by the bees, and still others are of plant origin (Anklam, 1998). Today, honey is more appreciated than in the past. Some honeys are sought after for their particular organoleptic or dietary qualities and are marketed under a specific floral name (Chefrour, 2007). To evaluate the different varieties of honey, it is necessary to consider their pollen, physicochemical, and microbiological characteristics. Among the important physicochemical parameters used to assess honey quality, 5-hydroxymethylfurfural (HMF) is a fructose degradation sugar naturally present in all honeys at harvest in trace amounts (1 to 3 mg/kg) (Fallico et al., 2004; Makhloufi et al., 2010). This factor is used to indicate honey purity (Khalil et al., 2012) and its freshness (Philippe, 1988; White, 1994). In contrast, proline, the main amino acid in honey, added by the bee (Von der Ohe et al., 1994), provides information on the honey's maturity and can be used to detect adulteration. Honey is considered mature when its proline content exceeds 183 mg/kg. Lower values indicate a lack of maturity or adulteration (Bogdanov, 1995). Furthermore, the diastase index of honey is another parameter; it expresses the enzymatic activity of amylase.

With the aging of honey, the content of diastases gradually decreases and tends towards zero (Louveaux, 1968).

In Algeria, the study of pollen, physicochemical, and microbiological characteristics has been the subject of several investigations. Among these, the study conducted by Mokhnache et al. (2025) examined a total of six honey samples, four of which came from the wilayas of Guelma, Setif, Medea, and Bejaïa, while the other two were commercially available. In contrast, the study by Tichati et al. (2025) used samples from two sites on the Edough peninsula in Annaba, in northeastern Algeria. Furthermore, the study by Kessi et al. (2024), which focuses on the physicochemical and palynological properties, as well as the composition of trace and toxic elements to evaluate the quality of twenty Algerian honeys including one sample from the wilaya of Guelma. Diafet et al. (2023) analyzed thirty honey samples from different regions of Bordj Bou Arreridj. Bahloul et al. (2022) performed a microscopic analysis on 41 honey samples from different localities in the Laghouat region. The study by Ayed et al. (2021) was conducted on two honey samples (H1 and H2) harvested in July 2019 in two different areas of the city of Jijel (Algeria). Ghorab et al. (2021) worked on 30 honey samples from the Babors Kabylie region (Northeast Algeria). Yabrir et al. (2021) worked on 10 samples that come from the wilaya of Djelfa. Homrani et al. (2020) conducted a study on 62 honey samples collected in different bioclimatic zones of Algeria, including semi-arid, sub-humid, and humid regions as well as the arid zones of the Sahara and the Saharan Atlas, including, among others, the wilayas of Guelma and Souk Ahras.

In the present study, we aim to characterize the selected physicochemical properties of honey (diastatic index, HMF, and proline content), as well as its pollen quantity and microbiological parameters, particularly mold and yeast counts. The study was conducted on samples from four wilayas (Guelma, Annaba, and Souk Ahras) in addition to a commercial honey (Saint Clara).

MATERIALS AND METHODS

Presentation of study areas

A total of seven samples, comprising six regional honey types from three northeastern Algerian wilayas (Souk Ahras, Guelma, and Annaba) and one commercial honey (Saint Clara brand) were studied (Figure 1).

The Wilaya of Souk Ahras is located in northeastern Algeria. It is characterized by a diverse topography (mostly mountains), resulting in significant forest cover and a semi-humid, rainy climate in winter and a hot, dry climate in summer. In this region, we collected two honey samples (Asaguia, City Center) (Figure 1). As for the Wilaya of Annaba, it is bordered by the Mediterranean Sea to the north, and its main vegetation consists of cork oaks. Zen oak, bramble, strawberry tree, herbaceous plants, chestnut, and maritime pine (Laouar et Tahar, 2017). A number of 3 samples are from the wilaya of Annaba, belonging to Hadjar, Berrahel, and Boukantas (Figure 1). The wilaya of Guelma is an agro-forestry region. It is bordered by the wilayas of Annaba and Souk-Ahras. Guelma has forests of cork oak, holm oak, kermes oak, eucalyptus, maritime pine, and cypress (Chefrour, 2007). Only one sample originates from Guelma (city center) (Figure 1).

Sampling

Honey sampling was carried out using a rigorous approach to ensure the representativeness and reliability of the analyses. Samples were collected from various geographical areas and production points between July and august, and each sample was carefully packaged in a clean, dry, and hermetically sealed glass vial, then stored at a constant temperature of 4 °C to prevent any chemical or biological alteration that could affect the honey's quality. These samples were used for all physicochemical and microbiological analyses.

Melissopalynological, physicochemical and microbiological analysis

Initially, to determine pollen quantity, we applied the Layka method (1989), which involves microscopic examination of a honey slide. Subsequently, the HMF content was determined using the Winkler method (1955), as adopted by the International Honey Commission (Bogdanov et al., 2002). This analysis is based on a spectrophotometric measurement of absorbance at 550 nm in the presence of barbituric acid and paratoluidine.

The proline concentration (expressed in mg/kg of honey) was then assessed using a colorimetric

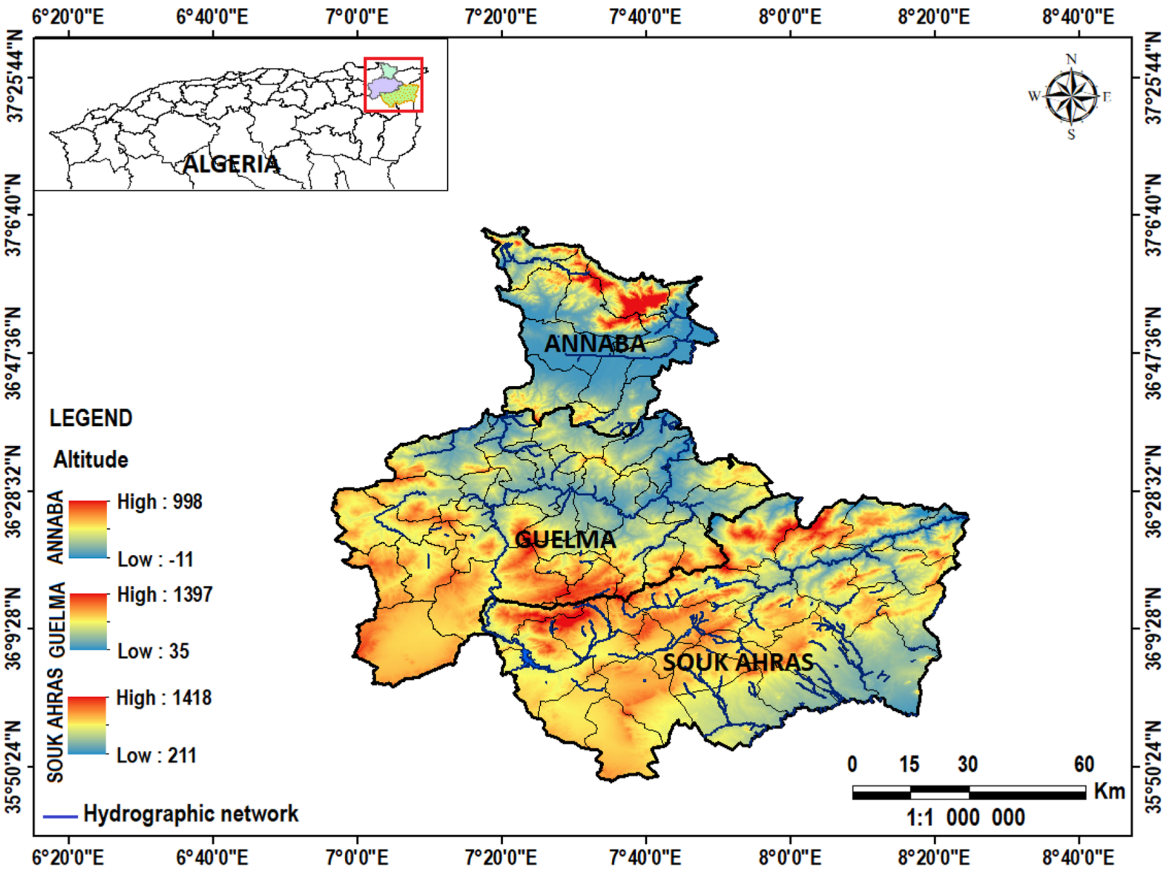

Figure 1. Location of the regions where the samples of honey were collected (ArcGIS, version 10.8.2)

method with ninhydrin, according to the protocol proposed by Ough (1969) and adapted by Bogdanov et al. (2002). This parameter is an indicator of honey maturity and also helps to detect potential adulteration: honey is generally considered mature when its proline content exceeds 183 mg/kg, while lower values may reflect early harvesting or spoilage (Meda et al., 2005).

The diastase index (DI) was also measured using the Phadebas method, which assesses diastase enzyme activity based on the amount of starch hydrolyzed under standardized conditions (1 g of honey, 1 hour, at 40 °C). Results are expressed in Goth units (or Schade units) per gram of honey. This determination was performed using a kit containing a modified starch substrate linked to a chromophore, along with a phosphate buffer (Phadebas Honey Diastase Test) (Bogdanov, 2002).

Finally, a microbiological analysis was conducted to assess the possible presence of yeasts and molds. To this end, successive decimal dilutions (10^0, 10^{-1}, and 10^{-2}) were prepared and then inoculated onto Saboraut medium for 5 days under 30 °C temperature. After incubation, colony counts were performed, and microbial loads were expressed using standard formulas.

Statistical analysis

Statistical analyses were performed to assess associations among the measured physicochemical parameters, pollen quantity and microbiological properties and to categorize samples according to their sampling location and type. Relationships between variables were examined using Spearman's rank correlation coefficient, allowing for the identification of significant associations between the studied parameters (the data are significant at $p < 0.05$). Furthermore, PCA was used to summarize the information and visualize the main sources of variability, while an ascending hierarchical classification (HCA) was performed using the HCPC function from the FactoMineR package in R. The classification was performed using the Euclidean distance as a dissimilarity measure and the Ward method (Ward.D2) as the aggregation criterion. The obtained groups were then projected onto the factorial plane of the PCA using the fviz_cluster function from the factoextra package.

RESULTS

Spatial evolution of the physico-chemical parameters of honey

The comparative study of samples from different sites (six wilayas producing natural honey and one sample of commercial honey) highlights a significant heterogeneity in the parameters analyzed. The pollen grain quantity (R.PL) shows a wide dispersion, with values ranging from a minimum observed in downtown Guelma (6000 grains/g) to a maximum recorded in downtown Souk Ahras (334600 grains/g), and followed by the locality of Asaguia in the wilaya of Souk Ahras (216200 grains/g). Furthermore, the diastatic index (DI) varies considerably, with extreme values recorded in Hadjar at 5.29 and in Berrahal at 22.5. In contrast, the proline concentration (PRL) remains generally high and relatively stable across the different samples, fluctuating between 370 mg/kg in Bougantas and 988 mg/kg in Berrahal.

Regarding hydroxymethylfurfural (HMF), a recognized indicator of honey quality and degree of spoilage, the lowest concentrations were found in Asaguia (Souk Ahras province) at 14.10 mg/kg and in Berrahal at 17.02 mg/kg. Conversely, high levels were observed in Santa Clara commercial honey (98.03 mg/kg) and in honey from downtown Guelma (89.88 mg/kg).

From a microbiological perspective, yeast counts are particularly high in Berrahal (1.36×10^4 CFU/g) and Hadjar (0.74×10^4 CFU/g), while remaining low in downtown Souk Ahras (0.33×10^4 CFU/g). Mold counts also show marked variability, with a very high peak in downtown Guelma (3.45×10^4 CFU/g) and a minimum value in Asaguia (Souk Ahras Province) (0.013×10^4 CFU/g) (Table 1, Figure 2).

Spearman correlation

Spearman correlation analysis revealed several significant associations between the measured honey parameters. A marked positive correlation was observed between HMF and yeasts (r = 0.67). Furthermore, a strong negative correlation was found between the diastatic index (DI) and yeasts (r = -0.90). Similarly, pollen quantity (R.PL) showed a significant negative correlation with molds (r = -0.86). Finally, a moderate to strong negative correlation was also observed between HMF and pollen quantity (r = -0.50), indicating an association between higher pollen quantity and lower HMF levels in the studied samples. Overall, these results highlight associations involving HMF and DI as measured indicators of honey condition, but they do not establish a causal or protective effect of pollen quantity on microbial contamination (Figure 3).

Principal component analysis (PCA)

PCA performed on the physicochemical and microbiological parameters of honeys from different wilayas, as well as the Santa Clara brand, shows a clear structuring of the samples along the first two axes, together explaining 73.3% of the total variability (Dim1 = 40.8%; Dim2 = 32.5%). The Dim1 axis is strongly positively correlated with the diastatic index (DI) (r = 0.719; cos2 = 0.517), proline (PRL) (r = 0.506; cos2 = 0.256), and pollen quantity (R.PL) (r = 0.515; cos2 = 0.265). Whereas, it is negatively correlated with HMF (r = -0.797; $\cos^2 = 0.635$) and yeast content (r = -0.880; $\cos^2 = 0.774$) (Figure 4-A).

Regarding the Dim2 axis, it is primarily structured by molds, which show a strong positive correlation (r = 0.942; $\cos^2 = 0.888$). Furthermore,

Table 1. Physico-chemical parameters, pollen quantity and microbiological analysis of studied honey

City			Pollen quantity (R.PL - pollen/gr)	Proline concentration (PRL)	Diastatic index (ID - mg/kg)	HMF (mg/kg)	Yeast (CFU/g × 10^4)	Mould (CFU/g × 10^4)
Annaba	Berrahel	Ber.An	14460.00	988.00	22.50	17.02	0.005	1.36
	Boukantas	Bou.An	95800.00	370.00	10.42	43.12	0.10	0.04
	Hadjar	Had.An	16720.00	892.00	5.29	52.90	0.74	0.12
Guelma	Guelma Province	Gue.Gu	6000.00	919.00	13.33	89.88	0.17	3.45
Souk Ahras	Asaguia	ASA.So	216200.00	933.00	8.15	14.10	0.10	0.01
	Souk Ahras Province	Sou.So	334600.00	902.00	10.80	39.88	0.03	0.06
Commercial	Santa Clara	San.Cl	14800.00	589.00	5.63	98.03	0.63	0.46

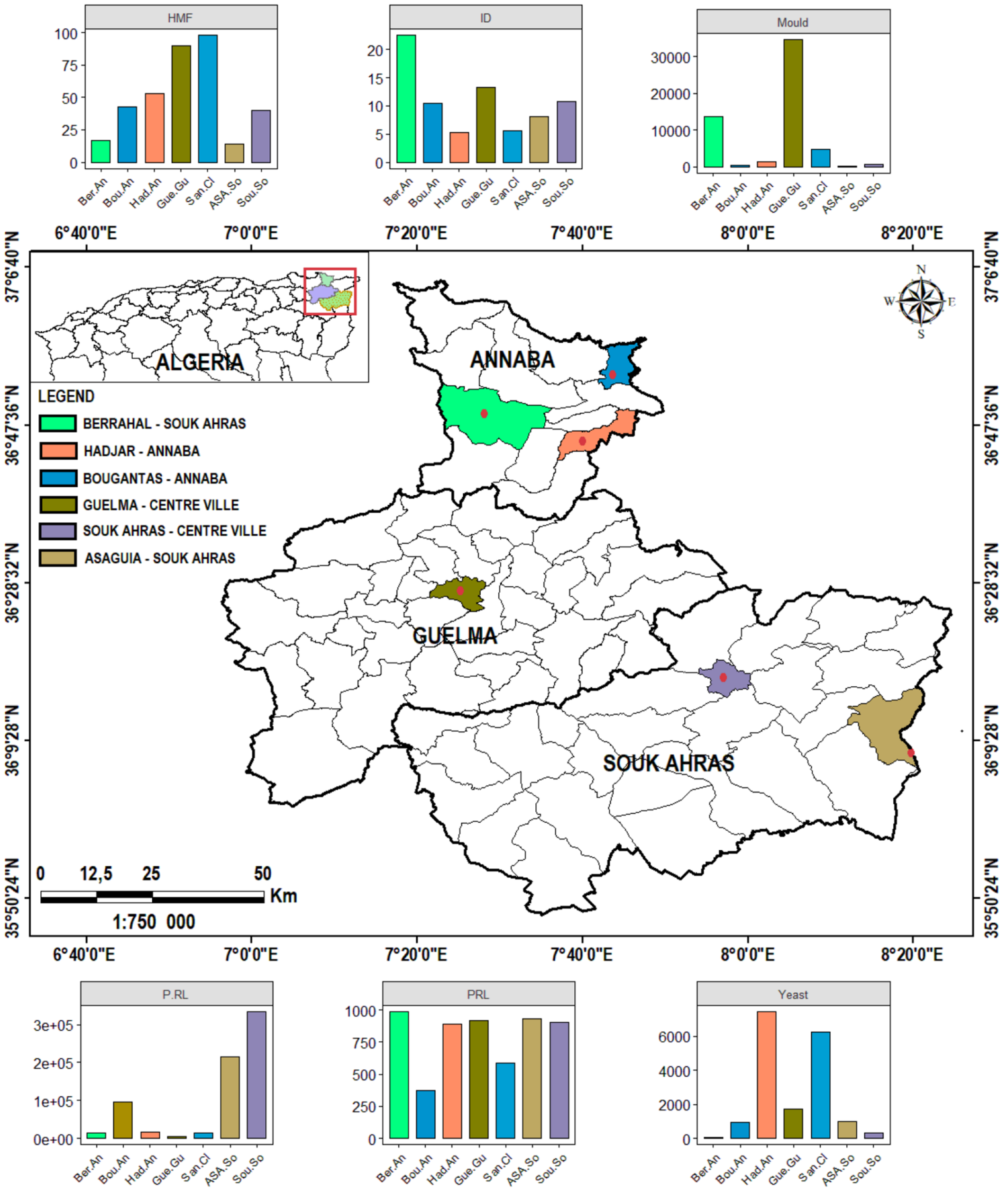

Figure 2. Variability of physicochemical and microbiological parameters of honey samples (ArcGIS, version 10.8.2). Diastatic index (DI), Proline concentration (PRL), HMF, Pollen quantity (R.PL)

this axis is negatively correlated with pollen quantity (R.PL) ($r = -0.684$; $\cos^2 = 0.468$). In contrast, a moderate positive correlation is observed with the diastatic index (ID) ($r = 0.572$; $\cos^2 = 0.327$) (Figure 4-A). Examination of the projection of the studied samples reveals well-structured spatial groupings, indeed:

- Berrahal Annaba honey is positioned in the positive quadrant of Dim1 and Dim2. It is characterized by high levels of mold, ID, PRL, and R.PL.
- The honeys from Souk Ahras city center, Asaguia, Souk Ahras, are positioned in the positive quadrant of Dim1. They are characterized by high levels of ID, PRL, and R.PL.
- Honey from Guelma city center is positioned in the negative quadrant of Dim1 and Dim2; it is characterized by high levels of mold, yeast, and HMF.
- The honeys from Bougantas, Annaba, and Hadjar, and the commercial Saint Clara honey are positioned in the positive quadrant of Dim1 and Dim2. They are characterized by high levels of yeasts and HMF (Figure 4-B).

In conclusion, the honeys from downtown Souk Ahras and Asaguia Souk Ahras showed the most favorable overall profiles among the studied samples based on the measured parameters, including higher DI, PRL, and R.PL values and lower mold, yeast, and HMF values. Conversely, honeys with higher levels of HMF, molds, and yeasts (downtown Guelma, the two municipalities of Annaba, and commercial honey) showed less favorable profiles with respect to these measured indicators (Figure 4-B).

Diastatic index, proline concentration, HMF, pollen quantity

Hierarchical ascending classification

The hierarchical ascending classification (HAC), illustrated by the factor map resulting from the PCA analysis, shows a clear structuring of the honey samples into three distinct groups, confirming the heterogeneity of their quality (Figure 5).

- Group 1: includes the honeys from San.Cl. (Saint Clara) and Had.An. (Hadjar Annaba).
- Group 2: includes honeys from Bou. An (Bougantas, Annaba), ASA.So (Asaguia, Souk Ahras), and Sou. So, Souk Ahras is a city center.
- Group 3: includes the honeys of Gue.Gu (Guelma city center) and Ber. An (Berrahal Annaba), which are clearly separated from the others.

These two analyses confirm a clear differentiation of the honeys according to their sampling location and measured analytical characteristics. Group 2 showed the most favorable profile among the groups based on the measured parameters, while cluster 3 included samples with comparatively higher indicators of deterioration, particularly HMF and/or microbial counts (Figure 5).

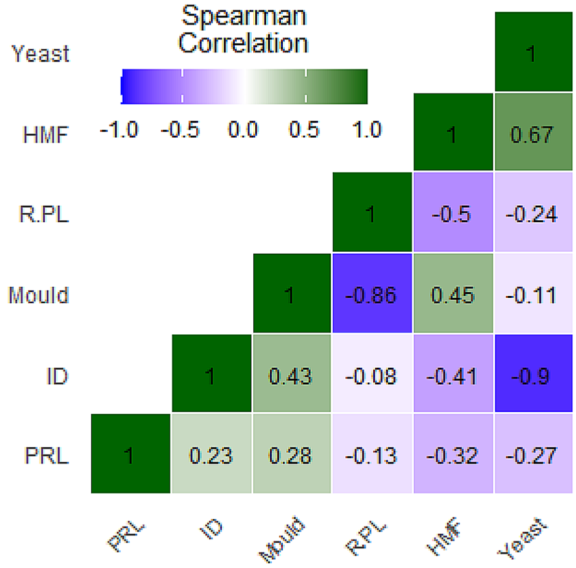

Figure 3. Spearman correlation between the physicochemical parameters of honey: diastatic index (DI), proline concentration (PRL), HMF, pollen quantity (R.PL)

DISCUSSION

Comparative analysis of pollen grains exhibits significant heterogeneity, with values ranging from 6.000 grains/g, recorded in downtown Guelma, to 334,600 grains/g in downtown Souk Ahras. High values are also observed in Asaguia (Souk Ahras province), with 216,200 grains/g.

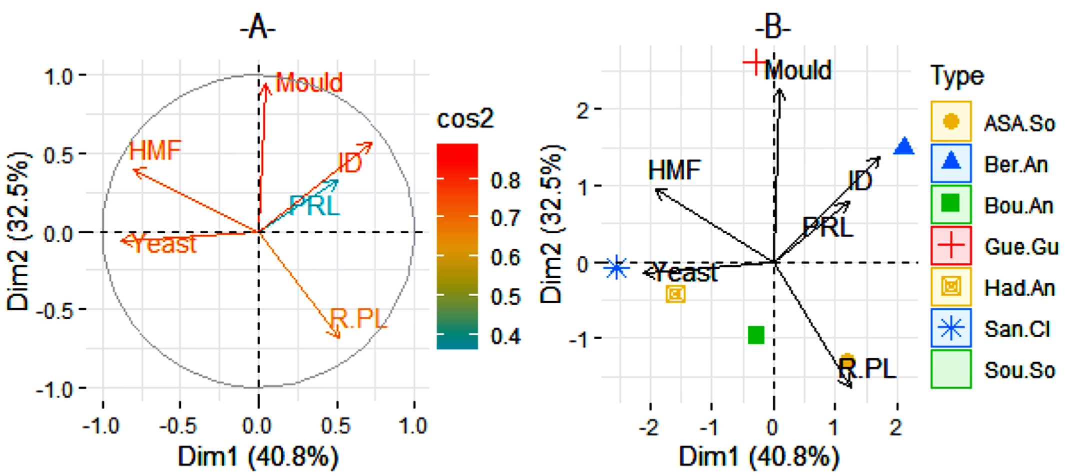

Figure 4. Principal component analysis (PCA): A—Correlation circle Dim1 vs. Dim2; B—Factorial plane according to the "Type" factor

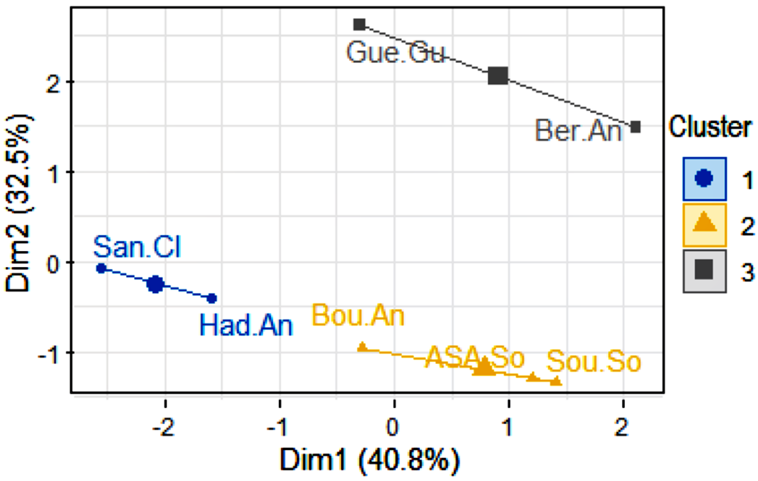

Figure 5. Hierarchical ascending classification of samples according to the physicochemical parameters of honey

Quantitative analysis by Bahloul et al. (2022) revealed pollen density ranging from 26,607 to 660,992 grains per 10 g of honey (average of 160,880 grains per 10 g). Ghorab et al. (2021) reported that their honey samples were characterized by relatively low pollen density compared to ours, with values ranging from 1.117 to 23,196 grains/g, for an average of 6,710 grains/g. Quantitative pollen analysis by Homrani et al. (2020) revealed a pollen quantity ranging from 2.000 grains/g to 100,000 grains/g of honey.

For the diastatic index (DI), the measured values ranged from 5.29 DI for the Hadjar sample to 22.5 DI for the Berrahal. These results are consistent with those reported by Ghorab et al. (2021), who indicated values between 4.8 and 29.0 DI, with a mean of 11.3 DI. The diastase analysis performed by Homrani et al. (2020) revealed values between 8.9° and 40.6°. Yabrir et al. (2021) reported values between 32.85 and 92.31 (Schad units).

Regarding hydroxymethylfurfural (HMF), commonly used as an indicator of honey quality and spoilage, the lowest concentrations were measured in samples from Asaguia (14.10 mg/kg) and Berrahal (17.02 mg/kg). Conversely, the highest values were recorded in Santa Clara commercial honey (98.03 mg/kg) and in the sample from downtown Guelma (89.88 mg/kg). The majority of the analyzed samples had levels exceeding the 40 mg/kg limit established by European and international standards, with the exception of the honeys from Asaguia and Berrahal. Furthermore, only two samples, Santa Clara and honey from downtown Guelma, exceeded the 80 mg/kg threshold permitted for honeys originating from tropical areas and their blends (Council Directive, 2002). HMF concentration is recognized as an indicator of honey freshness (Corbella and Cozzolino, 2006), an important criterion for detecting overheated honeys, especially since HMF is present in small quantities or absent in fresh honeys (Karabourniotti and Zervalaki, 2001), whose HMF content is influenced by various factors, including the type of sugar, its concentration, pH, floral origin, temperature, heating time, and storage conditions (Habib et al., 2014). HMF consumption can cause certain health problems, such as irritation of the mucous membranes of the upper respiratory tract, eyes, skin, etc., if the recommended limits are exceeded (Pastoriza de la Cueva et al., 2017; Shapla et al., 2018).

Our result falls within the range reported by Kessi et al. (2024), who found that HMF levels measured in all honey samples ranged from 0 to 184.7 mg/kg. Furthermore, Diafet et al. (2023) reported concentrations ranging from 0.1347 to 22.83 mg/kg, with an average of 9.462 mg/kg. In contrast, Ghorab et al. (2021) indicated that HMF values ranged from 1.3 mg/kg to 31.8 mg/kg. Ayed et al. (2021) even indicates that spectrometric analysis of honey samples revealed HMF levels of 3.19 ± 0.34 mg/kg for H1 and 3.79 ± 0.73 mg/kg for H2.

Our results measured in samples from Asaguia and Berrahal are consistent with that of Tichati et al. (2025) (HMF: 11.56–14.90 mg/kg).

Proline is a parameter commonly used to assess both the maturity and authenticity of honey. According to the *Codex Alimentarius* recommendations (2001), its content should generally not be less than 180 mg/kg. In our study, proline (PRL) concentrations ranged from 370 mg/kg for the Bougantas sample to 988 mg/kg for the Berrahal sample, indicating that our samples meet the maturity and authenticity criteria for honey established by the *Codex Alimentarius*. Emmanuelle et al. (1996) confirm that proline is the free amino acid produced in the bee's salivary secretions. It is considered the most important amino acid in honey. Its concentration in honey decreases significantly if it is adulterated, hence the importance of measuring it.

These values are within the range reported by Ayed et al. (2021) (553.29 mg/kg for H1 and 317.66 mg/kg for H2).

From a microbiological standpoint, yeast counts varied considerably among the sites studied. The highest values were recorded in Berrahal (1.36×10^4 CFU/g) and Hadjar (0.74×10^4 CFU/g), while significantly lower levels were observed in downtown Souk Ahras (0.04×10^4 CFU/g). A similar heterogeneity was noted for molds, characterized by a maximum in downtown Guelma (3.45×10^4 CFU/g) and a minimum in Asaguia (0.01×10^4 CFU/g). Mokhnache et al. (2025) indicate that honey from the Guelma province has mold and yeast counts ranging from 0.11×10^4 to 0.50×10^4 CFU/g. Furthermore, according to Campos et al. (2008), the European Union's microbiological quality standards recommend counts of less than 5×10^4 CFU/g for molds and yeasts.

Based on these thresholds, our results show low microbiological quality, particularly in Hadjar, downtown Souk Ahras, and Asaguia. This variability can be explained by the high nutritional value of pollen, which provides a favorable substrate for the growth of various microorganisms, including yeasts and molds. Their presence can be linked to different stages, from collection by bees to handling and storage conditions, which directly influence the final microbial load.

One of the most determining factors is the water content of the pollen grains. Indeed, the high moisture content of fresh pollen produced by bees, between 20 and 30% (Bogdanov, 2004; Campos et al., 2008), promotes the proliferation of microorganisms such as bacteria and yeasts. Drying and reducing water activity are therefore essential for preserving pollen collected by bees and preventing its deterioration (Fernández et al., 2021; Straumite et al., 2022; Ecem Bayram et al., 2023).

Correlation analysis and multivariate analyses performed using PCA and hierarchical clustering revealed differentiation among the honey samples according to their sampling location and measured analytical characteristics. Three distinct groups were identified, reflecting heterogeneity in the measured parameters among the samples. The group comprising honeys from Asaguia and downtown Souk Ahras showed the most favorable profile based on the measured indicators, while samples from downtown Guelma and certain communes of Annaba showed higher HMF and/or microbial counts.

CONCLUSIONS

The characterization of honey samples, based on the measured indicators, including diastase index, proline content, hydroxymethylfurfural (HMF) levels, pollen quantity, and microbial load (molds and yeasts) provides information on selected aspects of honey quality, and microbiological status.

The findings prove that honeys of Guelma and Annaba localities and the commercial honey presented in this study were characterized by high levels of HMF and microorganisms that could suggest an advanced deterioration level which could have occurred during storage, manipulation or conservation stages. The observed statistical correlations also confirm the role of HMF as an indicator of honey degradation and suggest a beneficial effect of pollen quantity on limiting microbial contamination.

The results of multivariate analyses clearly structured the samples into homogeneous groups and highlighted the combined influence of pollen, physicochemical, and microbiological factors on the final honey quality. These results contribute to a better understanding of Algerian honeys and underscore the importance of regularly monitoring their characteristics to guarantee their quality and compliance with international standards.

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