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Physicochemical and Palynological Characterization of the *Onobrychis* Miller (Fabaceae) Honey

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Abstract

The purpose of this study was to contribute toward characterizing the general properties of the *Onobrychis* Miller (sainfoin) honey.We analyzed 21 samples of *Onobrychis* honeys, which were collected in 2013, from Northeast Anatolia according to their microscopic and physicochemical features (fructose/glucose (F/G) ratio, 5-(hydroxymethyl) furan-2-carbaldehyde (HMF) value, and volatile compounds). The relationships were examined between these variables with statistical methods such as principal component, regression, and correlation analyses. Statistical analyses revealed certain correlation patterns between several parameters. *Onobrychis radiata* (Desf.) M. Bieb., *O. tournefortii* (Willd.) Desv., *O. oxyodonta* Boiss. and *Onobrychis* spp. pollens were identified.TPN-10 ranged from 6532 to 481157 while the HMF values were between 0.1–7.5 mg/kg and F/G ratios were between 0.8–1.3. Alcohols, aldehydes, aliphatic acids and their esters, carboxylic acids and their esters, hydrocarbons, flavonoids, ketones, sugars and vitamins were identified in the honey samples within ranges of 0–11.95%, 0–16.76%, 0–24.72%, 0–1.86%, 0–14.71%, 2.74–7.09%, 0–27.02%, 5.12–51.39%, and 0–21.36%, respectively.Although it was not significant, a positive correlation between flavonoids and *Onobrychis* pollen number was calculated via correlation analysis. There was no meaningful correlation between *Onobrychis* pollen number and other parameters. Although a negative trend between flavonoids and sugars was observed, it was not significant. F/G ratio was found to be positively correlated with sugars, HMF, and TPN-10 of which the latter two parameters had significant values. Furthermore, a significant positive correlation between TPN-10 and HMF was also detected.

1. INTRODUCTION

Honey is a natural food product processed by honeybees by blending the sweetened sap collected from flowers with metabolic gastric enzymes [1]. Honey contains particular components such as carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen, and wax. These components' source can be plants or honey bees [2].

The pollen grains found in honey can be derived from the foraging behaviour of honey bees (sucking up the nectar contaminated with pollen or dropping the pollen grains attached to her body into the honey combs accidentally), attitudes of beekeepers (inattentive honey harvest) or just from the air (pollen grains of anemophilous plants) [3]. Therefore, honey always includes numerous pollen grains that provide relevant information about the environment where the honey originated. Melissopalynology can be useful tool to

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determine the origin of a specific honey, which can also be attributed as the fingerprint of the honey; nevertheless, sensory and physicochemical analyses are also needed for a better identification [4].

Heating or storing honey for a long time can cause the decomposition of monosaccharides or the Maillard reaction in the honey. 5-(hydroxymethyl) furan-2-carbaldehyde (HMF) (a toxic compound depending on its concentration) is a product of the Maillard reaction [5]. According to international standards of the Codex Alimentarius (2001), the HMF content of honey should be below 40 mg/kg [6].

The sugar content of honey mainly depends on the composition of nectar collected by bees and the micro- and macro-environmental conditions of the hives. The concentration of monosaccharides (glucose and fructose) and the ratios between them are key characteristics for the classification of honey [7]. The fructose/glucose (F/G) ratio should be between the values of 0.9–1.4 according to the Codex Alimentarius (2001) and Turkish Food Codex Honey Notification (No: 2012/58) (2012) [6,8].

The studied honey samples were collected from Kars in Northeast of Turkey which belongs to Irano-Turanian phytogeographical region. The area is a pass between Caucasia and Anatolia, and a large part of this territory is on a plateau with an altitude ranging from 1500 to 2000 m [9]. A semi-arid and wet climate condition, which is defined as terrestrial, is dominant in this area [10]. A long period of winter season and a short and rainy summer season are key characteristics of this climate.

Onobrychis Miller is a Eurasian perennial herb that belongs to the Fabaceae family and includes about 170 species, of which 23 species are found in Europe. This genus is mainly distributed in Southwest Asia, the Mediterranean region, and in temperate Europe and Asia [11,12]. There are 55 species in Turkey, and 28 of them are endemic [13-18] The genus *Onobrychis* is a taxonomically difficult group in Anatolia, one of the main diversity centres of the genus [14]. These plants grow on grassland, agricultural land, and wasteland. In addition, they are major sources of food for farm animals and have potential for land conservation and rehabilitation [19,20].

The flowering period of the genus is between June and September and pollinated by honeybees and solitary bees. Sainfoin flowers are highly attractive for entomophily because they produce large amounts of pollen and nectar [21]. Knuth indicated that the cross-pollination of sainfoin flowers is required because automatic self-pollination does not occur [22]. Kropacova found that seed production in sainfoin is primarily dependent upon bee pollination [23].

There have not been any detailed studies on the *Onobrychis* honey in this region or in Turkey. Previous studies generally involved microscopic analysis [24, 25]. However, the aim of this study was to characterize and identify a fingerprint for the *Onobrychis* honey. For this purpose, pollen determination and physicochemical analyses were performed to identify the characteristics of the *Onobrychis* honey. Statistical analyses were also conducted to examine the relationships between variables.

2. MATERIAL AND METHODS

2.1. Honey samples

In the summer season of 2013, 21 honey samples were collected from Kars province (Northeast Anatolia in Turkey). Samples were stored at room temperature (22 ± 2 ° C) until analysis time.

2.2. Botanical Origin Determination of Honey Samples by Microscopic Analysis

The botanical origin of honey samples were determined by the melissopalynology. The materials were prepared for microscopic examination according to the Louveaux method [26].Pollen types were evaluated in three categories: predominant (D), secondary (S) and minor important (M). The key character is the percentage of the determined pollens. When one pollen type represented > 45% of the total number of pollen grains, the sample was classified as a monofloral honey [27]. After pollen identification of the honey

samples, the total pollen number in 10 g of honey (TPN-10) for each sample was calculated as described by Moar [28].

2.3. Headspace-Solid Phase Microextraction (HS-SPME)

The honey samples were prepared for HS-SPME by conducting the methodology of Wolski et. al [29].

2.3.1. Gas Chromatography-Cass Spectrometry (GC-MS)

A GC (Agilent 6890N Network GC System-G1530N-USA) combined with a mass detector (Agilent 5973 Mass Selective Dedector- G2577A-USA) was used for the chemical analysis of honey samples. A DB 5MS column (30 m × 0.25 mm, 0.25 μm) was used for the analysis, and the mobile phase was helium gas (He). The flow rate of the mobile phase was set at 1 mL/min. In the GC part, the initial oven temperature was 35 °C for 8 min, and the final temperature was set at 200 °C [30]. Organic compounds in the honey samples were identified in the Mass Spectral Library of Wiley and NIST. The values of the identified compounds in the honey extracts were reported as percentages to quantify most of the organic compounds in the samples.

2.4. Sugar Analysis by High-Performance Liquid Chromatography (HPLC)

Sugar analyses were performed according to the harmonized methods of the International Honey Commission [31]. Initially, 5 g of honey was weighed into a beaker and dissolved in 40 mL water. Then, 25 mL of methanol was added into a 100-mL volumetric flask, and the honey solution was transferred quantitatively to the flask. The flask was filled up to the mark with water, and the solution was poured through a 0. 45 μm membrane filter and collected in sample vials. The prepared solutions were analysed by HPLC with a refractive index detector (HPLC-RID) using a carbohydrate column [31].

2.5. Determination of HMF by HPLC

HMF analyses were performed according to the harmonized methods of the International Honey Commission [31]. Initially, 10 g of honey was weighed in a 50-mL beaker. Then, 25 mL of water was added and transferred quantitatively to a 50-mL volumetric flask. The solution was diluted with 50 mL water. After mixing the solution, it was filtered through a 0.45-μm membrane filter to produce a sample solution ready for chromatography. The samples were analyzed by HPLC with UV detector and C18-reversed phase column.

2.6. Statistical Analyses

Principal component analysis (PCA) was performed to exclude certain irrelevant physicochemical parameters (Table 2 and 3) from correlation analysis; 13 parameters (*Onobrychis* pollen numbers, alcohols, aldehydes, aliphatics, hydrocarbons, flavonoids, carboxylic acids, ketones, sugars, vitamins, F/G, HMF, and TPN-10) were examined. Because of the different measurements and zero values of the parameters, Box-Cox transformation was applied to the data before PCA. Based on PCA results, *Onobrychis* pollen numbers, flavonoids, sugars, F/G, HMF, and TPN-10 were selected as the most stable parameters for correlation analysis. Subsequently, these data were also analyzed via linear rank correlation analysis to examine the relationships between the variables. Spearman's rank correlation coefficient was used for analysis, a non-parametric method suitable for a poor sample size ($n < 30$). Correlation analyses were performed by using PAST software [32], and the significance was calculated for $P < 0.05$ and $P < 0.01$. The results were expressed both in tables and as multivariate linear regression graphs derived from the linear model analysis of PAST.

3. RESULTS AND DISCUSSION

3.1. Microscopic Analysis Results

During this study, 21 honey samples that were considered as unifloral were analyzed. *Onobrychis* was the dominant pollen source in all samples (Table 1). *Onobrychis radiata* (Desf.) M. Bieb. (n=21), *Onobrychis tournefortii* (Willd.) Desv. (n=14), *Onobrychis oxyodonta* Boiss. (n=9), and *Onobrychis* spp. (n=7) pollens were identified. Pollens from *Echium vulgare* L. (in sample 11), *Medicago sativa* L. (in sample 12), *Nigella arvensis* L. (in sample 15), *Lotus corniculatus* L. (in samples 17 and 20) constituted the secondary pollen types.

Table 1. *Results of the microscopic analysis (D: Dominant; S: Secondary; M: Important minor [27])*

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fabaceae	*Astragalus lagurus*	M		M	M		M	M			M		M		M	M			M			M
	Astragalus spp.													M								
	Hedysarum sp.		M	M	D		M	M	M	M	M	M	M	M	M	M	M			M	M	
	Lathyrus sp.					M							M			M						
	Lotus corniculatus	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	S	M	M	S	M
	Medicago falcata	M	M	M	M	M	M	M	M	M	M	M	M		M	M		M	M	M	M	M
	Medicago sativa										M		S	M	M	M	M			M		
	Onobrychis spp.		D					D		D		D			D		D			D		
	Onobrychis oxyodonta		D			D	D			D	D	D	D			D					D	
	Onobrychis radiata	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
	Onobrychis tournefortii		D			D	D	D		D	D	D	D	D	D	D	D			D	D	
	Medicago x varia																M					
	Melilotus officinalis			M																		

Table 1. *(Continued) Results of the microscopic analysis (D: Dominant; S: Secondary; M: Important minor [27])*

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Fabaceae	*Trifolium nigrescens*	M	M		M						M	M	M		M	M			M	M	M	M
	Trifolium ochrleucum		M	M		M					M					M						
	Trifolium pratense											M										
	Trifolium repens	M	M		M	M		M		M	M	M	M	M	M	M	M	M	M	M	M	
	Vicia sativa	M				M	M			M	M				M	M						
Lamiaceae	*Salvia spp.*										M		M		M		M		M			
	Teucrium orientalis				M			M	M				M	M	M	M			M		M	
	Teucrium chamaedrys					M	M										M					
	Teucrium polium												M	M		M	M			M		
	Teucrium spp.					M				M												
	Thymus longicaulis				M			M			M		M	M	M	M			M	M	M	M
Liliaceae	*Allium spp.*																					M
	Ornithagalum spp.	M																				
Malvaceae															M	M						

Table 1. *(Continued) Results of the microscopic analysis (D: Dominant; S: Secondary; M: Important minor [27])*

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Plantaginaceae	*Plantago lanceolata*			M					M		M		M		M					M		
Poaceae				M			M		M	M		M			M		M					
Polygonaceae						M								M								
Rhamnaceae																	M					
Rumex spp																						M
Ranunculaceae	*Nigella spp.*				M	M	M	M	M			M	M	M		S						
Rosaceae					M			M				M	M	M	M	M		M	M	M	M	
Rubiaceae	*Galium spp.*		M			M																
Salicaceae	*Salix spp.*	M								M					M	M						M

TPN-10 ranged from 6532 to 481157 in investigated honey samples. TPN-10 values, percentages of *Onobrychis* pollen in honey samples are shown in Table 2. The highest number of TPN-10 was in sample 1, while the lowest was in sample 17.

The determination of honey botanical origin is based on the relative frequencies of pollen types. Honey is considered as unifloral if the relative frequency of the pollen of that taxon exceeds 45% [4]. Unifloral honey characterization is necessary for both scientific and commercial interests [33].

In this study, 21 unifloral honey samples were examined and characterized according to their physicochemical parameters. *O. radiata* pollen was the most frequently observed pollen in all samples. It was the dominant pollen in all 21 samples, ranging from 46 to 78%. Similar to our results, Yurtsever and Sorkun (2005) reported that *Onobrychis* spp. pollen was dominant in honey samples from Erzincan, Turkey [34]. In addition, a characteristic feature of honey from the Lazdijai District is monofloral honey from *Onobrychis*; the pollen of this plant was the dominant pollen of the monofloral honey (52.1– 54.4%) and was the secondary or important minor of the polyfloral honey (9.2–17.8%) from this area [35].

TPN is a significant criterion in distinguishing natural honey samples from artificial ones [36]. Pollen concentration values per 10 g of < 20,000 were considered as ‘very poor’, 20,000–100,000 as ‘intermediate’, 100,000–500,000 as ‘rich’, and 500,000–1,000,000 as ‘very rich’ [37]. According to these values, the number of pollen grains per 10 g of honey varied from very poor to the rich category (6.532–481.157). Therefore, honey sample 1 would be more valuable in terms of pollen quantity compared with the other samples.

3.2. Sugar and HMF Contents

The HMF values of samples were between 0.1–7.5 mg/kg, and F/G ratios were between 0.8–1.3. Based on HMF contents, all the samples were suitable as honey according to the Turkish Food Codex. The F/G values of only three samples (< 0.9) were not compatible with the Codex values (Table 2).

Table 2. *TPN-10 values, percentages of Onobrychis pollen, the values of HMF and F/G ratio in honey samples*

	TPN-10	***Onobrychis* pollen (%)**	**HMF(mg/kg)**	**F/G**
1	481157	55.5	7.5	1.1
2	24770	54	0.9	0.83
3	55231	47.5	1.4	1.3
4	42303	51	1.2	1.2
5	22804	47	0.5	1.04
6	36058	62	0.4	1
7	7066	53.5	0.9	0.9
8	16461	70	1.2	1
9	58909	68	0.8	1.2
10	17482	75	0.3	0.8
11	9345	52.5	0.1	0.8
12	16230	53.5	0.4	1.1
13	6601	50	0.1	1
14	8466	56.5	0.3	0.96
15	24883	46	0.5	0.9
16	20381	54.5	0.1	0.9
17	6532	46.5	0.6	0.9
18	41807	55.5	1.7	1.2
19	14856	68.5	0.5	1.2
20	85442	76	0.3	1
21	28035	78	1.6	1.2

Physicochemical parameters such as moisture content, diastase number, HMF content, acidity, and sugar content were closely related to the quality of the honey and showed values in agreement with both national and international legal limits [38]. HMF is the most important and reliable criterion to detect if a honey sample was heated [39]. Our study found HMF values of 0.1 ppm to 7.5 ppm in the honey samples, while another study found an HMF value of 1.49 mg/kg [40]. Therefore, we can assume that no heating processes were applied to these samples.

3.3. Volatile compounds determined by GC-MS

The volatile compounds of honey samples were determined by SPME/GC-MS. The main classes of the compounds and their abundances in honey samples are listed in Table 3.

Table 3. *Results of GC-MS analysis*

	Alcohols	**Aldehydes**	**Aliphatic acids and their esters**	**Hydrocarbons**	**Flavonoids**	**Carboxylic acids and their esters**	**Ketones**	**Sugars**	**Vitamins**	**Others**
1	2.9	6.44	3.2	1.09	7.09	0.91	14.33	15.14	0.23	1.3
2	1.32	2.35	17.6	5.16	3.54	0	2.82	16.45	0.19	0
3	0.15	1.93	2.41	6.94	4.13	0.79	9.55	17.55	0	1.88
4	3.41	0.97	0.88	4.57	3.13	0.54	1.78	16.46	0	0.13
5	0.19	0	24.72	14.71	2.74	0	4.95	21.79	3.87	1.3
6	0.82	0	4.33	0.44	3.44	0	3.05	40.64	0	2.16
7	0	16.76	0.95	1.73	3.98	0	2.67	23.74	0	4.54
8	0.94	2.98	0	1.67	3.21	0	0	44.58	0	0.22
9	0	4.31	0	0	5.27	1.86	7.16	23.5	21.36	0
10	2.07	3.59	0	0.19	5.39	0.46	12.88	5.12	0	0.35
11	1.75	0.76	0.04	0.51	5.94	0.19	8.04	6.23	0	1.76
12	0.05	3.19	0	0	4.05	0.88	27.02	14.52	1.35	1.19
13	0	3.85	0	3.47	4.33	0.5	2.92	29.89	0	0
14	0.53	2.79	0	2.36	3.17	0	7.86	16.98	0	0.18
15	1.62	15. 7	0	6.31	4.58	0	2.21	18.64	0	9
16	2.02	2.95	0	0	5.48	0	13.19	9.16	0	1.03
17	11.95	4.74	0	5.17	3.29	0	7.95	10.18	0	0.81
18	0.5	2.18	0	5.07	3.52	0	0.38	51.39	0	2.15
19	9.09	4.75	0	1.12	5.96	0	5.48	16.28	0	0
20	3.26	2.16	0.06	7.08	4.33	0	11.77	7.3	0	0.31
21	1.13	1.68	0	0	5.96	0.32	6.03	19.8	0	8.57

Alcohols, aldehydes, aliphatic acids and their esters, carboxylic acids and their esters, hydrocarbons, flavonoids, ketones, sugars, and vitamins were identified in the honey samples within ranges of 0–11.95%, 0–16.76%, 0–24.72%, 0–1.86%, 0–14.71%, 2.74–7.09%, 0–27.02%, 5.12–51.39%, and 0–21.36%, respectively. Sugars were the main components in 15 of the 21 samples. The highest ratio of sugars was found in sample 18 (51.39 %).

3.4. Statistical Results

PCA analysis summarized the 13 parameters (*Onobrychis* pollen numbers, alcohols, aldehydes, aliphatics, hydrocarbons, flavonoids, carboxylic acids, ketones, sugars, vitamins, F/G, HMF, and TPN-10). From this analysis, the first three principal components that had the highest eigenvalues were able to describe nearly 74% of the total variance. Because of that, the parameters (*Onobrychis* pollen numbers, flavonoids, sugars, F/G, HMF, and TPN-10) with the lowest scores of each of the three components in the correlation analysis (Table 4) were selected for further analyses.

Table 4. *PCA loadings of the parameters for the first three components*

	PC 1	PC 2	PC 3
Onobrychis Pollen Number	-0.017	0.005	0.003
Alcohols	-0.154	-0.443	0.599
Aldehydes	-0.300	-0.175	-0.261
Aliphatics	0.734	0.216	0.337
Hydrocarbons	0.513	-0.475	-0.130
Flavonoids	-0.010	0.034	0.064
Carboxylics	-0.073	0.254	-0.040
Ketons	-0.197	0.326	0.581
Sugars	0.111	0.010	-0.264
Vitamins	0.087	0.574	-0.165
F/G	0.008	0.017	-0.024
HMF	0.111	-0.014	-0.045
TPN-10	0.008	0.008	0.008

Although it was not significant, a positive correlation between flavonoids and *Onobrychis* pollen number was calculated ($p > 0.05$; r: 0.323; Table 6 and Fig. 1a) via correlation analysis. There was no meaningful correlation between *Onobrychis* pollen number and other parameters. Although a negative trend between flavonoids and sugars was observed, it was not significant ($p > 0.05$; r: -0.402; Fig. 1b). F/G ratio was found to be positively correlated with sugars, HMF, and TPN-10 (Table 5; Fig. 1c, d), of which the latter two parameters had significant values ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, a significant positive correlation between TPN-10 and HMF was also detected (Table 5; Fig. 1e).

Table 5. *Spearman's correlation results of some physicochemical features of the honey (p, upper values; r below values)*

	O. Pollen Number	Flavonoids	Sugars	F/G	HMF	TPN-10
O. Pollen Number		0.154	0.922	0.549	0.840	0.256
Flavonoids	0.323		0.071	0.998	0.679	0.489
Sugars	-0.023	-0.402		0.076	0.061	0.737
F/G	0.139	0.001	0.396		0.008	0.017
HMF	0.047	-0.096	0.416	0.562		0.030
TPN-10	0.259	0.160	0.078	0.516	0.474	

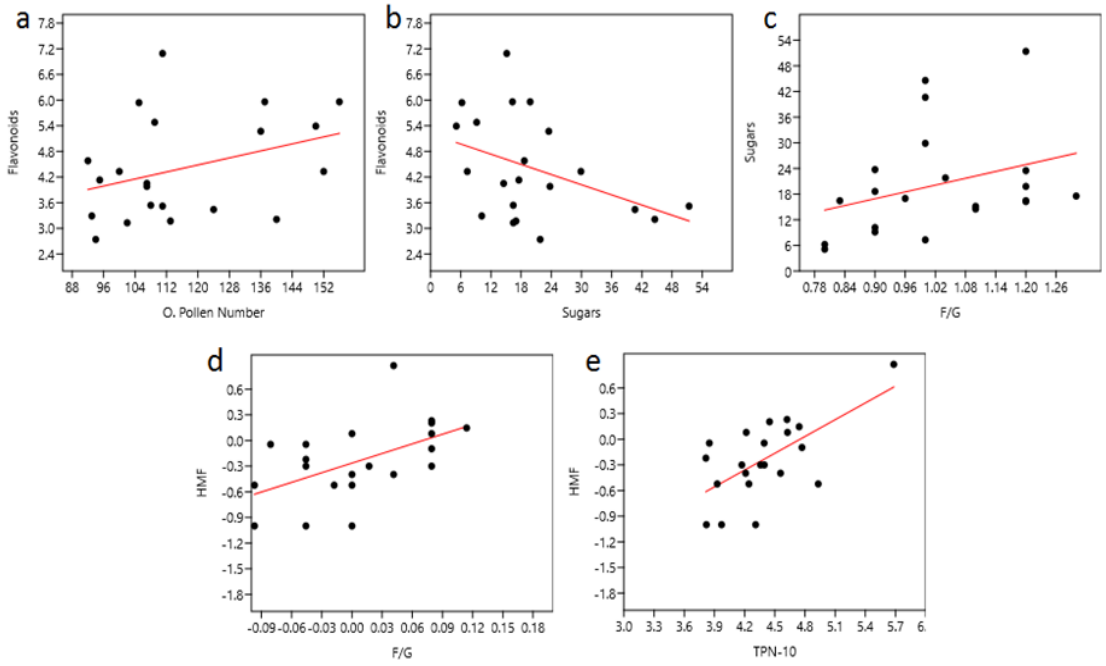

Figure 1. *Linear Multivariate Regression Model results of some physicochemical features of the honey*

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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