HPTLC Phenolic Profiles as Useful Tools for the Authentication of Honey

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Abstract

The present study reveals the utility of high-performance thin-layer chromatographic (HPTLC) fingerprinting of phenolic constituents for the authentication of monofloral honeys. The obtained data enables a more complete assessment of honey quality and the identification of emerging threats to honey quality. The developed procedure facilitates differentiation of varietal honeys and detection of honey adulterations. We used an HPTLC fingerprint analysis to determine the characteristic patterns of different honey types (willow, buckwheat, heather, pine honeydew, and manuka honey). The HPTLC chromatograms were used to determine the differences in the botanical origin of the honey samples on the basis of the band profiles, which are characteristic for each honey type. Identification of 11 polyphenols was performed by comparison of the color and R_f of the bands with available standards. Additionally, the results were confirmed by an HPLC analysis.

Keywords Honey · Phenolic compounds · HPTLC fingerprint · High-performance thin-layer chromatography

Introduction

Phenolic compounds rank among the most important compounds present in honey. They exhibit many biological effects, including their actions as antioxidant, antibacterial, anti-inflammatory, and anti-allergic agents. Therefore, honey, despite being considered a highly nutritious natural product, is also used for the prevention and treatment of many diseases. In recent decades, different methods have been implemented for the authentication of honey (Karabagias et al. 2014, Dinca et al. 2015, Ciulu et al. 2016, Pascual-Maté et al. 2017), and the analysis of phenolic compounds is well documented as a very promising method for determining the floral and geographical origins of honey. In general, an analytical procedure for the determination of phenolic compounds includes three steps: isolation, separation and identification, and the most common method of separation is high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) (Pyrzyńska and Biesaga 2009). In addition, statistical

methods, such as hierarchical cluster analysis, principal component analysis, or discriminant analysis are increasingly introduced for the study of the usefulness of different analytical technique used to authenticate honey (Kečkeš et al. 2013; Kuš et al. 2014; Kasprzyk et al. 2018).

This study presents the determination of the phenolic profiles of various honey samples by using high-performance thin layer chromatography (HPTLC) as a detection method. Although HPLC still remains the most dominant technique for the separation and identification of phenolic compounds (Badjah Hadj Ahmed et al. 2016, Pascual-Maté et al. 2017), HPTLC seems to be a very promising alternative for the analysis of these compounds in honey (Locher et al. 2017). This is because HPTLC is simpler, more flexible, more accessible, and cheaper than other commonly used methods. Furthermore, HPTLC allows a parallel separation and quantitative determination of many samples at the same time (Ristivojević et al. 2017). HPTLC chromatograms can be used to determine the differences in botanical origins of honey samples by exhibiting a characteristic set of common bands that is typical for each analyzed branch. Thus, the entire chromatogram is treated as a unique, distinctive fingerprint for each selected type of honey. Chromatograms could, thus, complement quality-control tools for honey. An equally important feature of the HPTLC analysis of phenolic compounds is the detection of individual chemical species that are present in certain honey. Separated substances can be detected by determining the color of the substance, the fluorescence of

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the substance in UV light, or the color reactions of separated substances. Individual classes of phenolic compounds exhibit different spot coloration under UV light with visualizing reagents. Specifically, flavonoids, like quercetin, show orange or yellow spots, while flavones appear dull brown. Anthocyanins are detected as purple-red-orange or sometimes yellow bands in visible light. Light and dark blue spots are characteristic for caffeic, *p*-coumaric, and ferulic acids. On the other hand, simple phenolics, such as gallic or salicylic acids, do not fluoresce under UV light and quench to give dark absorbing spots (Waterman 1994). Thus, Głowniak et al. 2005 analyzed phenolic compounds in the flowers of *Lavatera trimestris* L. The fluorescence colors of phenolic acid and flavonoid standards were blue or light blue for caffeic, *p*-coumaric, ferulic, and chlorogenic acids. Additionally, caffeic, ferulic, and chlorogenic acids, after spraying with a 2% aqueous solution of ferric chloride, produced brown-green red, light-orange, and gray-green spots, respectively. Milojković Opsenica et al. 2016 analyzed phenolic compounds in propolis samples. Propolis of so-called O-type showed orange (typical for flavonoids), light blue, and some green bands. Orange, green-brown, light blue, and green bands were further identified as quercetin, chrysin, caffeic acid, and naringenin, respectively. It is noteworthy that only one paper has been devoted to the analysis of the chemical composition of honey by means of HPTLC. Locher et al. 2017 explored HPTLC fingerprinting for the authentication of honey, such as jarrah (*Eucalyptus marginata*), marri (*Corymbia calophylla*), and manuka (*Leptospermum scoparium*), but they did not study the use of the HPTLC method for the determination of phenolic compounds.

The aim of the present work was fingerprinting of phenolics by HPTLC to determine their characteristic patterns in willow, buckwheat, heather, pine honeydew, and manuka honey.

Materials and Methods

Honey Samples

Seventeen honey samples of five different varieties of honey (willow, heather, buckwheat, pine honeydew, and manuka) were analyzed. The types of pollen present in honeys analyzed are shown in Table 1. Two categories of honey were distinguished: pure honeys (W2-W3, H1-H2, B1, M1-M2) and honeys with pollen content slightly lower than 45% (W1, B2-B4). In the case of pine honeydew honeys, the authentication is not carried out on the basis of the pollen grains, and their classifications is made by using electrical conductivity parameter. According to Polish Quality Standard (PN-88/A-77626 1998) and Bogdanov et al. 2004, the electrical conductivity should be higher than 800 $\mu\text{S}/\text{cm}$, then honey is a honeydew.

The all honeydew honey samples had an electrical conductivity in the range of 1160–1580 $\mu\text{S}/\text{cm}$. The honey samples were of different harvesting years (2015–2016) and geographical origins (Poland, New Zealand, and Australia) (Table 1). The products were stored at 4 °C in the dark.

Chemicals and Reagents

All chemicals and solvents used for the chromatography column, HPLC and HPTLC analyses were of analytical purity grade. Methanol, ethanol dibasic sodium phosphate heptahydrate ($\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$), 85% phosphoric acid, chloroform, ethyl acetate, and formic acid were purchased from POCH S.A. (Gliwice, Poland). The standards (chrysin, myricetin, 3,4-dihydroxybenzoic acid, caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, *p*-coumaric acid, rosmarinic acid, and ($\pm$)-abscisic acid) were purchased from Sigma-Aldrich (Poznań, Poland). Amberlite XAD-2, which was used as an adsorption resin for the extraction step, was obtained from Supelco (Bellefonte, PA, USA).

Physicochemical Properties

Selected physicochemical parameters: moisture content, pH, and electrical conductivity have been determined using the Polish Quality Standard (PN-88/A-77626).

Phenolic Extraction (Solid-Phase Extraction, SPE)

The extraction was performed using the method described by Jasicka-Misiak et al. (2017). The honey (25 g) was thoroughly mixed with five parts (125 mL) of acidified water (pH = 2 with HCl) and homogenized in an ultrasonic bath for 60 min. The solutions were then filtered through filter paper to remove any solid particles. Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3–1.2 mm) was packed into a glass column (2.5 cm $\times$ 55 cm) and washed with both methanol and acidified water. Next, the honey extract was transferred to a glass column and kept for 30–45 min. The solid phase was washed with acidified water (0.05 M HCl, 250 mL) and neutral distilled water (300 mL) to remove all sugars and other polar compounds. The polyphenolics were eluted from the sorbent with methanol (500 mL). The methanol extracts were evaporated under reduced pressure at 40 °C. The dried residue was then dissolved in methanol (HPLC-grade).

HPTLC Separation

The 10 μL of the honey extracts and 4 μL of the standard solutions (concentration of 1 mM) were applied as 8 mm bands on HPTLC sheets at rate of 150 nL s⁻¹ using a semiautomated HPTLC application device (Linomat 5,

Table 1 Characteristic of studied honey samples

Type of honey, floral origin	Place and year of production	Sample ID	Predominant pollen type [%]	Selected chemical parameters of honey (Polish Quality Standard PN-88/A-77626) $n = 3$		
				Moisture content [%]	Electric conductivity [mS/cm]	pH
Willow, <i>Salix</i> spp.	Poland, 2015	W1	27.0	16.3 ± 2.2	0.38 ± 0.15	3.98
	Poland, 2015	W2	59.0	16.1 ± 1.6	0.40 ± 0.09	3.97
	Poland, 2016	W3	65.0	16.4 ± 2.1	0.64 ± 0.11	3.92
Heather, <i>Calluna vulgaris</i> L.	Poland, 2016	H1	57.4	18.6 ± 1.5	0.66 ± 0.12	4.34
	Poland, 2016	H2	73.8	18.1 ± 1.3	0.64 ± 0.08	4.39
Buckwheat, <i>Fagopyrum esculentum</i> L.	Poland, 2016	B1	48.6	17.1 ± 1.9	0.43 ± 0.09	3.79
	Poland, 2016	B2	41.9	16.5 ± 3.1	0.46 ± 0.10	3.86
	Poland, 2016	B3	33.9	16.1 ± 1.9	0.39 ± 0.07	4.02
	Poland, 2016	B4	41.1	16.3 ± 1.7	0.44 ± 0.11	3.81
Pine honeydew, <i>Pinus sylvestris</i> L.	Poland, 2015	PH1	–	16.9 ± 2.2	1.24 ± 0.15	4.57
	Poland, 2015	PH2	–	16.2 ± 1.4	1.45 ± 0.13	4.53
	Poland, 2015	PH3	–	16.4 ± 2.1	1.29 ± 0.20	4.51
	Poland, 2015	PH4	–	16.9 ± 2.2	1.58 ± 0.19	4.62
	Poland, 2015	PH5	–	17.1 ± 2.4	1.21 ± 0.16	4.53
	Poland, 2015	PH6	–	16.5 ± 1.6	1.16 ± 0.12	4.50
Manuka, <i>Leptospermum scoparium</i>	New Zeland, 2015	M1	76.0	16.8 ± 1.5	0.47 ± 0.09	4.27
	Australia, 2016	M2	64.8	17.3 ± 1.9	0.54 ± 0.12	4.44

CAMAG, Muttentz, Switzerland). The chromatographic separations were performed on 20 × 10 cm (0.25 thick) TLC plates (HPTLC Sillica gel 60, Merck) using a mixture of chloroform:ethyl acetate:formic acid (5:4:1) as the mobile phase. Visualization was performed in two ways: (1) under long-wave UV light (254 nm, 366 nm) and (2) by spraying with 1% methanolic AlCl₃, Ce-P-Mo spraying reagent (10 g of Ce(SO₄)₂, and 20 g of phosphomolybdic acid in 1 L of 10% H₂SO₄) (Żyska et al. 2017) or 1% methanolic 2-aminoethyl diphenylborate (Neu's reagent), drying, and visualizing under both 254 nm and 366 nm. After spraying, the plates were dried for approximately 3 min in warm air until colored spots appeared. The obtained chromatographic results were analyzed using HPTLC software (visionCATS, CAMAG).

HPLC Separation

An UltiMate 3000 Dionex HPLC system was used to analyze the polyphenolic compounds in the honey samples. The chromatographic separation was achieved using a reserved-phase Gemini 5 µm C₁₈ column (250 × 4.6 mm, particle size 5 µm). The mobile phases were methanol (solvent A) and 0.01 M aqueous phosphate buffer at pH = 2.5 (solvent B) at a flow rate of 1 mL/min in a gradient elution mode. The gradient separation was as follows (A:B ratio): 10–90% $t = 0$; 40–60% $t = 13.5$ min; 90–10% $t = 39$ min; 100–0% $t = 42$ min; 10–90% $t = 55$ min. The standards and honey extracts were

injected into the system with an auto-sampler, and the phenolic compounds were detected using a photodiode array detector (PDA). Stock solutions of each standard were prepared with HPLC grade methanol at a concentration of 200 µg/ml and were stored at 4 °C while protecting from light.

Method of Validation

Calibration and Linearity

The calibration curves were generated using nine data points: 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, and 200 µg/ml. Standard solutions were prepared in HPLC grade methanol and analyzed in triplicate. Linear calibration plots were obtained over the calibration range of 0.05–200 µg/ml. The correlation coefficient (r^2) was found to be > 0.9972 (Table 2) for each regression equation.

Precision

The repeatability of the sample application and measurement of the peak area was tested by injecting three replicates of the standard (100 µg/ml) on the same day and over 3 days (three replicates each day). The intra- and inter-day variations were achieved with relative standard deviation (RSD) values lower than 3.3% for all the analyzed compounds (Table 2).

Table 2 Correlation coefficients, LOD, LOQ, and precision of standards

Standards	R^2	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)	Precision (RSD %)	
				Inter-day	Intra-day
Chlorogenic acid	0.9999	0.010	0.029	0.6	3.3
Gallic acid	0.9996	0.016	0.049	1.8	1.9
Caffeic acid	0.9993	0.046	0.140	1.4	3.1
Ferulic acid	0.9994	0.010	0.032	0.6	1.0
<i>p</i> -coumaric acid	0.9972	0.015	0.045	1.4	1.6
Rosmarinic acid	0.9995	0.011	0.034	0.5	2.7
Ellagic acid	0.9981	0.175	0.531	0.3	1.4
3,4-dihydroxybenzoic acid	0.9997	0.011	0.033	0.9	2.5
Abscisic acid	0.9996	0.028	0.084	0.5	1.7
Myricetin	0.9999	0.218	0.662	1.1	1.6
Chrysin	0.9995	0.014	0.044	2.1	2.0

Limit of Detection and Limit of Quantitation

To estimate the limit of detection (LOD) and limit of quantitation (LOQ), a blank sample (methanol) was spiked three times with each standard at low concentration levels, and the signal-to-noise ratio was determined. The LOD was considered as 3:1, and the LOQ as 10:1. The LOD values were between 0.010 and 0.218 $\mu\text{g/ml}$, while the LOQ values were between 0.029 and 0.662 $\mu\text{g/ml}$ for each standard compound (Table 2).

Statistical Analysis

Statistical analyses were performed using the Statistica 13.1 software (StatSoft). Means were separated by least significant difference test (LSD) and statistical significance was set at $\alpha = 0.05$. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to compare concentrations of identified compounds of honey samples (phenolic acids, flavonoids, and abscisic acid).

Results and Discussion

Physicochemical Parameters

The physicochemical parameters studied are moisture content, pH, and electrical conductivity. The mean values for each physicochemical parameter are presented in Table 1. The honey moisture content is one of the most important parameters, and its level should not exceed 20% in accordance with international regulations of quality (Codex Alimentarius 2001) and the Polish Quality Standard (PN-88/A-77626 1998). The levels higher than 20% are indicative of the acceleration of fermentation processes during storage (Semkiw et al. 2008, Muruke 2014).

The electrical conductivity values in honey samples are in the range of 0.38–1.58 mS/cm. The highest values (1.16–1.58 mS/cm) obtained for pine honeydew honeys are according to Polish Quality Standard and Codex Alimentarius. The significant difference in electrical conductivity is used to differentiate honeydew honeys from nectar honeys.

Generally, honeys have an acidic character. In the case of honey samples analyzed, the pH ranged between 3.92 and 4.62. These values were similar to those previously reported in other honeys (Fechner et al. 2016, Chuttong et al. 2016). The highest levels of pH were observed in the case of honeydew honeys while willow had the lowest level of pH. Some authors suggest that pH has an effect on the extraction and storage of honey, but not on the botanical origin (Oroian and Sorina 2017).

HPTLC Fingerprints of Honey

Each of the received HPTLC images was evaluated to assess whether they provided useful information for the discrimination of the honey brands. The HPTLC chromatograms showed differences in the chemical composition of the honey samples with different origins. All of the honey extracts were enriched in phenolic compounds, and their patterns were dominated by light blue, blue, navy blue, yellow, and black bands. The HPTLC fingerprint analysis allowed the differentiation of honey samples depending on the fluorescent color of the phenolic compounds and showed that these compounds were present in some honey samples in varying amounts (Table 3).

The fingerprint of certain honeys exhibits a unique profile of bands that correspond to the phenolics and can be considered unique because they visually differ from each other. Fig. 1 shows the typical HPTLC fingerprints of willow honey samples (W1–W3). Major bands at R_f of 0.03 (light blue), 0.05 (light blue), 0.13 (blue), 0.40 (yellow), 0.45 (blue), 0.50 (blue), 0.59 (navy blue), and 0.61 (yellow) under 366 nm irradiation can be seen. Under 254 nm, the most prominent

Table 3 Average R_f values of identified standards separated by HPTLC and their respective colors

Standard	Honey samples	Fluorescence		R_f
		$\lambda = 254 \text{ nm}$	$\lambda = 366 \text{ nm}$	
Chlorogenic acid	W1-W3, H1-H2, PH1-PH6	Light blue	Blue	0.03
Gallic acid	PH1-PH6	Navy blue	Black	0.29
Caffeic acid	W1-W3, H1-H2, PH1-PH6	Light blue	Light blue	0.41
Ferulic acid	W1-W3, PH1-PH6	Blue	Navy blue	0.51
<i>p</i> -Coumaric acid	B1-B4, H1-H2, M1-M2	Navy blue	Navy blue	0.58
Rosmarinic acid	M1-M2	Blue	Blue	0.28
Ellagic acid	M1-M2	Navy blue	Navy blue	0.44
3,4-Dihydroxybenzoic acid	W1-W3	Green	Blue	0.50
Abscisic acid	W1-W3, H1-H2	Black	–	0.49
Myricetin	W1-W3, H1-H2, B1-B4, PH1-PH6, M1-M2	Green	Yellow	0.39
Chrysin	H1-H2	–	Yellow	0.66

bands are at R_f of 0.03 (light blue), 0.05 (light blue), 0.13 (blue), 0.40 (green), 0.45 (blue), 0.50 (green), 0.52 (black), 0.59 (blue), and 0.61 (green) (Fig. 2). After derivatization with NEU and 1% methanolic AlCl_3 spraying reagent, the bands at R_f 0.40 and 0.61 are intensely yellow, and the appearance of new bands at 0.70 (orange) and 0.71 (yellow) can be observed. It is also seen that the phenolic compounds in willow honey change their composition over time from the harvest period. The track profiles for samples of differing harvesting years exhibited the same band profiles, but the older samples (W1 and W2) exhibit bands with a lower intensity ($R_f = 0.40$). We

have identified the following phenolic compounds (based on the similarity of the R_f and the color of the bands): $R_f = 0.03$ chlorogenic acid, $R_f = 0.40$ myricetin, $R_f = 0.45$ caffeic acid, $R_f = 0.50$ 3,4-dihydroxybenzoic acid, $R_f = 0.52$ abscisic acid, and $R_f = 0.59$ ferulic acid, and the most intense signals were derived from chlorogenic acid (light blue), myricetin (yellow), and abscisic acid (black).

The heather honey samples (H1-H2) exhibit notable bands at R_f 0.03 (light blue), 0.07 (light blue), 0.09 (yellow), 0.13 (yellow), 0.16 (blue), 0.19 (yellow), 0.29 (yellow), 0.34 (yellow), 0.37 (yellow), 0.40 (blue), 0.47 (blue), 0.55 (dark blue),

Fig. 1 HPTLC chromatogram at 366 nm, W1-W3 willow honey samples, H1-H2 heather honey samples, M1-M2- manuka honey samples, R references, FA ferulic acid, DHBA 3,4-dihydroxybenzoic

acid, M myricetin, ChA chlorogenic acid, Ch chrysin, *p*-CA *p*-coumaric acid, CA caffeic acid, EA ellagic acid, RA rosmarinic acid

Fig. 2 HPTLC chromatogram at 254 nm. W1-W3 willow honey samples, H1-H2 heater honey samples, M1-M2 manuka honey samples, R references, FA ferulic acid, DHBA 3,4-dihydroxybenzoic acid, M

myricetin, ChA chlorogenic acid, *p*-CA *p*-coumaric acid, CA caffeic acid, EA ellagic acid, RA rosmarinic acid, AA abscisic acid

0.58 (navy blue), and 0.66 (yellow) under 366 nm (Fig. 1). Additionally, visualization with 254 nm results in spots of R_f of 0.03 (light blue), 0.07 (light blue), 0.09 (yellow), 0.13 (yellow), 0.16 (yellow), 0.19 (yellow), 0.29 (yellow), 0.34 (intense yellow), 0.37 (yellow), 0.40 (blue), 0.47 (black), 0.55 (blue), 0.58 (blue), and 0.66 (yellow) (Fig. 2). Based on the color reactions of polyphenols before and after the derivatization, the following components were identified: $R_f = 0.03$ chlorogenic acid, $R_f = 0.34$ myricetin, $R_f = 0.40$ caffeic acid, $R_f = 0.47$ abscisic acid, $R_f = 0.58$ *p*-coumaric acid and $R_f = 0.66$ chrysin. It is noteworthy that the chromatogram was dominated by the intense yellow and black bands that were identified as myricetin and abscisic acid, respectively. In addition, the heather honey profiles at 366 nm irradiation showed great similarity in the phenolic signals, which indicated that all of the samples belong to the same type of honey (Fig. 5). The strongest signal was observed at $R_f = 0.34$, which originated from myricetin.

The buckwheat honey samples (B1-B4) exhibited bands of interest at R_f of 0.03 (light blue), 0.05 (dark blue), 0.08 (blue), 0.14 (dark blue), 0.18 (blue), 0.25 (yellow), 0.36 (navy blue), 0.38 (yellow), 0.42 (dark blue), 0.46 (dark blue), 0.54 (dark blue), 0.57 (navy blue), and 0.68 (dark blue) under 366 nm (Fig. 3). UV irradiation at 254 nm, there are bands at R_f of 0.03 (light blue), 0.05 (dark blue), 0.08 (white), 0.10 (dark blue), 0.14 (dark blue), 0.25 (yellow), 0.36 (light blue), 0.38 (yellow), 0.42 (dark blue), 0.46 (dark blue), 0.54 (dark blue), 0.57 (navy blue), and 0.68 (dark blue) (Fig. 4). Two phenolic

compounds were identified here: $R_f = 0.38$ myricetin and $R_f = 0.57$ *p*-coumaric acid (Fig. 4).

Figure 3 shows the various pine honeydew extract chromatograms. Visualization at 366 nm unveils notable bands at R_f values of 0.03 (light blue), 0.05 (blue), 0.11 (dark blue), 0.13 (dark blue), 0.30 (black), 0.34 (orange), 0.38 (yellow), 0.39 (blue), 0.46 (black), 0.51 (navy blue), and 0.66 (dark blue). The derivatized samples (NEU reagent) have a pronounced band at $R_f = 0.54$ (yellow). UV irradiation with 254 nm, the samples contain additional spots at $R_f = 0.08$ (light blue), 0.17 (light blue), 0.43 (yellow), 0.51 (dark blue), and 0.54 (yellow) (Fig. 4). HPTLC led to the identification of four phenolic acids, chlorogenic ($R_f = 0.03$), gallic ($R_f = 0.30$), caffeic ($R_f = 0.39$) and ferulic ($R_f = 0.51$), and one flavonoid, myricetin ($R_f = 0.38$). As observed in Fig. 5, various pine honeydew honey sample profiles under 366 nm display very similar profiles with key peaks at $R_f = 0.03$, 0.13, 0.46, and 0.51.

Figures 1 and 2 illustrate the chromatograms of manuka honey obtained from different suppliers and locations with varying ages (Table 1). Still, the manuka honey extract under 366 nm irradiation exhibits a very similar fingerprint with key peaks at $R_f = 0.03$ (light blue), 0.06 (light blue), 0.10 (light blue), 0.14 (blue), 0.21 (light blue), 0.24 (white), 0.34 (light blue), 0.38 (yellow), 0.44 (navy blue), 0.47 (dark blue), 0.57 (dark blue), 0.69 (navy blue), 0.78 (dark blue), 0.89 (dark blue), and 0.28 (dark blue), which are characteristic for only the M1 honey samples (Fig. 1). Under 254 nm, there are prominent additional bands at $R_f = 0.24$ (yellow), 0.47 (dark

Fig. 3 HPTLC chromatogram at 366 nm. B1–B4 buckwheat honey samples, PH1–PH6 pine honeydew honey samples, R references, *p*-CA *p*-coumaric acid, M myricetin, FA ferulic acid, CA caffeic acid

blue), 0.50 (light blue), and 0.62 (light blue), while bands at $R_f=0.78$ and $R_f=0.89$ are not visible (Fig. 2). Manuka samples contained rosmarinic acid ($R_f=0.28$), ellagic acid ($R_f=0.44$), *p*-coumaric acid ($R_f=0.57$), and myricetin ($R_f=0.38$). As is illustrated in Fig. 5, despite very similar profiles, differences between the samples M1 and M2 are observed. Under 366 nm irradiation, a loss in the band intensity at 0.10, 0.21, 0.34, and 0.47 and an intense band appearance at 0.14, 0.38, and 0.57 in sample M1 are observed, which is in contrast to the M2 sample. These insignificant differences are most likely the result of the geographical origin of these honey samples.

Identification of Phenolic Compounds by HPLC

Eleven polyphenolics were identified in honey samples by HPTLC, and the obtained results were further confirmed by HPLC analysis. HPLC was used for the quantification of phenolic compounds in all honey samples. Initially, the validation parameters, such as linearity, precision, limit of detection, and quantification of the developed method (HPLC-DAD), were established (Table 2). The concentrations of all of the identified phenolic compounds in the honey samples are given in Table 4. The HPLC analysis of willow honey (W1–W3)

Fig. 4 HPTLC chromatogram at 254 nm. B1–B4 buckwheat honey samples, PH1–PH6 pine honeydew honey samples, R references, *p*-CA *p*-coumaric acid, M myricetin, FA ferulic acid, CA caffeic acid, GA gallic acid

Fig. 5 HPTLC honey extract profiles at 366 nm. H1–H2 heather honey samples, PH1–PH6 pine honeydew honey samples, M1–M2 manuka honey samples

confirmed the presence of chlorogenic acid, myricetin, caffeic acid, 3,4-dihydroxybenzoic acid, abscisic acid, and ferulic acid. Their content varied in the range from 5.276 to 7.643 mg per 100 g of honey. Abscisic acid was found in high quantities. In relation to the literature data, abscisic acid was found in Polish (Jerkovic et al. 2014) and Croatian willow honey (Tuberoso et al. 2011) in concentrations of 25.8–35.6 and 31.7–98.2 mg/kg, respectively. A similar dependence between the composition of the willow honey samples and the harvest year was observed by both HPTLC and HPLC. The highest amounts of caffeic acid, ferulic acid, and myricetin were found in the W3 sample collected in 2016 compared with the W1 and W2 samples that were collected in 2015. The level of these compounds gradually decreased with the age of the honey (caffeic acid content decreased to 60%, ferulic acid to 81%, and myricetin to 73%). In the case of other identified phenolic compounds, no significant differences in their concentrations were found.

The heather honey exhibited the highest level of abscisic acid (8.959–11.772 mg/100 g of honey) and myricetin (0.639–0.861 mg/100 g of honey). Similar content values of these compounds were observed by Jasicka-Misiak et al. 2012 (abscisic acid 8.272–12.061 mg/100 g of honey; myricetin 0.161–1.485 mg/100 g of honey). Additionally, a high level of abscisic acid (2.5–16.66 mg/100 g honey) in heather honey was identified by Ferreres et al. 1996 who considered this compound as a potential marker for this honey.

Myricetin and *p*-coumaric acid dominated in buckwheat honey. The content of these phenolics ranged from 0.243 to 0.606 and 0.267 to 0.489 mg/100 g of honey, respectively. A similar phenolic profile was found by Jasicka-Misiak et al. 2012 with a myricetin level of 0.082–1.907 mg/100 g of honey and *p*-coumaric acid of 0.026–4.551 mg/100 g of honey. Moreover, according to Pasini et al. (2013), the quantified levels of *p*-coumaric acid in buckwheat honey were 0.058–1.297 mg/100 g of honey. In another study, carried out by Karabagias et al. (2014), it was reported that myricetin and chrysin were determined in thyme honeys. Myricetin ranged from 0.74 to 244.67 mg/kg and chrysin ranged from 0.01 to 5.60 mg/kg.

The most prominent components of pine honeydew honey were chlorogenic acid (0.179–0.824 mg/100 g), gallic acid (1.315–4.793 mg/100 g), caffeic acid (0.049–0.337 mg/100 g), ferulic acid (0.039–0.620 mg/100 g), and myricetin (0.179–0.842 mg/100 g). Gallic acid was found to be in the largest amounts, whereas caffeic acid, chlorogenic acid, ferulic acid, and myricetin were at lower levels. The level of phenolic compounds in honeydew honey was higher than those obtained in other studies (Oroian and Sorina 2017; Socha et al. 2011), but the values obtained may vary depending on the type of honeydew used by bees for production of certain honey.

The chromatography data confirmed the already described presence (Yao et al. 2003) of rosmarinic, ellagic, *p*-coumaric acids, and myricetin in manuka honey. The *Leptospermum* honey, including Australian and New Zealand manuka honey,

Table 4 Quantitative determination of phenolic compounds in honey samples by HPLC-DAD

Phenolic compounds and abscisic acid (mg/100 g honeys $\pm$ RSD, $n = 3$)												
Honey samples	ChA	GA	CA	FA	p-CA	RA	EA	3,4-DHBA	AA	M	Ch	
W1	0.493 $\pm$ 0.017	–	0.063 $\pm$ 0.003	0.091 $\pm$ 0.016	–	–	–	0.717 $\pm$ 0.004	5.276 $\pm$ 0.014	0.184 $\pm$ 0.003	–	
W2	0.351 $\pm$ 0.013	–	0.070 $\pm$ 0.012	0.101 $\pm$ 0.024	–	–	–	1.323 $\pm$ 0.006	7.643 $\pm$ 0.034	0.172 $\pm$ 0.005	–	
W3	0.301 $\pm$ 0.035	–	0.159 $\pm$ 0.011	0.486 $\pm$ 0.027	–	–	–	1.049 $\pm$ 0.017	6.404 $\pm$ 0.013	0.641 $\pm$ 0.039	–	
H1	0.172 $\pm$ 0.027	–	0.316 $\pm$ 0.085	–	0.690 $\pm$ 0.117	–	–	–	11.772 $\pm$ 0.002	0.861 $\pm$ 0.008	0.190 $\pm$ 0.095	
H2	0.113 $\pm$ 0.057	–	0.142 $\pm$ 0.031	–	0.354 $\pm$ 0.046	–	–	–	8.959 $\pm$ 0.014	0.639 $\pm$ 0.049	0.121 $\pm$ 0.076	
B1	–	–	–	–	0.300 $\pm$ 0.006	–	–	–	–	0.606 $\pm$ 0.039	–	
B2	–	–	–	–	0.489 $\pm$ 0.052	–	–	–	–	0.532 $\pm$ 0.037	–	
B3	–	–	–	–	0.397 $\pm$ 0.009	–	–	–	–	0.243 $\pm$ 0.025	–	
B4	–	–	–	–	0.267 $\pm$ 0.026	–	–	–	–	0.264 $\pm$ 0.014	–	
PH1	0.260 $\pm$ 0.001	2.010 $\pm$ 0.006	0.102 $\pm$ 0.010	0.104 $\pm$ 0.003	–	–	–	–	–	0.260 $\pm$ 0.001	–	
PH2	0.221 $\pm$ 0.096	2.842 $\pm$ 0.003	0.060 $\pm$ 0.009	0.051 $\pm$ 0.027	–	–	–	–	–	0.221 $\pm$ 0.096	–	
PH3	0.214 $\pm$ 0.020	1.315 $\pm$ 0.059	0.049 $\pm$ 0.024	0.039 $\pm$ 0.010	–	–	–	–	–	0.214 $\pm$ 0.020	–	
PH4	0.824 $\pm$ 0.018	3.255 $\pm$ 0.068	0.337 $\pm$ 0.036	0.620 $\pm$ 0.035	–	–	–	–	–	0.824 $\pm$ 0.018	–	
PH5	0.447 $\pm$ 0.093	4.793 $\pm$ 0.011	0.083 $\pm$ 0.002	0.093 $\pm$ 0.035	–	–	–	–	–	0.447 $\pm$ 0.093	–	
PH6	0.179 $\pm$ 0.003	2.357 $\pm$ 0.003	0.079 $\pm$ 0.005	0.042 $\pm$ 0.030	–	–	–	–	–	0.179 $\pm$ 0.003	–	
M1	–	–	–	–	2.938 $\pm$ 0.005	0.262 $\pm$ 0.007	0.525 $\pm$ 0.001	–	–	1.261 $\pm$ 0.370	–	
M2	–	–	–	–	2.879 $\pm$ 0.006	0.291 $\pm$ 0.011	0.519 $\pm$ 0.026	–	–	1.052 $\pm$ 0.062	–	

ChA chlorogenic acid, GA gallic acid, CA caffeic acid, FA ferulic acid, p-CA p-coumaric acid, RA rosmarinic acid, EA ellagic acid, 3,4-DHBA 3,4-dihydroxybenzoic acid, AA abscisic acid, M myricetin, Ch chrysin

Fig. 6 Hierarchical cluster analysis dendrograms (a) and principal component analysis (b) of the tested honey samples. W1–W3 willow, H1–H2 heather, B1–B4 buckwheat, PH1–PH6 pine honeydew, M1–M2 manuka honey

presented standard phenolic profiles with only slight differences between the varieties. Thus, the quantified levels of *p*-coumaric, rosmarinic, and ellagic acid in Australian manuka honey were slightly higher than those in New Zealand manuka honey, which additionally exhibited lower values of myricetin.

Multivariate Data Analysis

This study aimed to determine the compounds such as phenolic acids, flavonoids, and abscisic acid which could be very useful when classifying honeys according to their variety. HCA and PCA were used to establish the relationship between the chemical composition of honeys and their floral origin. The results of HCA are presented as dendrograms in Fig. 6 a. All honey samples were classified into three clusters (A, B, and C). The cluster A contains of pine honeydew samples. This group clearly differed from other tested honey. The samples manuka and buckwheat honeys formed cluster B, which showed weak similarity with samples heather and willow honeys, distributed cluster C. Among the samples in the third group, willow honey samples clearly differed from heather honey samples. Moreover, this tendency was repeated in the PCA classification. The components explained 74.95% of the total variance (PC1 39.16% and PC2 35.79%). Figure 6b show the clustering of honey samples into three main groups, such as willow and heather (first), buckwheat and manuka (second), and pine honeydew honeys (third). Principal component analysis clearly differentiates pine honeydew located on the right side from other honey samples. Willow and heather honeys samples are grouped into one cluster. This is probably due to the fact that these samples were characterized by large amounts of abscisic acid. The cluster buckwheat and manuka honey samples partly

overlap, but a certain separation can be observed. In both honeys, *p*-coumaric acid and myricetin were identified, but the manuka honeys were additionally characterized by the occurrence of rosmarinic acid and ellagic acid. Comparing HCA and PCA technique obtained results that exhibited possible relationship to botanical origin of honey samples. Accordingly to the phenolic properties, honeydew honey samples were perfectly classified, while manuka and buckwheat or willow and heather samples partly overlap. Additionally, the similarity of honeys with large percentage of pollen type (W2–W3, B1) and with pollen of less than 45% (W1, B2–B4) was observed, which shows that the slightly lower value of pollen in honeys is not different from honey with dominance of one type pollen.

Conclusions

The low instrumentation price, short analysis time, wide range of adsorbents and solvents, small sample requirements for analysis, good precision, and accuracy make HPTLC a method of choice for the differentiation of the botanical origin of honey samples. In the present study, the HPTLC fingerprint analysis allowed the identification of the type of the following samples of honey: willow, buckwheat, heather, pine honeydew, and manuka. This showed that on the basis of the similarities and differences in the observed bands, it was possible to determine the botanical origin of certain honey. Additionally, 11 phenolic compounds were identified, and their presence was independently confirmed by an HPLC analysis. HPTLC has been proven to be a good method for the rapid separation of honey components and well suited for the identification of phenolic compounds present in honey samples. It should also be emphasized

that the HPTLC analysis of honey might assist in developing HPTLC-supported quality control tools and might improve consumer confidence and support to the product.

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Compliance with Ethical Standards

Conflict of Interest Natalia Stanek declares that she has no conflict of interest. Izabela Jasicka-Misiak declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

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References

- Badjah Hadj Ahmed AY, Wabaidur SM, Siddiqui MR, Alothman ZA, Obeid MS, Khan MR, Tamrah AL (2016) Simultaneous determination of twenty-five polyphenols in multifloral and cactus honeys using solid-phase extraction and high-performance liquid chromatography with photodiode array detection. *Eur Food Res Technol* 242:943–952
- Bogdanov S, Ruoff K, Oddo L (2004) Physico-chemical methods for the characterisation of unifloral honeys: a review. *Appidologie* 35:S4–S17
- Chuttong B, Chanbang Y, Sringarm K, Burgett M (2016) Physicochemicals profiles of singles bee (Apidae: Meliponini) honey from South East Asia (Thailand). *Food Chem* 192:149–155
- Ciulu M, Spano N, Pilo MI, Sanna G (2016) Recent advances in the analysis of phenolic compounds in unifloral honeys. *Molecules* 21:451–482
- Codex Alimentarius (2001) revised codex standards for honey. *Codex standard* 12–1981
- Dinca RF, Ionete RE, Popescu R, Costinel D, Radu GL (2015) Geographical and botanical origin of Romanian honey using complex stable isotope data and chemometrics. *Food Anal Methods* 8:401–412
- Fechner DC, Moresi AL, Diaz JDR, Pellerano RG, Vazquez FA (2016) Multivariate classification of honeys from Corrientes (Argentina) according to geographical origin based on physicochemical properties. *Food Biosci* 15:49–54
- Ferreres F, Andrade P, Tomas-Barberan FA (1996) Natural occurrence of abscisic acid in heather honey and floral nectar. *J Agric Food Chem* 44:2053–2056
- Główniak K, Skalicka K, Ludwiczuk A, Jop K (2005) Phenolic compounds in the flowers of *Lavatera trimestris* L. (Malvaceae). *J Planar Chromatogr* 18:264–268
- Jasicka-Misiak I, Poliwoda A, Dereń M, Kafarski P (2012) Phenolic compounds and abscisic acid as potential markers for the floral origin of two Polish unifloral honeys. *Food Chem* 131:1149–1156
- Jasicka-Misiak I, Makowicz E, Stanek N (2017) Polish yellow sweet clover (*Melilotus officinalis* L.) honey, chromatographic fingerprints and chemical markers. *Molecules* 22:138
- Jerkovic I, Kuś PM, Tuberoso CIG, Šarolic M (2014) Phytochemical and physical–chemical analysis of Polish willow (*Salix* spp.) honey: identification of the marker compounds. *Food Chem* 145:8–14
- Karabagias IK, Vavoura MV, Badeka A, Kontakos S, Kontamias MG (2014) Differentiation of Greek thyme honeys according to geographical origin based on the combination of phenolic compounds and conventional quality parameters using chemometrics. *Food Anal Methods* 7:2113–2121
- Kasprzyk I, Depciuch J, Grabek-Lejko D, Parlinska-Wojtan M (2018) FTIR-ATR spectroscopy of pollen and honey as tool for unifloral honey authentication. The case study of rape honey. *Food Control* 84:33–40
- Kečkeš S, Gašić U, Veličković TC, Milojković-Opsenica D, Natić M, Tešić Ž (2013) The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry. *Food Chem* 138:32–40
- Kuś PM, Congiu F, Teper D, Sroka Z, Jerković I, Tuberoso CIG (2014) Antioxidant activity, color characteristics, total phenol content and general HPLC fingerprints of six Polish unifloral honey types. *LWT Food Sci Technol* 55:124–130
- Locher C, Neumann J, Sostaric T (2017) Authentication of honeys of different floral origin via high-performance thin-layer chromatographic fingerprinting. *J Planar Chromatogr* 30:57–62
- Milojković Opsenica D, Ristivojević P, Trifković J, Vovk I, Lušić D, Tešić Ž (2016) TLC fingerprinting and pattern recognition methods in the assessment of authenticity of poplar-type propolis. *J Chromatogr Sci* 54:1077–1083
- Muruke MH (2014) Assessment of quality of Tanzanian honey based on physicochemical properties. *Food Sci* 33:61–72
- Oroian M, Sorina R (2017) Honey authentication based on physicochemical parameters and phenolic compounds. *Comp Electron Agric* 138:148–156
- Pascual-Maté A, Osés SM, Fernández-Muñoz MA, Sancho MT (2017) Analysis of polyphenols in honey: extraction, separation, and quantification procedures. *J Sep Purif* 16:1–17
- Pasini F, Gardini S, Marcuzzan GL, Caboni MF (2013) Buckwheat honeys: screening of composition and properties. *Food Chem* 141:2802–2811
- PN-88/A-77626 (1998) Miód pszczele. [Polish Quality Standard “Honey”] Dziennik Norm i Miar nr 8, Wydawnictwo Normalizacyjne Alfa
- Pyrzyńska K, Biesaga M (2009) Analysis of phenolic acids and flavonoids in honey. *Trends Anal Chem* 28:893–902
- Ristivojević P, Trifković J, Vovk I, Milojković-Opsenica D (2017) Comparative study of different approaches for multivariate image analysis in HPTLC fingerprinting of natural products such as plant resin. *Talanta* 162:72–79
- Semkiw P, Skowronek W, Skubida P (2008) Changes in water content of honey during ripening under controlled condition. *J Apic Sci* 52:57–63
- Socha R, Juszczak L, Pietrzyk S, Gałkowska D, Fortuna T, Witeczak T (2011) Phenolic profile and antioxidant properties of Polish honeys. *Int J Food Sci Technol* 46:528–534
- Tuberoso CIG, Jerkovic I, Bifulcoa E, Marijanovic Z (2011) Biodiversity of *Salix* spp. honeydew and nectar honeys determined by RP-HPLC and evaluation of their antioxidant capacity. *Chem Biodivers* 8:872–879
- Waterman PG (1994) *Methods in Ecology. Analysis of Phenolic Plant Metabolites*. Oxford, pp 151–154
- Yao L, Datta N, Tomas-Barberan FA, Ferreres F, Martos I, Singanusong R (2003) Flavonoids, phenolic acids and abscisic acid in Australian and New Zealand Leptospermum honeys. *Food Chem* 81:159–168
- Żyska B, Anioł M, Lipok J (2017) Highly effective, region specific reduction of chalcone by cyanobacteria leads to the formation of dihydrochalcone: two steps towards natural sweetness. *Microb Cell Fact* 16:136–151