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PHYSICO-CHEMICAL PROPERTIES, RHEOLOGICAL BEHAVIOR AND MINERAL CONTENTS OF HONEY VARIETIES FROM OLTENIA REGION

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ABSTRACT. The honey samples analysed in this study were obtained from a private producer located in Olt County who requested a qualitative characterization of his products depending on honey sources. The first stage of the investigation involved key physicochemical parameters: moisture content, free and total acidity, initial and total invert sugars, sucrose levels, and the presence of hydroxymethylfurfural (HMF) and metallic elements. Differential Scanning Calorimetry (DSC) was additionally used to characterize the thermal properties of the samples. The final part of the study examines the rheological behavior of four honey varieties under different temperature conditions, providing insights into their flow characteristics and structural stability. All the obtained values fall within the ranges established by European regulations, except for Pb; the exceedances reflect the influence of anthropogenic factors in the mentioned region.

Keywords: *acidity, differential scanning calorimetry, heavy metals, honey, invert sugar, hydroxymethylfurfural, moisture, sucrose, viscosity*

INTRODUCTION

The use and production of honey have a long and complex historical trajectory. After more than six thousand years of written history, honey is still widely recognized for its nutritional and medicinal properties. Honey was the first sweetener used by humans and has a high nutritional value, providing

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simple sugars, organic acids, amino acids, macro- and microelements, as well as biologically active compounds beneficial to human health [1,2]. It is consumed directly, used as an ingredient in various food products, or applied in preventive medicine [2].

The physical, chemical, and sensory characteristics of honey are strongly influenced by its botanical and geographical origin. Carbohydrates, particularly the reducing sugars-collectively known as “invert sugar”-represent the most important constituents of honey. Small amounts of sucrose are also present. The determination of invert sugar and sucrose content is a key criterion for the authenticity of bee products and relies on the reducing properties of sugars [3,4].

Despite its high nutritional and therapeutic value, honey is also among the food products most susceptible to adulteration. Evaluating the quality and authenticity of honey is a significant research area with implications for industry, consumer protection, and regulatory compliance. Honey authenticity is defined by the Codex Alimentarius Committee, the EU Honey Directive, and national legislation. The concept of “authenticity” concerns two main aspects: production authenticity (prevention of adulteration) and authenticity related to geographic or botanical origin (prevention of mislabelling) [5].

The measurement of 5-hydroxymethylfurfural (HMF) is widely used to assess honey quality, as its concentration increases during processing and improper storage. Heating honey to reduce viscosity or prevent crystallization can also increase HMF levels, depending on the type of honey [6]. HMF is produced through the acid-catalyzed dehydration of hexoses [7] and its accumulation is influenced by the chemical properties of the honey [8]. Codex Alimentarius [9,10] sets a maximum HMF content of 40 mg/kg after processing and/or blending. The European Union [11] has also set the maximum limit of 40 mg/kg, with exceptions of 80 mg/kg for honeys from tropical regions and 15 mg/kg for honeys with low enzymatic activity [12].

Some of metals (K, Ca, Na, Fe, Cu, Zn) are commonly present in honey attending the route: soil-plants-bees-honey and may increase its nutritional value. Metals as Pb, Cd, Ni, Cr, Al are contaminants from industrial and logistics activities, agrochemicals (cadmium-based fertilizers, organic mercury compounds, and arsenic-containing pesticides), polluted soil and water [13,14]. From these points of view, honey is an effective biomarker reflecting environmental quality over large areas, due the foraging activity of honeybees [15-17].

Floral honeys typically contain 0.1–0.2% minerals, while honeydew honeys can reach 1% or more [18]. Recent research has quantified metal concentrations in honey from several European countries [19].

This study also examines the rheological behaviour of honey as a function of temperature and presents results from DSC analyses. Viscosity, an important physical parameter of honey, is correlated with its other physicochemical properties [20,21]. Understanding honey rheology is necessary for its production, processing, and storage. Most honey varieties exhibit Newtonian behaviour [22,23]; however, some studies report non-Newtonian behaviour associated with the presence of high molecular weight compounds, such as proteins or polysaccharides [24].

The viscosity of honey depends primarily on water content, temperature and chemical composition. Honey typically contains between 13 g and 29 g of water per 100 g. Higher water content results in lower viscosity [25]. Temperature is another determining factor: as temperature increases, viscosity decreases due to reduced molecular friction and hydrodynamic forces [26,27]. The temperature-viscosity relationship can be described using the Arrhenius model [22,26].

The thermal behaviour of authentic honeys has been investigated using Differential Scanning Calorimetry (DSC), with glass transition temperatures (Tg) reported between -46°C and -32°C, depending on honey variety [28]. Understanding the properties of honey at low temperatures is essential for proper storage. The present study aims to characterize the rheological properties of Romanian honey varieties and to describe the influence of temperature on their viscosity.

RESULTS AND DISCUSSION

Moisture

Moisture content, along with storage temperature and any heat treatments, are parameters that influence the quality of honey.

Table 1. The moisture content of the honey samples

Honey	n_1	n_2	n_3	Refractive index (mean ± SD, n = 3)	Moisture %
Robinia pseudoacacia	1.4925	1.4924	1.4923	1.4924±0.0001	17.6
Linden	1.5008	1.5006	1.5004	1.5006±0.0002	14.4
Polyfloral	1.4990	1.4994	1.4992	1.4992±0.0002	15.0
Fir honeydew	1.4915	1.4911	1.4913	1.4913±0.0002	18.1
Rapeseed + Robinia pseudoacacia	1.4972	1.4972	1.4972	1.4972±0.0000	15.8

The moisture content of honey is directly associated with its botanical origin, harvesting and processing conditions and, implicitly, varies from year to year. Low moisture content prevents fermentation, the development of microorganisms and inhibits the formation of HMF, increasing shelf life and maintaining the quality of honey unaltered.

The results are expressed as mean ± standard deviation (n = 3). Each sample was analysed in triplicate and results are expressed as mean ± standard deviation (n = 3)

As shown in Table 1, the moisture content of the analysed honey samples ranges from 14.4% to 18.1%, with all values remaining below the 20% limit established by Codex Alimentarius [9], confirming their good quality and stability against fermentation.

Determination of pH, free and total acidities and lactone content

The lactone content of honey reflects the contribution of esterified organic acids to its overall acidity profile, serving as an indicator of both botanical origin and the degree of freshness or chemical stability of the product.

Table 2. pH values, acidity and lactone content

Honey	pH (mean ± SD, n = 3)	Free acids, meq/kg (mean ± SD, n = 3)	Lactone, meq/kg (mean ± SD, n = 3)	Total acidity, meq/kg (mean ± SD, n = 3)
Robinia pseudoacacia	4.72±0.01	20.20±0.01	14.41±0.02	34.61±0.02
Linden	4.28±0.02	22.62±0.01	14.60±0.02	37.22±0.02
Polyfloral	5.08±0.01	28.51±0.02	12.52±0.01	41.03±0.02
Fir honeydew	3.92±0.01	27.50±0.01	19.49±0.03	46.99±0.03
Rapeseed + Robinia pseudoacacia	4.92±0.02	23.41±0.01	20.51±0.01	43.92±0.01

Data from the literature shows that good quality honey has an acceptable pH range between 3.5 and 4.5 [29,30]. The lower value of pH in honey inhibits the growth of microorganisms. An increase in pH above the typical range could indicate adulteration or the beginning of a fermentation process. From Table 2, it can be observed that Robinia pseudoacacia honey, polyfloral honey and the rapeseed-Robinia pseudoacacia mixture have slightly higher, but insignificant, pH values. The pH values, ranging from 3.92 to 5.08, remain within acceptable quality limits, confirming the stability of the samples and the absence of fermentation-related alterations.

The data in Table 2 show that all honey samples fall within the expected acidity range for authentic honeys, according with UE regulation [31], with total acidity varying between 34.6 and 47.0 meq/kg. Also, the experimental values are in accordance with other studies [32]. Polyfloral, fir honeydew and the rapeseed-Robinia pseudoacacia mixture exhibit the highest acidity values, consistent with their botanical origin. The lactone content follows a similar pattern, indicating a balanced contribution of free and lactonic acids to the overall acidity profile.

Determination of proline

Based on the calibration curve it was established the equation that characterized the variation of the absorbance (A) with the concentration of proline solution (c, gx10^{-4}/g sample): $A=0.013 + 89.851 \cdot c$, $R^2= 0.9924$.

The results in Table 3 show that, among the analysed honey samples, the proline content varies significantly, reflecting their botanical origin. Fir honeydew has the highest percentage of proline (0.06%), consistent with its generally richer nitrogen profile.

Table 3. The proline (Pro) content in honey samples

Honey	Absorbance (520 nm) (mean ± SD, n = 3)	g Pro x 10^{-4} / g sample (mean ± SD, n = 3)	Pro, % (mean ± SD, n = 3)
Robinia pseudoacacia	0.1270±0.0002	4.388±0.002	0.01±0.0002
Linden	0.0765±0.0003	4.658±0.003	0.02±0.0003
Polyfloral	0.0765±0.0003	4.658±0.003	0.02±0.0003
Fir honeydew	0.1065±0.0004	9.524±0.004	0.06±0.0004
Rapeseed + Robinia pseudoacacia	0.0720±0.0003	7.763±0.003	0.03±0.0003

Linden honey and polyfloral honey have intermediate levels of proline (0.02%), while Robinia pseudoacacia honey has the lowest value (0.01%), in agreement with literature data indicating naturally lower amino acid concentrations in acacia varieties [33]. The rapeseed-Robinia pseudoacacia mixture shows a moderate proline content (0.03%), suggesting contributions from both floral sources. Overall, all samples fall within the expected ranges for authentic honey, which supports their natural origin.

Determination of HMF content

The HMF content of honey samples is presented in Table 4.

Table 4. HMF content in honey samples

Honey	Absorbance (550 nm) (mean ± SD, n = 3)	HMF, mg/kg (mean ± SD, n = 3)
Robinia pseudoacacia	0.070±0.0012	13.5±0.2
Linden	0.067±0.0013	12.8±0.2
Polyfloral	0.089±0.0017	17.2±0.3
Fir honeydew	0.157±0.003	30.2±0.6
Rapeseed + Robinia pseudoacacia	0.080±0.0015	15.4±0.3

The HMF values presented in Table 4 are low for all analysed honey samples, remaining well below the maximum limits established by Codex Alimentarius and EU legislation. Fir honeydew exhibits the highest HMF content (3.02 mg/100g), which is still characteristic of minimally processed and properly stored honey. The slightly elevated values from the polyfloral and rapeseed-Robinia pseudoacacia samples probably reflect natural variability associated with floral origin. Overall, the low HMF concentrations confirm that all honeys were fresh, unheated, and stored under appropriate conditions [34].

Invert sugar and sucrose determination

The content of invert sugar and sucrose is an essential indicator of the authenticity and maturity of honey, as high levels of invert sugar reflect the enzymatic activity of bees, while low values of sucrose confirm the absence of adulteration and minimal processing [34,35].

The values of reducing sugar direct before inverting (RSDBI), reducing sugar direct after inverting (RSDAI) and sucrose content are presented in Table 5.

Table 5. Invert sugar and sucrose content of honey samples

Honey	RSDBI		RSDAI		Sucrose %[a)]
	Invert sugar (mg)*, [a)]	Initial invert sugar, %[a)]	Invert sugar (mg)*, [a)]	Total invert sugar, %[a)]	
Robinia pseudoacacia	45.9±0.8	75.25±0.8	48.5±0.9	79.5±0.9	4.25±1.2
Linden	43.7±0.7	72.69±0.7	46.1±0.8	76.77±0.8	4.08±1.06
Polyfloral	45.5±0.8	75.70±0.8	43.4±0.7	80.51±0.7	4.81±1.13
Fir honeydew	41.4±0.8	68.95±0.8	45.5±0.8	75.73±0.8	6.78±1.13
Rapeseed + Robinia pseudoacacia	45.9±0.8	75.25±0.8	48.5±0.9	79.5±0.9	4.25±1.2

* in accordance with [4,36]

a) the results are expressed as mean ± standard deviation (n = 3). Each sample was analysed in triplicate and the results are expressed as mean ± standard deviation (n = 3).

The data in Table 5 show that all honey samples contain levels of invert sugar characteristic of authentic, unadulterated honey. Total invert sugar values exceed 75% in all samples, well above the minimum of 60% required for floral honey, confirming their natural origin. Fir honeydew has the lowest percentage of invert sugar, as expected for honeydew varieties, that typically contain higher amounts of oligosaccharides. Sucrose levels remain low in most samples, except the fir honeydew sample, whose higher sucrose content (6.78%) remains within acceptable limits and reflects botanical specificity rather than adulteration. Overall, the carbohydrate profiles support the authenticity and good quality of the analysed honeys.

Ash content

Ash content is a direct measure of the inorganic mineral content in honey. The ash content of honey is generally low and influenced by the chemical composition of the nectar, which varies depending on the different botanical sources involved in the formation of the honey. It can vary between 0.02% and 1.0%, and the maximum limit allowed by legislation for honey from floral sources is 0.6%. Normally, however, ash contents between 0.1% and 0.3% are found for these honeys. The very high mineral content (around 1.0%) is only found in honeydew, and the ash content is often used to identify this type of honey [37].

Table 6 presents the ash content of honey samples.

Table 6. Ash content of honey samples

Honey	**Ash content, % (mean ± SD, n = 3)**
Robinia pseudoacacia	0.20±0.004
Linden	0.14±0.003
Polyfloral	0.51±0.01
Fir honeydew	0.30±0.006
Rapeseed + Robinia pseudoacacia	0.24±0.005

In all samples, the ash content is low, indicating that the organic fraction (primarily sugars) is predominant, while the levels of microminerals, macrominerals and heavy metals are minimal.

Determination of metal content

The content of macrominerals and microminerals in the five honey samples is presented in Tables 7a and 7b.

Table 7a. Macrominerals content in honey samples

Honey	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Al^{3+}	Mn^{2+}
	mg/kg					
Robinia pseudoacacia	0.46	1.86	0.17	0.25	0.02	< 0.01
Linden	1.40	2.49	0.45	2.28	0.01	< 0.01
Polyfloral	0.36	1.54	0.25	3.85	1.17	10.00
Fir honeydew	1.18	3.31	0.27	1.71	0.21	0.84
Rapeseed + Robinia pseudoacacia	0.25	0.46	0.24	13.72	15.25	155.59

*the detection limit of the method (LOD) was 0.01

Variations in the content of microminerals and macrominerals (not considered contaminants) do not negatively influence the quality of honey, giving it a therapeutic effect specific to its content and implicitly, to the honey variety.

Table 7b. Microminerals content in honey samples

Honey	Ag^{+}	Co^{2+}	Cr^{n+}	Li^{+}	Ni^{2+}	Sr^{2+}	Ti^{2+}	Se^{2+}	V^{3+}
	mg/kg								
Robinia pseudoacacia	<0.01	0.11	0.19	0.10	0.36	29.14	0.99	1.69	<0.01
Linden	0.08	0.04	0.17	1.38	0.32	42.75	0.25	0.68	0.14
Polyfloral	0.04	<0.01	0.34	0.89	1.12	14.96	0.24	<0.01	0.05
Fir honeydew	0.11	0.01	0.14	5.20	0.32	55.22	0.74	<0.01	<0.01
Rapeseed + Robinia pseudoacacia	0.04	<0.01	0.10	0.88	0.23	16.66	<0.01	<0.01	<0.01

*the detection limit of the method (LOD) was 0.01

Table 8 contains the heavy metal content in honey samples.

Table 8. Heavy metal content in honey samples

Honey	Cd^{2+}	Cu^{2+}	Fe^{n+}	Pb^{2+}	Zn^{2+}
	mg/kg				
Robinia pseudoacacia	0.046	1.03	0.07	0.183	< 0.01
Linden	0.16	2.68	0.09	0.17	0.01
Polyfloral	0.28	5.30	0.09	0.198	0.01
Fir honeydew	0.027	3.24	0.11	0.213	0.00
Rapeseed + Robinia pseudoacacia	0.084	0.64	0.04	0.29	0.01
MPC* according to [31]	**0.2**	**-**	**1.00**	**0.20**	**1.0-3.0**

*MPC-Maximum Permitted Concentration; the detection limit of the method (LOD) was 0.01

The values for Cu^{2+}, Fe^{n+}, and Zn^{2+} are within the permitted limits or close to the detection limit [9,11].

The analysis of heavy metal content shows variations depending on the botanical origin of the honey. Pb^{2+} concentrations slightly exceed the maximum allowed values in some samples, especially in the mixture (rapeseed + Robinia pseudoacacia) and in fir honeydew, suggesting the influence of anthropogenic factors (industrial pollution, road traffic, soil contamination) [31,38,39]. Due to its high mobility in the environment and plant affinity, lead is sequestered from the soil by melliferous flora and translocated into nectar [40,41]. Through the maturation process, the honeybee acts as a secondary concentrator, ensuring that the final matrix provides an accurate spatial reflection of geogenic and anthropogenic contamination in the foraging area. These elevated levels have also been reported in other scientific studies [42].

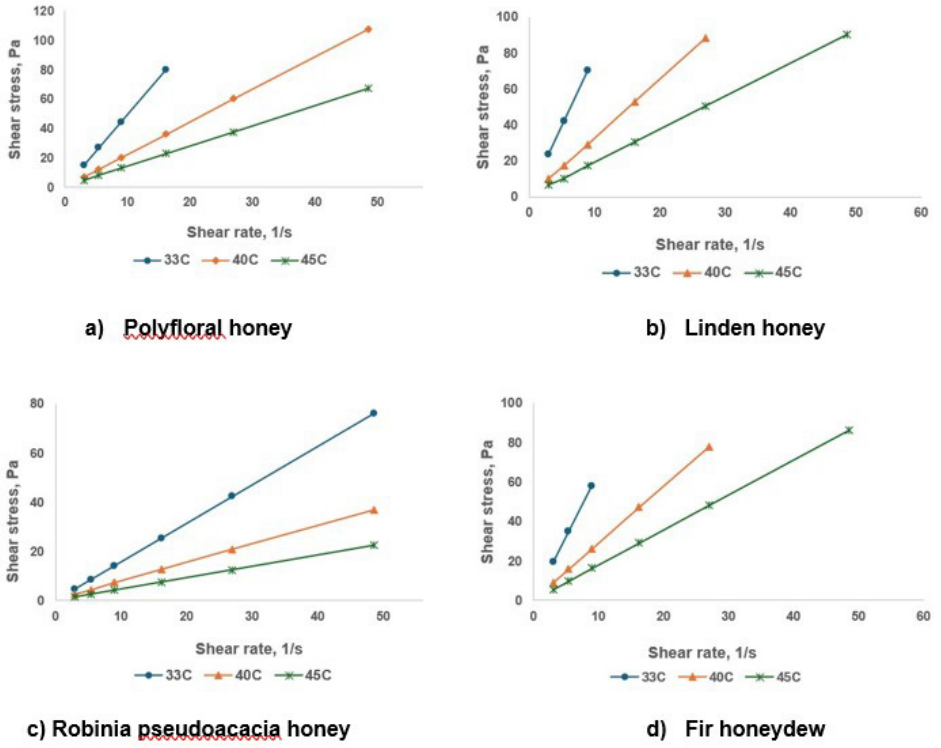

Figure 1. Shear stress vs. shear rate

The results confirm that honey can be used as a bioindicator of environmental pollution, and periodic monitoring of heavy metals is necessary to ensure food safety [43].

Rheological behavior

The dependence between shear stress (t=) and shear rate ($\dot{\gamma}$), at three temperature values, is presented in Figures 1 (a-d). It is observed that, regardless of temperature values, these dependences are linear, which suggests a Newtonian behavior. Also, the values of dynamic viscosity decrease with increasing temperature.

The experimental data were fitted using the software package Table Curve 2D. Thus, the model used to describe the rheological properties of honey samples is defined by Newton's equation (1):

$$\tau = \eta \cdot \dot{\gamma} \tag{1}$$

where η is the dynamic viscosity of the fluid [22, 44-46].

The values of dynamic viscosity are presented in Table 9.

Table 9. The values of dynamic viscosity of the honey samples

Honey	Dynamic viscosity, Pa.s		
	33°C	40°C	45°C
Polyfloral	4.94	2.23	1.38
Linden	7.79	3.26	1.84
Robinia pseudoacacia	1.57	0.75	0.46
Fir honeydew	6.46	2.89	1.78

The Arrhenius model (eq. (2)) has been that most often used to adequately describe the dependence of viscosity on temperature [22,44].

$$\eta = A \cdot e^{\frac{E_a}{R \cdot T}} \tag{2}$$

where E_a is the activation energy reflecting the sensitivity of viscosity to temperature variation and the pre-exponential factor (A) represents viscosity at a temperature close to infinity.

The particular form of equation (2) for the honey samples is presented in Table 10, together with the percentage of decrease in dynamic viscosity with increasing temperature from 33°C to 45°C.

Table 10. Particular forms of Arrhenius type equation

Honey	Eq. (2)	E_a, kJ/mol	Viscosity decrease, %
Polyfloral	$\eta = 7.2 \cdot 10^{-15} \cdot exp(^{10454}/_{T})$	86.9	72.1
Linden	$\eta = 2.3 \cdot 10^{-16} \cdot exp(^{11647}/_{T})$	96.8	76.4
Robinia Pseudoacacia	$\eta = 1.4 \cdot 10^{-14} \cdot exp(^{9888.3}/_{T})$	82.2	70.7
Fir honeydew	$\eta = 6.5 \cdot 10^{-15} \cdot exp(^{10567}/_{T})$	87.8	72.5

The values of the activation energy of the viscous flow are relatively close. However, from these values and the percentage of viscosity decrease, it is confirmed that the linden honey with the highest activation energy value exhibits the greatest change (decrease) in viscosity with temperature.

It is also confirmed that for linden, polyflora and Robinia pseudoacacia honeys, increasing the moisture content leads to a decrease in viscosity, regardless of the temperature value, according to [25].

DSC analysis

For honey, DSC analysis can be used to study the melting point or crystallization phenomena and to determine the glass transition temperature (T_g) [46-49]. Because honey is normally used below its melting point, it is a supercooled liquid and, although it appears liquid, it is in a metastable state in which sugar crystals can spontaneously form. Generally, honey has glass transition temperature values between -30°C and -50°C. Below these values, it is in a vitreous state, becoming an amorphous, non-crystalline solid [50].

DSC thermograms are presented in Figures 2 (a-e) and the values of glass transition temperature (T_g) in Table 11.

It is observed that all the glass transition temperatures have negative values from -32°C to -46°C, what is typical for natural honeys with normal moisture content (15÷20%). The values show that Robinia pseudoacacia honey has the lowest T_g value and the highest water content (Table 1), which denotes a high fructose content, and therefore, the honey has a low tendency to crystallize.

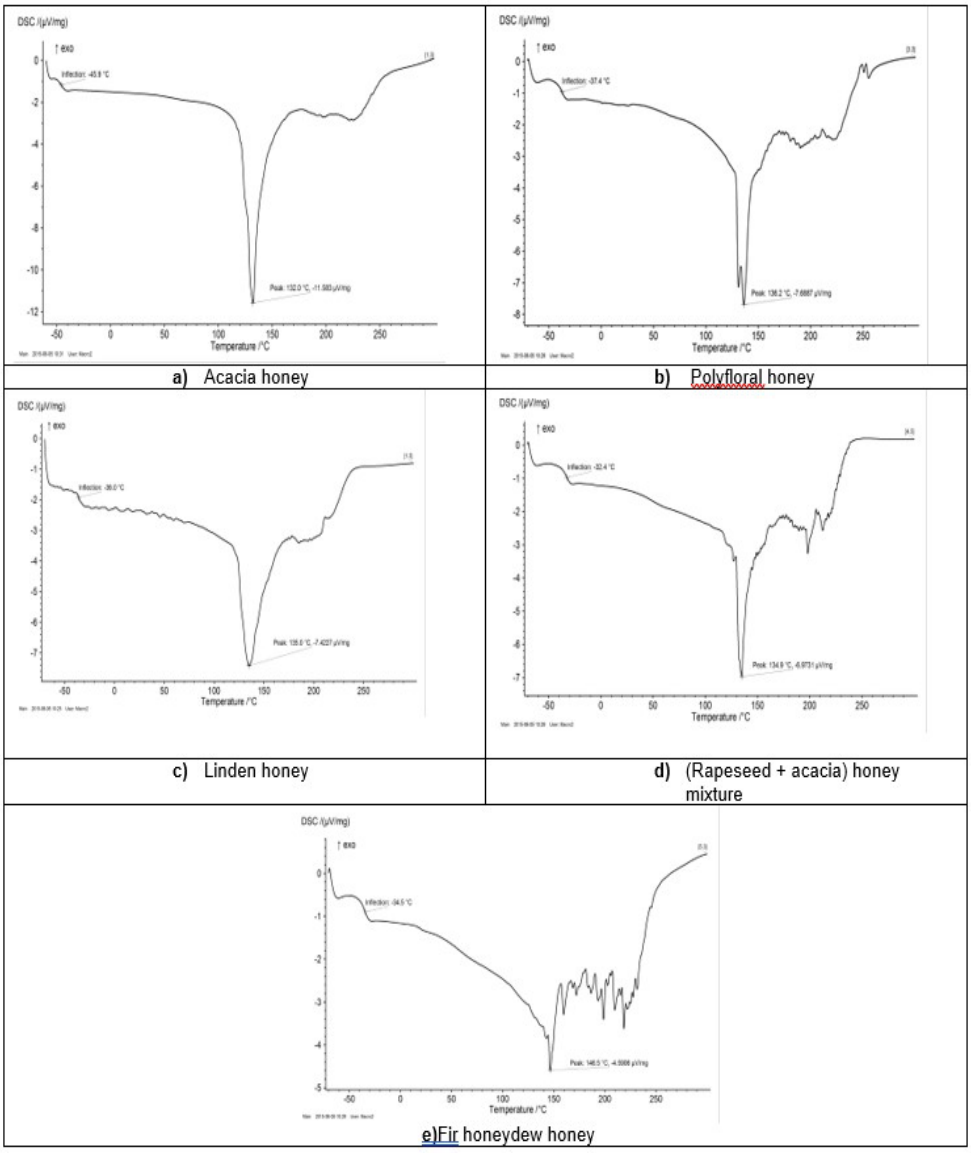

Figure 2. DSC thermograms of honey samples

Table 11. The values of glass transition temperatures

Honey	**T_g, °C**
Robinia pseudoacacia	- 45.9
Linden	- 36.0
Polyfloral	- 37.4
Fir honeydew	- 34.5
Rapeseed + Robinia pseudoacacia	- 32.4

In contrast, the honey mixture (rapeseed + Robinia pseudoacacia) has the highest T_g value (-32.4°C), suggesting a more rigid amorphous structure. Rapeseed honey is typically glucose-rich and prone to rapid crystallization, which is consistent with a higher T_g value and reduced molecular mobility of the supercooled sugar phase. The increased T_g value observed in the blended sample indicates that the presence of rapeseed honey significantly influences the thermal behavior of the blend, despite the contribution of Robinia pseudoacacia honey. Linden, polyfloral and fir honeys exhibited intermediate T_g values (-36°C to -34°C), reflecting more balanced fructose-glucose ratios and heterogeneous botanical composition.

In addition, in all cases, an endothermic peak appears in the range (130÷145)°C, corresponding to thermal decomposition (in fact, rather the decomposition of sugars which are the major constituents, honey being a supersaturated sugar solution).

CONCLUSIONS

Five honey samples from the Oltenia region were studied in terms of physico-chemical parameters.

The obtained results indicated the following:

· The Newtonian behavior observed across all samples indicates a predictable and consistent flow, enhancing consumer experience through uniform texture, ease of pouring, and stable sensory attributes. This rheological linearity also serves as a physical marker of product homogeneity and purity, confirming the absence of advanced crystallization or structural additives

· The carbohydrate composition, characterized by high invert sugar levels and low sucrose contents, demonstrates the authenticity, proper maturation, and overall quality of the analysed honey samples.

· In all samples, the ash content is low, demonstrating the preponderance of the organic phase.

· The content of micro- and macrominerals does not negatively influence the quality of honey. However, a slightly higher lead content indicates a high degree of pollution, due to the natural background of the soil, industrial areas or other factors.

· The DSC data classify the studied honeys in the value ranges mentioned in the literature.

EXPERIMENTAL SECTION

Materials

The five honey samples produced from different floral origins were purchased from beekeepers in the Oltenia region, Romania, in 2023. The honeys were stored at 16÷18°C throughout the entire period of the experimental determinations.

Determination of moisture content

The moisture content of honey samples was obtained by measuring the refractive index (Method 969.383) according to [51] with a Carl Zeiss 16531 refractometer at 20°C, using refractive index of distilled water as a reference. The refractive index was converted to moisture content (%) based on a Chataway Table [3,52].

Determination of pH, free and total acidities and lactone content

pH measurements were performed potentiometrically at 20°C, using a MultiMeter MM 41 in a solution prepared by dissolving 10 g of honey in 75 mL of CO_2-free distilled water. All measurements were performed in triplicate after a preliminary calibration at pH 3.0, 7.0 and 9.0 [3]. The content of free acids and lactone was determined by a titrimetric method. The previously prepared solution was titrated with 0.05 M NaOH solution to pH 8.3 (free acidity). Immediately, 10 mL of 0.05 M NaOH solution was added and the mixture was titrated again with 0.05 M HCl solution to pH 8.3 (lactone acidity) [53]. Total acidity was obtained by adding the two values.

Determination of proline

Proline content was determined using ninhydrin spectrophotometric method (Method 979.20) according to [51]. This method is based on the reaction of proline (a proteinogenic amino acid) with ninhydrin, with the formation of a colored complex with a characteristic maximum at 520 nm [3].

a) Calibration curve for proline: 0.0, 0.1, 0.2, … 0.9 mL of proline standard solution (0.05 mg/mL) are introduced into 10 test tubes to which distilled water is added up to 5.5 mL. To each test tube is added 1 mL of 3% ninhydrin solution in ethylene glycol and 0.25 mL of formic acid. The samples were kept in a boiling water bath for 15 minutes, then cooled for 5 minutes in a water bath at 22°C. 5 mL of solvent (2-propanol and water, ratio 1:1) was added to each sample under stirring. After 35 minutes, the absorbance is determined at 520 nm with a blank containing 0.5 mL of distilled water.

b) Proline determination: 2.5 g honey are transferred to a 50 mL volumetric flask with distilled water to obtain working solutions. 0.5 mL of sample, 5 mL of distilled water, 0.25 mL of formic acid and 1 mL of 3% ninhydrine solution in ethylene glycol are placed in a test tube (triplicate for each honey sample). Each sample is treated according to the instructions above, and the results are calculated as an average value.

Determination of hydroxymethylfurfural - HMF (Winkler method)

HMF forms with barbituric acid, in the presence of p-toluidine, a red compound that exhibits a characteristic absorption maximum at 550 nm.

5 g of honey were weighted and transferred to a 25 mL volumetric flask. The appropriate volume of distilled water was added and homogenized. 2 mL of the honey solution prepared above were transferred to 2 test tubes. 5 mL of p-toluidine solution (10% in 2-propanol) was added to each test tube. 1 mL of distilled water was added to the control tube, and 1 mL of 0.5% aqueous solution of barbituric acid was added to the other tube and homogenized. After 4 minutes, the absorbance was determinated at 550 nm [3, 54].

Determination of invert sugar and sucrose (Elser method)

The method is based on the fact that the reducing sugars (glucose and others) in honey can reduce Cu (II) at Cu (I) in the presence of heat. The amount of red precipitate of copper (I) oxide (Cu_2O) is proportional to the invert sugar content in the sample [36].

a) *Reducing sugar direct before inverting (RSDBI)*: 20 mL Fehling I solution, 20 mL Fehling II solution and 20 mL water are added in a 250 mL Erlenmeyer. The mixture is brought to boiling. The honey stock solution was prepared by diluting about 3 g honey with distilled water in a 200 mL volumetric flask. 20 mL of honey work solution (prepared by diluting 20 mL of stock solution in a 100 mL volumetric flask with distilled water) is added to the previously boiling mixture and boiled for another 5 minutes. To dissolve the red precipitate (Cu_2O), 35 mL of saturated acidified NaCl solution and 2-3 g of $NaHCO_3$ were used to alkalinize the mixture (finally, the mixture becomes clear blue with $NaHCO_3$ crystals). The solution is finally titrated with 0.05 N iodine solution. Since the chemical reaction occurs slowly and the equivalence point is difficult to observe, a small excess of iodine is used and back-titrated with 0.05 N $Na_2S_2O_3$ solution in the presence of a 1% starch solution until the colour turns light blue (the change is green – dark blue – light blue). The effective volume of iodine solution is the difference between the initial and final volumes used in the titration.

$$RSDBI(\%) = \frac{m \cdot 10 \cdot 5}{m_1 \cdot 1000} \cdot 100 \quad (3)$$

where: m – the amount of sugar corresponding to the volume of iodine, mg [4,55]; 10 – volumetric ratio between stock solution and working solution (200/20); 5 – volumetric ratio between working solution and sample solution (100/20); m_1 – the amount of honey sample, g.

b) *Sucrose determination*: in a 100 mL volumetric flask, 20 mL of working solution, 30 mL of distilled water and 1.0 mL of HCl solution were added and maintained for 30 minutes in a boiling water bath for sucrose hydrolysis. To the cooled mixture was added 1.0 mL of 1 N NaOH solution and the volume was adjusted with distilled water. The reducing sugar direct after inverting (RSDAI) was determined as before, using the final mixture as a working solution.

$$Sucrose(\%) = [RSDAI(\%) - RSDBI(\%)] \cdot 0.95 \quad (4)$$

0.95 represents the ratio between the molar mass of sucrose and the molar mass of glucose and fructose.

Determination of ash content

The ash content was determined according to the method of incineration of honey samples (Method 920.181) according to [51].

To remove moisture and prevent foaming, about 5 g of honey was preheated with an infrared lamp, after which the sample was incinerated in a muffle furnace at 600°C, until constant weight. The sample was weighed again after cooling to room temperature [29].

Determination of metal content

For sample preparation, 5 g of honey was weighed and incinerated at a temperature of 450°C for 13 hours. The resulting ash was dissolved in 10 mL of 0.5 M HNO_3, quantitatively filtered through filter paper and brought to a final volume of 100 mL. The content of macrominerals, microminerals, and heavy metals in honey was determined by inductively coupled plasma mass spectrometry (ICP-MS), using a BRUKER Aurora M90 inductively coupled plasma spectrometer equipped with a mass detector.

Rheological behavior

Viscosity measurements were performed on honey samples at different temperature values (33°C, 40°C and 45°C), with a Rheotest-2 rotational viscometer equipped with a thermostatic control bath and a coaxial cylinder system (S/S1). Experimental determinations were performed at the shear rate ramp-up (from 3 s^{-1} to 81 s^{-1}) and ramp-down (from 81 s^{-1} to 3 s^{-1}). Each measurement was taken in duplicate.

DSC analysis

DSC analysis were performed using a DSC 204F1 Phoenix differential scanning calorimeter produced by Netzsch. Honey samples were placed in aluminum crucibles and heated from -50°C to 300°C with a heating rate of 10 K/min. Thermograms were interpreted using Netzsch Proteus Thermal Analysis software version 6.1.0.

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