

## APPLICATION OF SPECTROSCOPY IN ANALYSIS OF SOME FLORAL HONEY SAMPLES COLLECTED FROM FOUR SOUTH ROMANIAN COUNTIES IN 2021

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### Abstract

Honey is a concentrated aqueous solution of sugars, especially glucose and fructose, and minor amounts of dextrin, enzymes, volatile oils, organic acids, ethers, and minerals. Honey samples vary in quality according to various factors, such as climate diversity, geographical characteristics, floral supply period, and packaging and storage conditions, which can compromise the quality of the hive's final product. The assessment of honey quality is a major concern that has gained interest internationally because honey has always been subjected to various fraudulent practices which become more sophisticated in the present day. Consumers, beekeepers, and regulatory bodies are interested to have reliable analytical tools and information to allow the detection of adulterated honey. The UV-VIS spectroscopy and FTIR spectroscopy techniques are two analytical methods used to analyze the honey quality nowadays, with hydroxymethylfurfural being used as a standard for testing honey's freshness. The present study was conducted to assess the quality of some floral honey samples (rapeseed, acacia, and polyfloral honey) produced in Argeș, Călărași, Giurgiu, and Teleorman counties from the south of Romania. Differences between honey botanical and geographical origins were analyzed and highlighted with One-way ANOVA ( $p < 0.05$ ), followed by Duncan's Multiple Range Test. Our results indicated that honey is a valuable source of bioactive compounds.

**Keywords:** honey quality, UV-VIS spectroscopy, FTIR spectroscopy, hydroxymethylfurfural, sugars, polyphenols, flavonoids, tannins

### 1. INTRODUCTION

Containing about 200 compounds, such as phenolic compounds, vitamins, enzymes, amino acids, and minerals, with the major content being water and sugars (Pasupuleti et al., 2016) that act synergistically, honey has antioxidant, anti-inflammatory, anti-bacterial, anti-diabetic, and anti-cancer well-documented character (Erejuwa et al., 2012; Moniruzzaman et al., 2012). Even their minor constituents, such as enzymes, are involved in a wide range of biological activities, among which antimicrobial activity and calcium absorption were reported (Ariefdjohan et al., 2008). Many studies (Habib et al., 2014; Can et al., 2015; Flores et al., 2015) have reported that the antioxidant capacity of honey is dependent on total phenolic compounds, especially flavonoids which play an important role in ameliorating oxidative stress. However, the honey composition varies depending on the plant species or cultivar, climatic conditions, and geographical region (Vit et al., 2015).

FTIR spectroscopy is one of the most widely used methods to identify chemical constituents and elucidate the structure of the compounds. Owing to the fingerprint characters and extensive applicability to the samples, FTIR has played an important role in plant analysis in recent years. Analysis of honey with infrared spectroscopy can be used to determine the crystalline structure, the purity (Martos et al., 2000; Kędzierska-Matysek et al., 2018), and to classify the honey samples (Sivakesava and Irudayaraj, 2001). FTIR spectra of honey samples may significantly differ with some wavelength ranges, due to the different bands overlapping (in samples) due to the presence of constituents like polysaccharides, water, and many other pollutants.

The aim of this study was to assess the quality of some floral honey samples (rapeseed, acacia, and polyfloral honey) produced in Argeş, Călăraşi, Giurgiu, and Teleorman counties from the south of Romania, using both UV-VIS and IR spectroscopy.

## 2. MATERIALS AND METHODS

In 2021, the following nine samples of Romanian honey types were collected directly from three beekeepers: acacia (A), three samples, rapeseed (R), two samples, and multifloral (M), four samples. The honey samples were collected from four Southeastern Romanian counties (Figure 1).

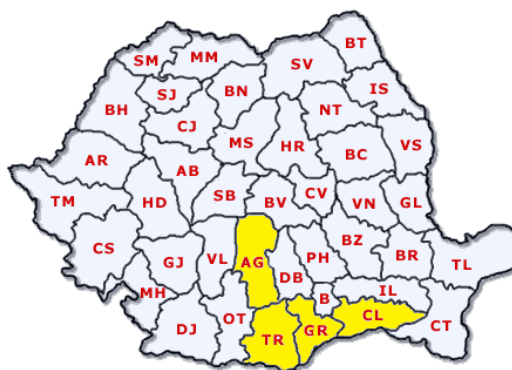


Figure 1. Geographic regions of collected honey samples (Argeş County; Călăraşi County; Giurgiu County; Teleorman County)

The acacia honey samples were collected from Argeş - Poienari (AG-P), Călăraşi - Luica (CL-L), Teleorman - Alexandria (TR-A). The rapeseed honey samples were collected from Călăraşi - Frumuşani (CL-F), Teleorman - Alexandria (TR-A). The multifloral honey samples were collected from Argeş - Boţeşti (AG-B), Costeşti (AG-C), Piteşti (AG-P), Giurgiu - Bolintin (GR-B).

*5-hydroxymethylfurfural* (HMF) expressed in mg HMF/kg honey was determined colorimetrically in aqueous honey solutions with 20 % dry matter by the methodology suggested by White, 1979.

*Total sugars content* (TSC) expressed as g glucose (Glu)/100 g was determined colorimetrically by the methodology suggested by Dubois et al., 1956.

*Total polyphenols content* (TPC) expressed as mg gallic acid equivalent (GAE)/100 g was determined colorimetrically according to the methodology suggested by Matić et al., 2017.

*Total flavonoid content* (TFC) expressed as catechin equivalent (CE)/100 g was determined colorimetrically by the methodology suggested by Tudor-Radu et al., 2016.

*Total tannin content* (TTC) expressed as mg gallic acid equivalent (GAE)/100 g was determined colorimetrically according to the methodology suggested by Giura et al., 2019.

Measurements were made with a UV-VIS spectrophotometer PerkinElmer Lambda25 and an FTIR spectrometer Jasco 6300. An ATR accessory equipped with a diamond crystal (Pike Technologies) allows the collection of FTIR spectra directly on a sample without any special preparation.

The FTIR spectra were recorded in the region of 4000-400  $\text{cm}^{-1}$ , detector TGS, and apodization Cosine. Samples were scanned at 4  $\text{cm}^{-1}$  resolution, accumulation of 100 scans. Background reference spectra were recorded using air after every sample to minimize the interference due to carbon dioxide and water vapor in the atmosphere. Between measurements, the ATR crystal was carefully cleaned using pure acetone (Sigma-Aldrich Co.) and then dried with soft tissue. All measurements were taken at room temperature. Infrared Spectra were exported from Spectra Manager, in ASCII (dx) format, into the Unscrambler Software (Edition X 10.4, Camo. Oslo Norway) for chemometric analysis. Spectra were preprocessed using the second-derivative transformation, the Savitzky-Golay derivation. The use of spectra derivatives with the Savitzky-Golay algorithm as a chemometric pre-processing technique is widely reported in most classifications based on FTIR spectroscopy (Chatfield and Colins, 1980; Topală et. al., 2020). The principal component analysis (PCA) model was developed using cross-validation. PCA was performed both on the entire spectral range (4000 to 400  $\text{cm}^{-1}$ ), and on the MIR 'fingerprint' (1700 to 750  $\text{cm}^{-1}$ ), Validation: Cross Validation, Algorithm: Singular Value Decomposition (SDV). Honey quality data were statistically analyzed. One-way analysis of variance and Duncan's Multiple Range test were used ( $\alpha=95\%$ ).

### 3. RESULTS AND DISCUSSIONS

The descriptive analysis of the data (Table 1) indicated that in acacia, multiflora, and rapeseed honey, collected from the four counties in the southern part of Romania, the HMF content showed an average value of 3.6 mg/100 g, while the average sugar level was 62.47 g Glu/100 g, and showed the lowest variability (CV 10.02%). As can be observed, the highest coefficient of variation (CV 37.92%) was determined in the case of TPC, whose level oscillated around 89.62 mg GAE/100 g. Of the total phenolic compounds, flavonoids and tannins accounted for about 16.3% and 73.6% and showed mean levels of 14.57 mg CE/100 g and 65.95 mg GAE/100 g, respectively.

*Table 1. Statistical indicators for hydroxymethylfurfural, sugar, total phenolic compounds, total flavonoid, and total tannin in determined in honey*

	HMF (mg/100 g)	TSC (g Glu/100 g)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	TTC (mg GAE/100 g)
<b>Mean</b>	3.60	62.47	89.62	14.57	65.95
<b>Std. Deviation</b>	0.66	6.26	33.99	2.92	21.71
<b>Variation coefficient %</b>	18.27	10.02	37.92	20.01	32.92
<b>Range</b>	2.07	17.14	115.49	8.77	69.85
<b>Minimum</b>	2.22	52.30	55.30	10.87	41.61
<b>Maximum</b>	4.29	69.44	170.79	19.64	111.46

The analysis of the correlations between the components dosed in the honey samples analyzed in this study (Table 2) indicated that HMF was significantly positively correlated with TPC and tannins. In general, the correlation of sugars with phenolic components was negative, and of these,

the correlation with tannins only showed statistical significance. Last but not least, all determined phenolic components correlated positively and very significantly.

**Table 2. The correlation matrix between HMF, TSC, TPC, TFC, and TTC determined in the acacia, multiflora, and rapeseed honey (Pearson correlation coefficients and their significance)**

	TSC (g Glu/100 g)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	TTC (mg GAE/100 g)
HMF (mg/100 g)	-0.279	<b>0.506**</b>	0.320	<b>0.558**</b>
TSC (g Glu/100 g)	1	-0.260	-0.237	<b>-0.402*</b>
TPC (mg GAE/100 g)		1	<b>0.823**</b>	<b>0.913**</b>
TFC (mg CE/100 g)			1	<b>0.921**</b>

\* - significant correlation at 0.000 level

\*\* - significant correlation at 0.010 level

\*\*\* - significant correlation at 0.050 level

As shown in Table 3, HMF fluctuated insignificantly and had the highest concentration in rapeseed honey (3.85 mg/100 g), about 13% more compared to acacia honey. Multiflora honey presented the highest amplitude of HMF oscillation, respectively 2.07 mg/100 g. The total sugar content varied significantly between 60.23 and 68.84 g Glu/100 g, determined for multiflora and rapeseed honey, respectively and the highest TSC oscillation amplitude was observed for acacia honey (13.94 g Glu/100 g). TPC, similar to TFC, was distinctly significantly influenced by honey botanical origin. The highest TPC was recorded for multiflora honey (111.41 mg GAE/100 g), 23.3% and 75.8% more than rapeseed and acacia honey. Moreover, TPC registered the largest oscillations among all the components dosed in the study, and the largest amplitude was in multiflora honey (90.6 mg GAE/100 g).

**Table 3. Influence of the botanical origin on the content of hydroxymethylfurfural, sugar, total phenolic compounds, total flavonoid, and total tannin in the honey samples (mean, standard deviation, and extreme values are shown)**

Botanical origin	HMF (mg/100 g)	TSC (g Glu/100 g)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	TTC (mg GAE/100 g)
Acacia	3.32±0.36 <sup>a*,**</sup>	61.02±6.64 <sup>b</sup>	63.37±10.29 <sup>b</sup>	12.85±2.3 <sup>b</sup>	50.99±9.95 <sup>b</sup>
	(3.01-3.81)	(52.30-66.24)	(55.30-77.03)	(10.87-15.98)	(41.61-63.83)
Multiflora	3.68±0.86 <sup>a</sup>	60.23±5.62 <sup>b</sup>	111.41±37.61 <sup>a</sup>	16.74±2.65 <sup>a</sup>	82.55±22.21 <sup>a</sup>
	(2.22-4.29)	(55.83-69.44)	(80.19-170.79)	(13.13-19.64)	(57.14-111.46)
Rapeseed	3.85±0.41 <sup>a</sup>	68.84±0.09 <sup>a</sup>	85.43±18.03 <sup>ab</sup>	12.81±0.16 <sup>b</sup>	55.2±1.92 <sup>b</sup>
	(3.46-4.24)	(68.69-68.94)	(68.94-101.90)	(12.60-13.03)	(53.44-56.97)
<b>Sig.</b>	0.272	0.011	0.002	0.001	0.000

\*Means and standard deviation are presented

\*\*Means on the column followed by the same letter are not significantly different at 5% level.

Flavonoids showed the lowest concentration in rapeseed (12.81 mg CE/100 g) and were determined in the highest amount also in multiflora honey (16.74 mg CE/100 g), in which the oscillation amplitude was the biggest. The strongest effect of botanical origin was registered on tannins. Tannin maximum level was determined in multiflora honey, exceeding by approximately 38% the

minimum level determined in acacia. Also, similar to TPC and TFC, tannins showed the highest amplitude of oscillation in multifloral (54.3 mg GAE/100 g).

The effect of geographical origin on the analyzed honey samples was highly significant ( $p=0.000$ ), except for TFC ( $p=0.015$ ) and TSC ( $p=0.364$ ), both in rapeseed honey (Table 4). It can thus be observed that the multifloral honey from Argeş was distinguished by its high level of HMF, TPC, TFC, and TTC, while sugars showed a maximum level in the multifloral honey from the GR-B area, surpassing the two rapeseed samples.

**Table 4. Influence of geographical origin on the content of hydroxymethylfurfural, sugars, total phenolic compounds, total flavonoid, and total tannin, depending on honey botanical origin**

Geographical origin	HMF (mg/100 g)	TSC (g Glu/100 g)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	TTC (mg GAE/100 g)
Acacia-AG-P	3.04±0.03 <sup>c**</sup>	52.37±0.06 <sup>c</sup>	57.79±0.03 <sup>b</sup>	10.94±0.07 <sup>c</sup>	41.62±0.01 <sup>c</sup>
Acacia-CL-L	3.13±0.02 <sup>b</sup>	66.17±0.1 <sup>a</sup>	55.31±0.01 <sup>c</sup>	11.74±0.35 <sup>b</sup>	47.53±0.02 <sup>b</sup>
Acacia-TR-A	3.79±0.02 <sup>a</sup>	65.07±0.07 <sup>b</sup>	77.01±0.02 <sup>a</sup>	15.88±0.1 <sup>a</sup>	63.81±0.03 <sup>a</sup>
<i>Sig.</i>	0.000	0.000	0.000	0.000	0.000
Multifloral-AG-B	3.97±0.05 <sup>c</sup>	55.91±0.07 <sup>d</sup>	109.41±0.02 <sup>b</sup>	18.76±0.12 <sup>b</sup>	93.53±0.01 <sup>b</sup>
Multifloral-AG-C	4.29±0.01 <sup>a</sup>	58.88±0.1 <sup>b</sup>	80.21±0.02 <sup>d</sup>	13.24±0.11 <sup>d</sup>	68.08±0.01 <sup>c</sup>
Multifloral-AG-P	4.21±0.01 <sup>b</sup>	56.77±0.1 <sup>c</sup>	170.78±0.02 <sup>a</sup>	19.5±0.15 <sup>a</sup>	111.43±0.03 <sup>a</sup>
Multifloral-GR-B	2.26±0.05 <sup>d</sup>	69.37±0.13 <sup>a</sup>	85.25±0.05 <sup>c</sup>	15.45±0.16 <sup>c</sup>	57.15±0.01 <sup>d</sup>
<i>Sig.</i>	0.000	0.000	0.000	0.000	0.000
Rapeseed-CL-F	4.22±0.02 <sup>a</sup>	68.88±0.07 <sup>a</sup>	101.89±0.02 <sup>a</sup>	12.94±0.09 <sup>a</sup>	56.96±0.02 <sup>a</sup>
Rapeseed-TR-A	3.48±0.02 <sup>b</sup>	68.81±0.1 <sup>a</sup>	68.98±0.03 <sup>b</sup>	12.67±0.08 <sup>b</sup>	53.44±0.01 <sup>b</sup>
<i>Sig.</i>	0.000	0.364	0.000	0.015	0.000

\*Means of three determinations are presented

\*\* Means on the column followed by the same letter are not significantly different at 5% level.

An efficient method of distinguishing the honey samples is based on ATR-FTIR spectra. Characteristic differences were observed for FTIR spectra wavelengths of natural honey.

The O–H stretching vibration band in carboxylic acids and alcohols is very broad and occurs in the field of 3300-2500  $\text{cm}^{-1}$  the maximum at 3000  $\text{cm}^{-1}$  (Nickless et. al., 2016). It represents the same area for the stretching vibration region of carbon and aromatic C–H groups (Svečnjak et al., 2017). The peaks around 2930  $\text{cm}^{-1}$  are characteristics of C–H stretching in carboxylic acids and  $\text{NH}_3$  stretching in free amino acids (Tewari and Irudayaraj, 2004; Gok et. al., 2015).

The Romanian honey samples used in this study presented an absorption band around 1640  $\text{cm}^{-1}$ , due to both water and a small number of protein molecules (Gok et. al., 2015; Bunaciu and Aboul-Enein, 2022), due to the difference in water content, water-carbohydrate interactions and protein content.

The peaks from 1175 to 940  $\text{cm}^{-1}$  corresponding to C–O stretching in carbohydrates, are as follows: 1148  $\text{cm}^{-1}$  was specific to sucrose, 1087  $\text{cm}^{-1}$  and 1043  $\text{cm}^{-1}$  to the presence of glucose and fructose, and 983  $\text{cm}^{-1}$  and 965  $\text{cm}^{-1}$  to fructose (Anjos et.al., 2015; Bunaciu and Aboul-Enein, 2022).



The stretching vibration band of the C=O carboxylic acids group occurs between 1760-1690  $\text{cm}^{-1}$ . The exact position of the band depends on whether the acid is saturated or unsaturated, dimerized or associated, etc.

The contribution of sucrose, glucose, and fructose, show characteristic bands in the region between 1500 and 900  $\text{cm}^{-1}$ . Another important spectral region located at 900-750  $\text{cm}^{-1}$  is characteristic of the saccharide configuration. Authors in many publications define the following spectral peaks as important to sugar characterization (Kędzierska-Matysek et. al., 2018).

Figure 1 shows the comparative infrared spectra of honey samples in the 4000-650  $\text{cm}^{-1}$  region.

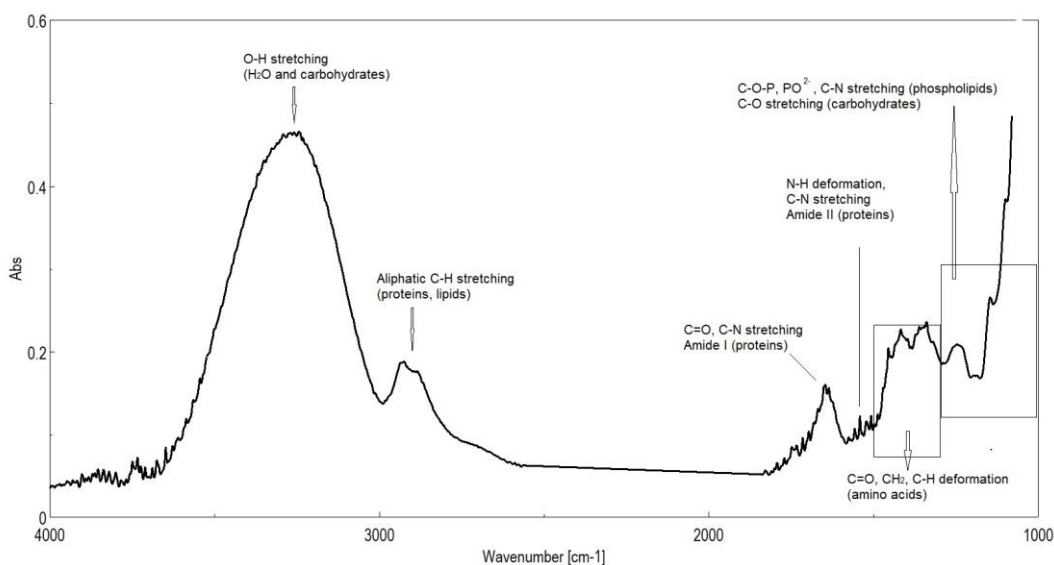


Figure 1. Characteristic FTIR-ATR spectrum of the honey (acacia Calarasi sample)

Figures 2-4 show ATR-FTIR spectra of the tested honey with major high bands.

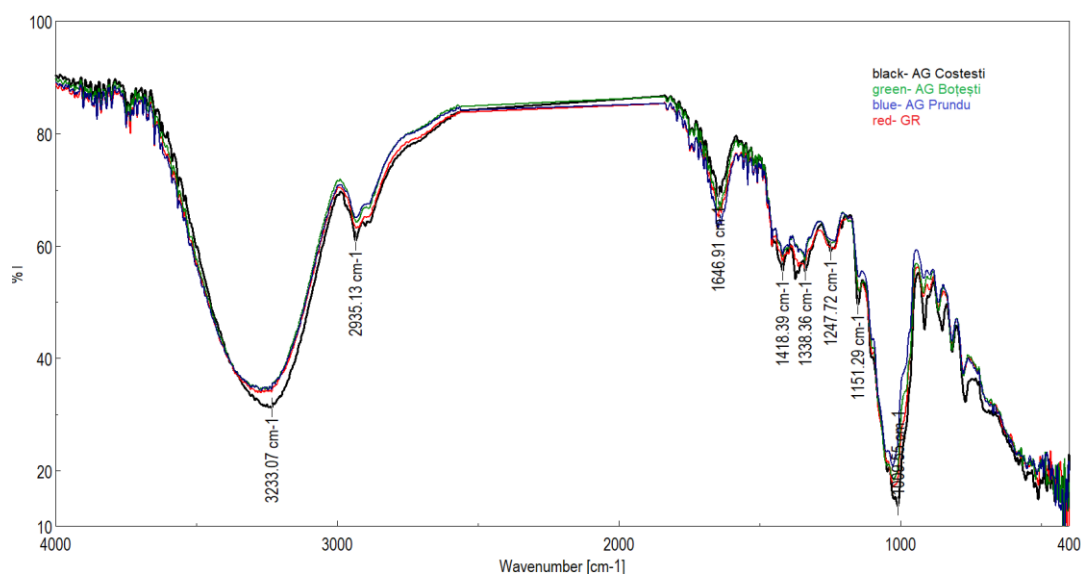
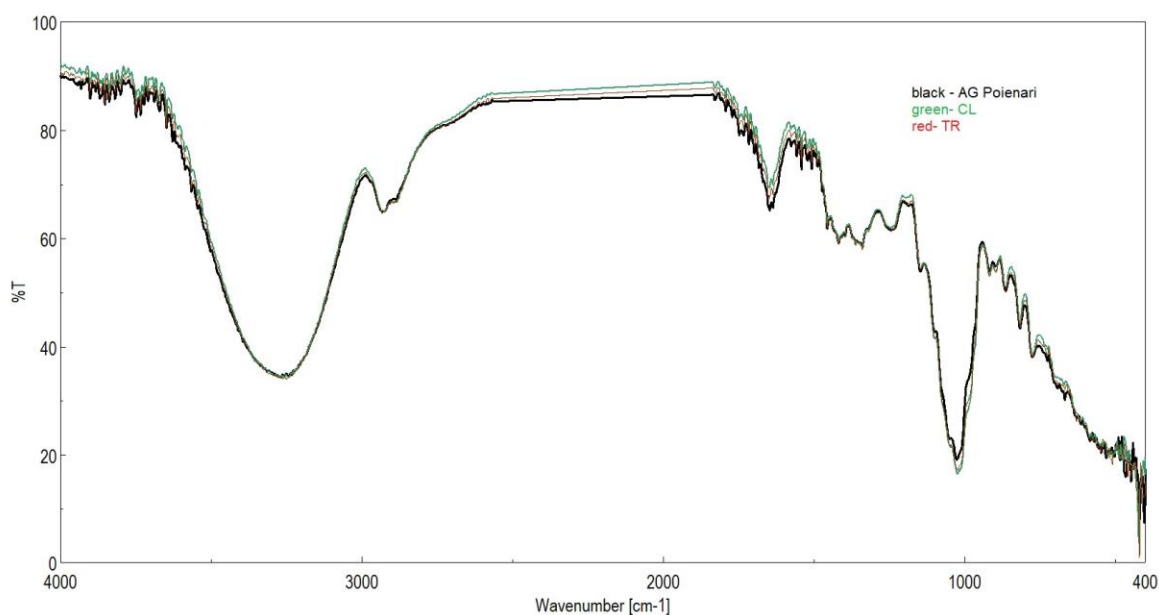
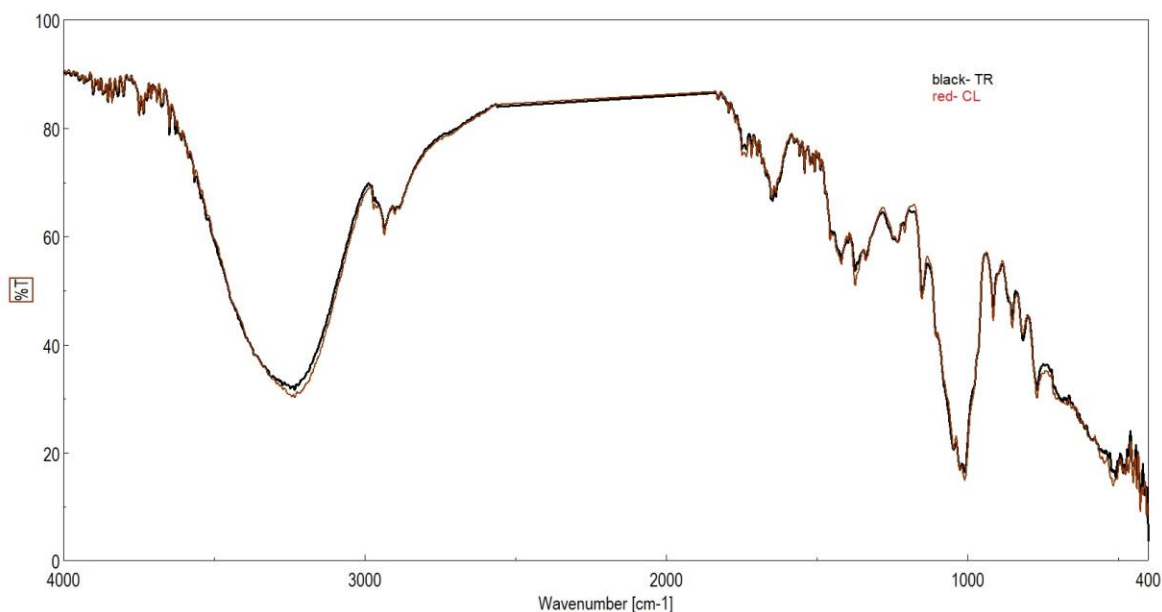


Figure 2. ATR-FTIR spectra of four honey multifloral samples



**Figure 3. ATR-FTIR spectra of three honey acacia samples**



**Figure 4. ATR-FTIR spectra of two honey rapeseed samples**

Table 1 shows the exact position of the bands together with the assignment of relevant vibration to specific functional groups.

For the selected regions 1700-750  $\text{cm}^{-1}$  good results have been seen in discrimination between multifloral honey from acacia (Figure 5).

For the discussed honey samples, 99% of the total variance was represented by the first three PCs (PC1=93%, PC2=4%, and PC3=2%), indicating that these first three components can offer a good delimitation of groups.

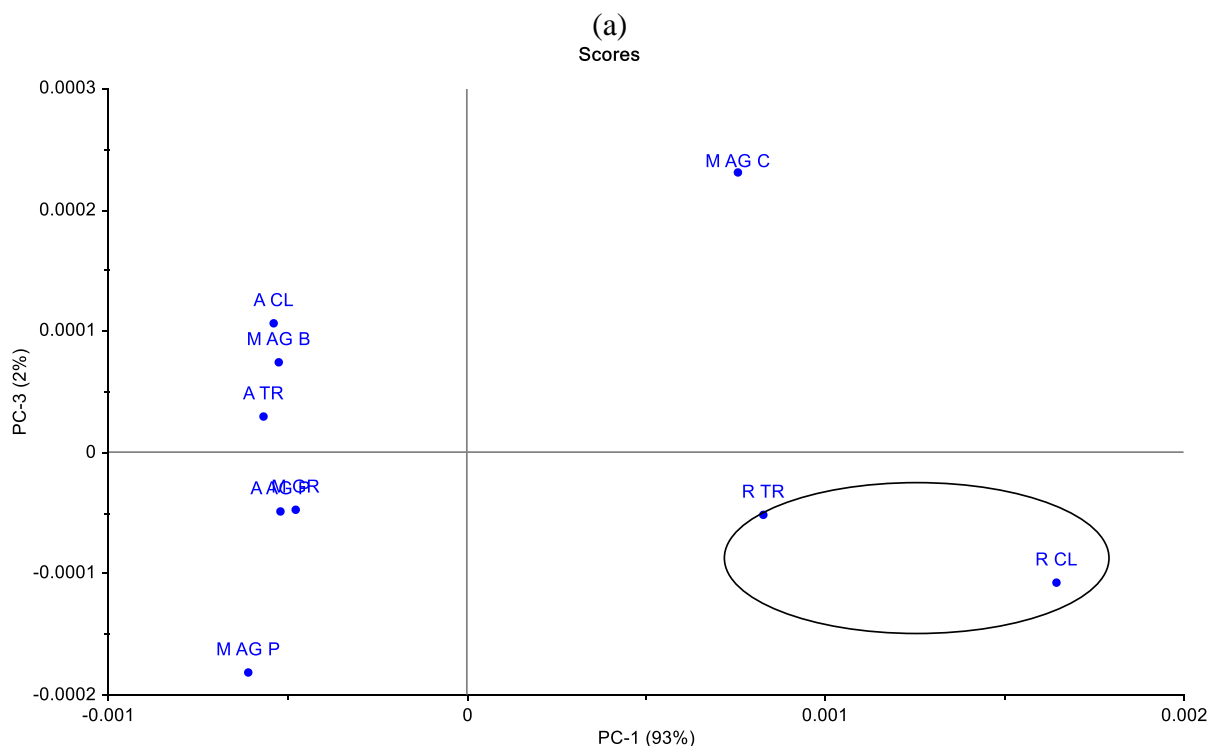
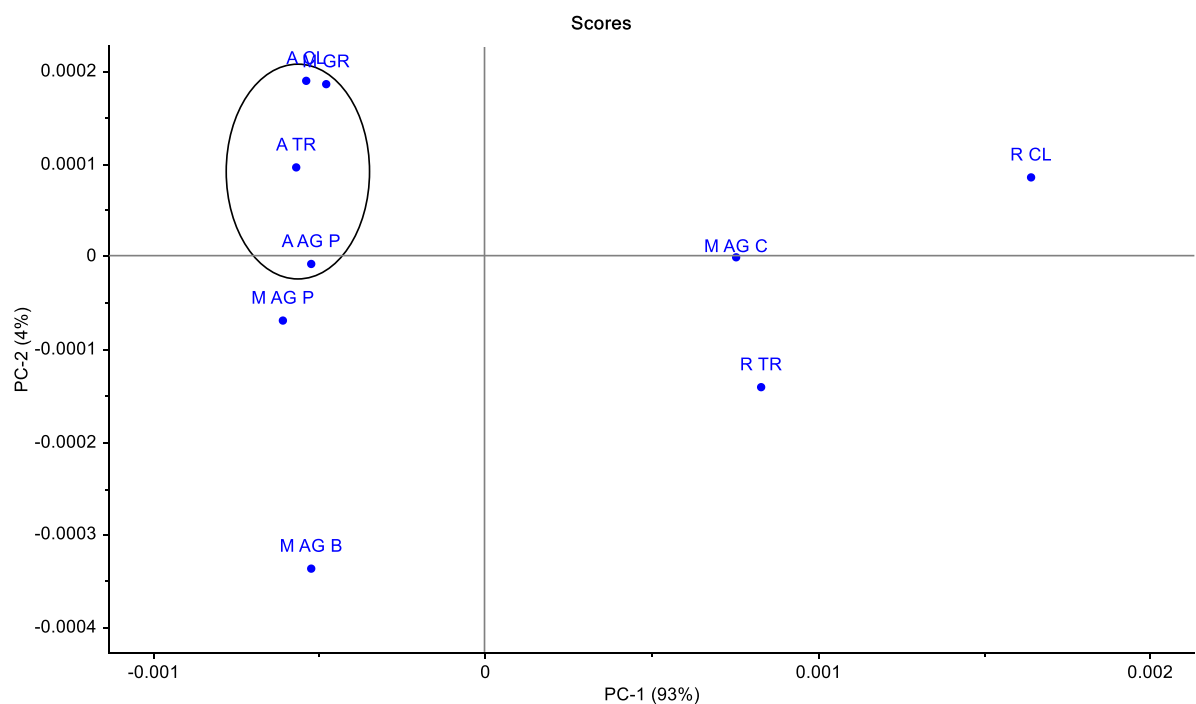


Figure 5. 2-D scores of FTIR PCA spectra for honey: the first two PCs (a), and PC3 versus PC1 (b)



**Table 5. Absorption bands FTIR, together with the assignment of appropriate vibration for selected honey: in terms of spectral 3800-800 cm<sup>-1</sup>**

Position of bands [cm <sup>-1</sup> ]									Type and origin of vibrations
Rapeseed		Acacia			Multifloral				
TR-A	CL-F	AG-P	CL-L	TR-A	AG-C	AG-B	AG-P	GR-B	
3233	3232	3268	3243	3251	3233	3276	3270	3271	v (O-H) in H <sub>2</sub> O
2935	2934	2930	2924	2933	2935	2932	2935	2932	v (C-H) or/and v (NH <sub>3</sub> ) of free amino acids
1646	1647	1645	1646	1646	1646	1646	1646	1646	δ (O-H) from H <sub>2</sub> O
1418	1418	1418	1418	1418	1418	1418	1418	1418	δ (O-H) in the C-OH group + δ (C-H) in the alkenes
1338	1338	1339	1339	1339	1338	1339	1339	1339	δ (-OH) in the C-OH group
1232	1232	1242	1246	1245	1247	1244	1234	1234	v (C-H) in carbohydrates + v (C-O) in carbohydrates
1151	1151	1146	1146	1146	1151	1146	1147	1146	v (C-H) in carbohydrates + v (C-O) in carbohydrates
1105	1105	1105	1102	1100	1102	1102	1100	1100	v (C-O) in the C-O-C group
1046	1046	1025	1024	1024	1043	1024	1027	1024	v (C-O) in the C-OH group + v (C-C) in the carbohydrates
1009	1009	1010	1009	1009	1009	1009	1009	1009	
915	915	916	917	917	914	917	917	917	δ (C-H)
852	851	862	862	863	851	864	864	863	anomeric region of carbohydrates + δ (C-H)

#### 4. CONCLUSIONS

Sugar, phenolic compounds, flavonoids, and tannin levels fluctuated significantly depending on the botanical origin, while the effect of the geographical origin was also significant, except for the sugar in rapeseed honey. Multifloral honey was noted for its high content of phenolic compounds, flavonoids, and tannins. Although the botanical origin highlighted rapeseed honey for its sugar content, the highest level of sugar was determined in multifloral honey from GR-B. The analysis of the geographical origin influence highlighted the multifloral honey from AG-C for its high HMF content.

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