

Quantitative and qualitative determination of phenolic compounds in honey

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The research on bioactive compounds of natural origin has gained much interest in the last years. The aim of the present study was a quantitative and qualitative analysis of honey phenolic compounds. Four honey samples (collected in Poland) from lime trees, rape, buckwheat and heather were selected for the analysis. Phenolic compounds were extracted with methanol by solid phase extraction. The total content of phenolic compounds was tested by the Folin–Ciocalteu method, and the total content of flavonoids was also determined. The qualitative analysis of honey phenolic compounds was performed by high performance liquid chromatography (HPLC).

The total content of phenolic compounds in honey samples varied from 71.7 to 202.6 $\mu\text{g/g}$. Not unexpectedly, the highest content of phenolic compounds was found in darker honeys (buckwheat and heather samples). The range of the total content of flavonoids was lower compared to the total content of phenolic compounds and varied from 13.5 to 44.5 $\mu\text{g/g}$. The ratio between the total content of phenolic compounds and flavonoids in buckwheat, heather, lime, and rape honey was 4.8, 4.5, 4.7 and 5.3, respectively, i.e. the major part of phenolic compounds in honey samples consisted of compounds other than flavonoids. Chromatographic analysis revealed that non-flavonoid compounds – (phenolic acids) prevailed in the samples. The phenolic compounds identified in the honey samples were caffeic, ferulic and benzoic acids, rutin, quercetin, kaempferol. Some peaks in the chromatograms were not identified.

Introduction

The research on bioactive compounds of natural origin has gained much interest in the last years. It is generally accepted that a natural product is healthier than that of synthetic origin. However, this question is still open for discussions, and many studies on this subject are carried out [1, 2]. Nevertheless, some natural products (bee products, some herbs, garlic, etc.) have been used in folk medicine since ancient times, and it is important to ascertain the origin of biological activity in order to apply products for human wellbeing.

Honey was selected for the analysis because it has been produced in many countries since olden times. It is the most popular and the easiest available bee product highly appreciated by people. Honey consists of carbohydrates, amino acids, proteins (including enzymes), organic acids, vitamins, minerals and various phytochemicals [3]. Honey is an important and unique food product containing bioactive compounds derived from bees and plants. It is rich in phenolic acids and flavonoids, which exhibit a wide range of biological effects and act as natural antioxidants. Honey was found to exhibit peroxyl-scavenging capacity [4], radical scavenging capacity [5], anticancer and wound healing properties [6]. The composition of honey phenolic compounds depends on the floral source used to collect nectar, seasonal and environmental factors, geographic origin, storage conditions;

also, the processing may also have an effect on honey antioxidant activity which is due to the composition of phenolic compounds [7, 8].

The variation of the botanical composition of honey is very wide; for this reason, honeys differ not only by their chemical composition (volatile compounds, carbohydrates and phytochemicals), physical properties (colour, viscosity, hygroscopic properties and pH) and taste, but also by biological activity – some honeys have a stronger biological activity than others. Consequently, it can be reasonably expected that honey composition and properties from various locations may be different. For example, honey from the blossoms of the manuka bush, a native of New Zealand, because of its specific antibacterial properties became well-known all over the world and is called “active manuka honey” [9]. It is considered that plant pollen and climatic conditions have the highest effect on the properties and phytochemical composition of honey. The main phytochemicals reported in honey are phenolic compounds. The total content of phenolic compounds, depending on the botanical source of honey and collection region, varies from 46.0 to 753.0 $\mu\text{g/g}$ [4, 6, 10]. For example, total polyphenols in Portugal honeys (from rosemary, viper’s bugloss and heather) varied from 132.0 to 728.0 $\mu\text{g/g}$ depending on sample preparation (entire honey or honey extract); the lowest values were found in honey extracts, probably due to the lost of some compounds during extraction [11]. In herbhoneys from Romania, the total content of phenolic compounds was

20.0–450.0 µg/g [12], Gheldof et al. [4] tested non-European honeys and found that phenolic compound extracts and total phenolic compound content in them varied from 46.0 to 456.0 µg/g (the highest content was obtained in buckwheat honey). Flavonoids mostly pinobanksin, pinocembrin, quercetin, chrysin, galangin, luteolin and kaempferol are present in honey [4, 13], while pinocembrin, pinobanksin and chrysin are characteristic flavonoids of propolis; these flavonoids were determined in most of the previously analysed European honey samples [14].

The aim of this research work was to perform a quantitative and qualitative analysis of phenolic compounds in honey samples collected from four different botanical sources in Poland.

Materials and methods

RAW MATERIALS. Honey samples were collected in Poland in 2008 during the flowering season in Krajanka (in the north-west part of the country).

Four samples of monofloral rape (*Brassica napus*), lime tree (*Tilia* sp.), buckwheat (*Fagopyrum esculentum*) and heather (*Calluna vulgaris*) honey were selected for the analysis. The botanical origin was selected according to the claim of apiarists. The honey samples were stored at room temperature before analysis.

EXTRACTION OF PHENOLIC COMPOUNDS. Solid phase extraction (SPE) was used for the extraction of phenolic compounds. In brief, 5 g of honey was dissolved in 50 ml of acidified deionised water (pH 2.0 achieved with trifluoroacetic acid), and the phenolics were adsorbed into preconditioned C18 cartridge (Supelco, Supelclean LC-18, 0.5 g, Bellefonte PA, USA). The cartridges were preconditioned by sequentially passing 3 ml each of methanol and acidified water (pH 2.0) at a drop-wise flow rate. 10 ml of aqueous honey solution was passed through the preconditioned cartridges at a drop-wise flow rate to ensure an efficient adsorption of phenolic compounds. The adsorbed phenolics were then eluted from the cartridges by passing 1.5 ml of 90% (v/v) methanol/water solution at a drop-wise flow rate. Extraction was repeated 3 times. Methanol extracts of honey phenolic compounds were stored in a refrigerator and analysed by spectrophotometric methods and by high performance liquid chromatography.

SPECTROPHOTOMETRIC METHODS. The total content of phenolic compounds. Determination of the total content of phenolic compounds in honey extracts was determined by the Folin–Ciocalteu method [15]. A volume of 2.5 ml of distilled water and 0.1 ml of a honey extract were added to a test tube, followed by addition of 0.1 ml of undiluted commercially available Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA). The solution was mixed well and then allowed to stand for 6 min before 0.5 ml of a 20% sodium carbonate solution was added. The colour was developed during 30 min at room (20 °C) temperature, and the absorbance was mea-

sured at 760 nm using a spectrophotometer (Milton Roy Spectronic 1201, USA). A blank sample was prepared using 0.1 ml of methanol instead of extract. The measurement was compared to a calibration curve of gallic acid solutions and expressed as gallic acid equivalents per gram of honey. The total content of phenolic compounds in honey samples was calculated according to the formula:

$$C = \frac{c \cdot V}{m};$$

where C is the total content of phenolic compounds, mg/g honey, expressed as gallic acid equivalent; c is the concentration of gallic acid established from the calibration curve, mg/ml; V is the volume of honey solution, ml; m is the weight of honey, g.

Determination of the content of flavonoids. The total content of flavonoids in honey extracts was determined by the Pharmacopoeia method [16]. The total content of flavonoids was determined using rutin as a reference compound. 0.08 ml of honey phenolic compound extract in methanol was mixed with 0.8 ml methanol, 0.04 ml of 33% acetic acid, 0.16 ml of 5% hexamethylene tetramine, 0.12 ml of 10% $AlCl_3$ and 0.8 ml of water. The absorption at 407 nm was read after 30 min at 20 °C. A blank sample was also prepared from 0.08 ml of plant extract, 0.8 ml of methanol, 0.04 ml of 33% acetic acid and 1.08 ml of water. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 25 mg of rutin. The amount of flavonoids in honey in rutin equivalents was calculated by the following formula:

$$X = \frac{m_r \cdot A \cdot V}{m \cdot A_r \cdot V_r};$$

where X is flavonoid amount, mg/g honey; A is the absorption of honey extract solution; A_r is the absorption of standard rutin solution; m is the weight of honey, g; m_r is the weight of rutin in the solution, mg; V is total volume of phenolic compounds extract, ml; V_r is volume of rutin solution, ml.

Honey colour. Honey samples were heated up to 40 °C to dissolve sugar crystals, and the colour was determined by spectrophotometric measurement of the absorbance of a 50% honey solution (w/v) at 635 nm. The honeys were classified according to the Pfund scale after conversion of the absorbance (Abs) values:

$$mmPfund = -38.70 + 371.39 \times Abs; \quad [17]$$

where $mmPfund$ is the intensity of honey colour in the Pfund scale; Abs is the absorption of honey solution.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC). The analysis of honey extracts was performed employing the Shimadzu HPLC system with a UV detector. Honey phenolic compounds were separated on a LiChro-CART RP-18 analytical column, 150 × 4.0 mm i.d. (Merck, Dortmund, Germany) packed with C_{18} stationary phase, particle size 5 µm. The linear

gradient was used at a flow rate of 0.5 ml/min. The time of HPLC run was over 35 min. The binary mobile phase consisted of a solvent A (ultra pure water with 0.1% of phosphoric acid) and solvent B (pure methanol with 0.1% of phosphoric acid). Elution from the column was achieved with the following gradient: 0 min to 10 min B increased from 35% to 55%; 10–25 min B increased to 62%; 25–30 min B increased to 85% and kept constant till 35 min. The UV detector was operating at a wavelength of 265 nm.

The identification of phenolic compounds was performed by comparing the retention time of analytes and reference compounds. Phenolic acids (benzoic, caffeic, chlorogenic, ferulic, and gallic) and flavonoids (rutin, quercetin, kaempferol, isorhamnetin, apigenin, pinocembrin, sakurin, rhamnetin, chrysin, galangin, 7-methyloether pinocembrin, tectochrysin), commonly found in honey samples, were used as reference compounds. All reagents were obtained from Sigma (St. Louis, MO, USA).

STATISTICAL ANALYSIS. All experiments were repeated in triplicate, except for colour measurement (two

times). All values are expressed as the mean \pm standard deviation. Standard deviations were calculated using spreadsheet software (Excel[®]). The correlation coefficient to determine the relationship between honey colour and phenolic compound content was calculated using MS Excel[®] software (CORREL statistical function).

Results and discussion

Natural honey varies in colour from water white (clover, sage honeys) to dark (honeydew, heather, chestnut honeys), depending on its floral source [3]. The colour of honey is related to its mineral content. The colour of the test honeys varied from white (rape honey) to amber (buckwheat and heather honey) (Table 1). Several methods exist for the quantitative evaluation of honey colour. In the industry, honey colour is usually expressed in mm Pfund scale. The colour of honey can be assessed using a Lovibond 2000 visual comparator system [17], CIEXYZ or CIELAB methods [18].

Table 1. Colour of tested honey samples

Honey origin	Visual evaluation of colour	Colour intensity (mm Pfund scale)	Literature data of colour intensity (mm Pfund scale)
Buckwheat	Amber	72.7	n. a.
Heather	Amber	77.4	63.8–90.0 [3]
Lime	Light amber	69.8	11.0–55.0 [3]
Rape	Light (white)	18.1	20.0–34.3 [3]

n. a. – not available.

As mentioned before, the colour of honey depends on its floral source due to minerals and other minor components. The colour of honey is closely related to its chemical composition, primarily to the presence of pigments such as chlorophylls, carotenoids, flavonoids and derivatives of tannins and polyphenols. Darker honeys possess a higher antioxidant activity [5, 10] which mostly is due to the content and composition of phenolic compounds. The phenolic compounds were assessed quantitatively by the Folin–Ciocalteu method and qualitatively by using the HPLC analysis.

The total content of phenolic compounds in the honey samples varied from 71.7 to 202.6 $\mu\text{g/g}$ (Table 2). The highest content of phenolic compounds, as expected, was found in darker honeys (buckwheat and heather), while the lowest amount of phenolic compounds was found in rape honey (lightest honey from the tested samples). A correlation was found between the colour of honey (in mm Pfund scale) and the content of phenolic compounds (correlation coefficient 0.95).

Table 2. Total content of phenolic compounds (expressed as gallic acid equivalent) and flavonoids (expressed as rutin equivalent) in the honey samples

Honey origin	Total content of phenolic compounds, $\mu\text{g/g}$	Total content of flavonoids, $\mu\text{g/g}$	Content of phenolic compound / Content of flavonoids
Buckwheat	201.6 \pm 16.8	41.7 \pm 2.1	4.8
Heather	201.2 \pm 5.5	44.5 \pm 3.2	4.5
Lime	153.1 \pm 5.5	32.0 \pm 1.7	4.7
Rape	71.7 \pm 1.3	13.5 \pm 1.3	5.3

In another study from Poland, the pure herbhoneys the content of phenolic compounds ranged from 217.0 to

753.0 $\mu\text{g/g}$ (with the lowest content in camomile honey and the highest in raspberry honey) [10]. In the same

study, the total content of flavonoids varied from 69.0 to 285.0 $\mu\text{g/g}$ expressed as a quercetin equivalent (the lowest and the highest values were also found for camomile and raspberry honeys, respectively). Remarkably lower values of flavonoids were found in Romanian herbhoneys (9.1–153.3 $\mu\text{g/g}$ expressed as a quercetin equivalent). In our study, the range of the total content of flavonoids was lower than in other honeys originated from Poland, as reported by Socha et al. [10] and similar to honeys from Romania [8], and varied from 13.5 to 44.5 $\mu\text{g/g}$ (Table 2). Nevertheless, it is difficult to compare the results, since in our study the total content of flavonoids is expressed as a rutin equivalent

As can be concluded from the results, honey is a source of antioxidants; however, the content of polyphenols in honey is not very high in comparison with some fruits or vegetables (for example, plums contain 3686.6 $\mu\text{g/g}$ of polyphenols, strawberries 2250.0 $\mu\text{g/g}$, apples 1183.0 $\mu\text{g/g}$, asparagus 641.5 $\mu\text{g/g}$ [19]), because carbohydrates (mainly fructose and glucose) are dominant in honeys. Nevertheless, some types of honey exceed the content of phenolic compounds in some fruits or vegetables. For example, in our case, buckwheat and heather honeys had a higher content of phenolic compounds compared with honeydew melon (114.5 $\mu\text{g/g}$), carrots and mushrooms (84.0 and 112.5 $\mu\text{g/g}$, respectively) [19].

The ratio of the total content of phenolic compounds and flavonoids was calculated in order to evaluate the

distribution of flavonoids and non-flavonoid compounds in honey. The ratio was about 5 in all samples (Table 2). It means that the content of non-flavonoid compounds (mostly various phenolic acids) in the honey samples was about five times higher than the content of flavonoids. The literature data show that the ratio of the total content of polyphenols and flavonoids varies from 2 to 20 depending on the floral source of honey [10, 12]. The differences between the ratio of the total content of phenolic compounds and flavonoids suggest that the composition of phenolic compounds in honey may have a strong correlation with the botanical source of honey. The composition profile of phenolic compounds was analysed by HPLC.

Chromatographic analysis also showed that non-flavonoid compounds – phenolic acids – prevailed in the samples. The composition and content of individual phenolic compounds in the samples was slightly different and depended on the botanical origin of honeys. Figures 1 and 2 represent HPLC chromatogram profiles of rape and heather honeys. As one can see, first of all the intensity of peaks is different, which means a different content of phenolic compounds in these kinds of honey. These results coincide with the results obtained by the Folin–Ciocalteu method (Table 2): heather honey contained a high amount of phenolic compounds, while in rape honey was the lowest.

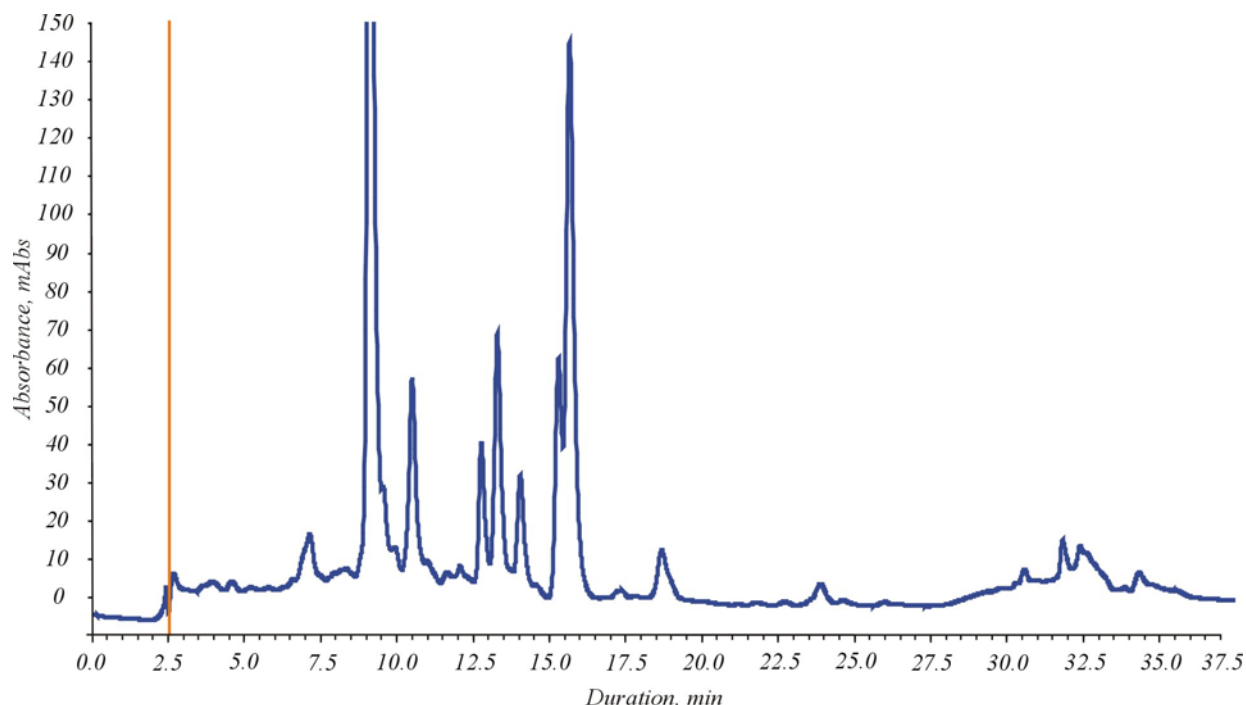


Fig. 1. HPLC chromatogram of heather honey extract at 265 nm

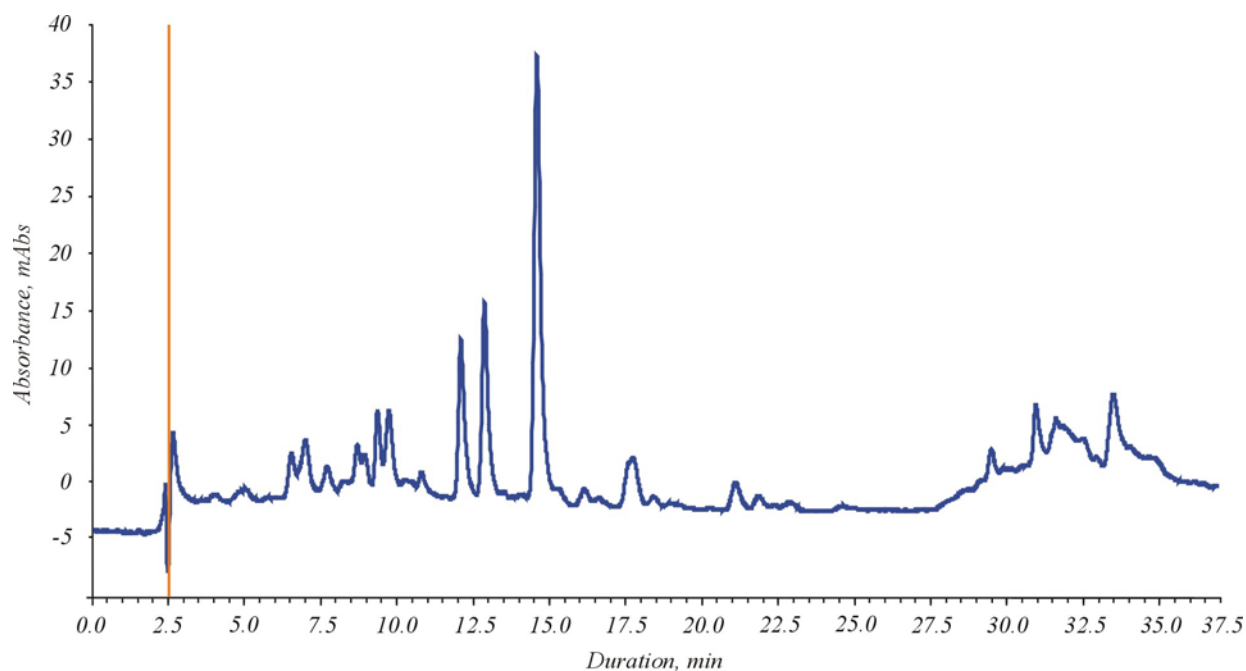


Fig. 2. HPLC chromatogram of rape honey extract at 265 nm

Twelve different compounds from 17 reference compounds were identified in the honey samples. Some peaks in the chromatograms were not identified due to the lack of standards. The composition of phenolic compounds in the honey samples was different: some compounds (for example, gallic and chlorogenic acids, quercetin, isorhamnetin and galangin) were found only in one sample, while several compounds were identified in two or three honeys, and only two compounds (caffeic acid and kaempferol) were found in all samples (Table 3). Lime-tree,

rape and heather honeys had a similar composition of phenolic acids. Rutin and kaempferol were found in these sorts of honey. Lime-tree honey was distinguished by isorhamnetin and rape honey by galangin. Buckwheat honey contained three compounds that were not identified in the rest of samples: gallic and chlorogenic acids and quercetin. The chemical structure of the determined phenolic acids and flavonoids is shown in Figures 3 and 4, respectively.

Table 3. Phenolic compounds identified in the honey samples

Reference compound	Honey origin			
	Lime-tree	Rape	Heather	Buckwheat
Gallic acid	-	-	-	+
Chlorogenic acid	-	-	-	+
Caffeic acid	+	+	+	+
Ferulic acid	+	+	+	-
Benzoic acid	+	+	+	-
Rutin	+	+	+	-
Quercetin	-	-	-	+
Kaempferol	+	+	+	+
Isorhamnetin	+	-	-	-
Apigenin	-	-	-	-
Pinocembrin	-	+	+	+
Sakurin	-	-	-	-
Rhamnetin	-	-	-	-
Chrysin	-	-	+	+
Galangin	-	+	-	-
7-methyloether pinocembrin	-	-	-	-
Tectochrysin	-	-	-	-

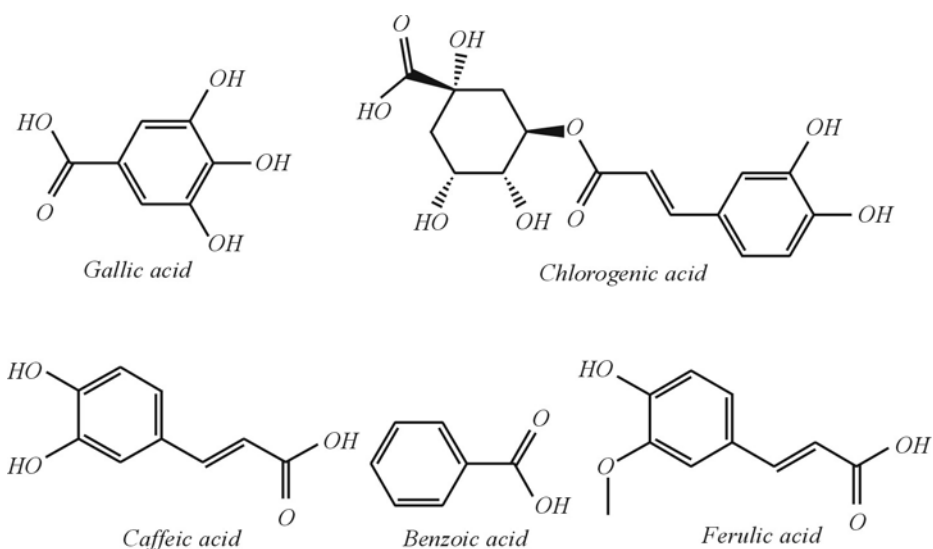


Fig. 3. Structure of phenolic acids identified in the honey samples

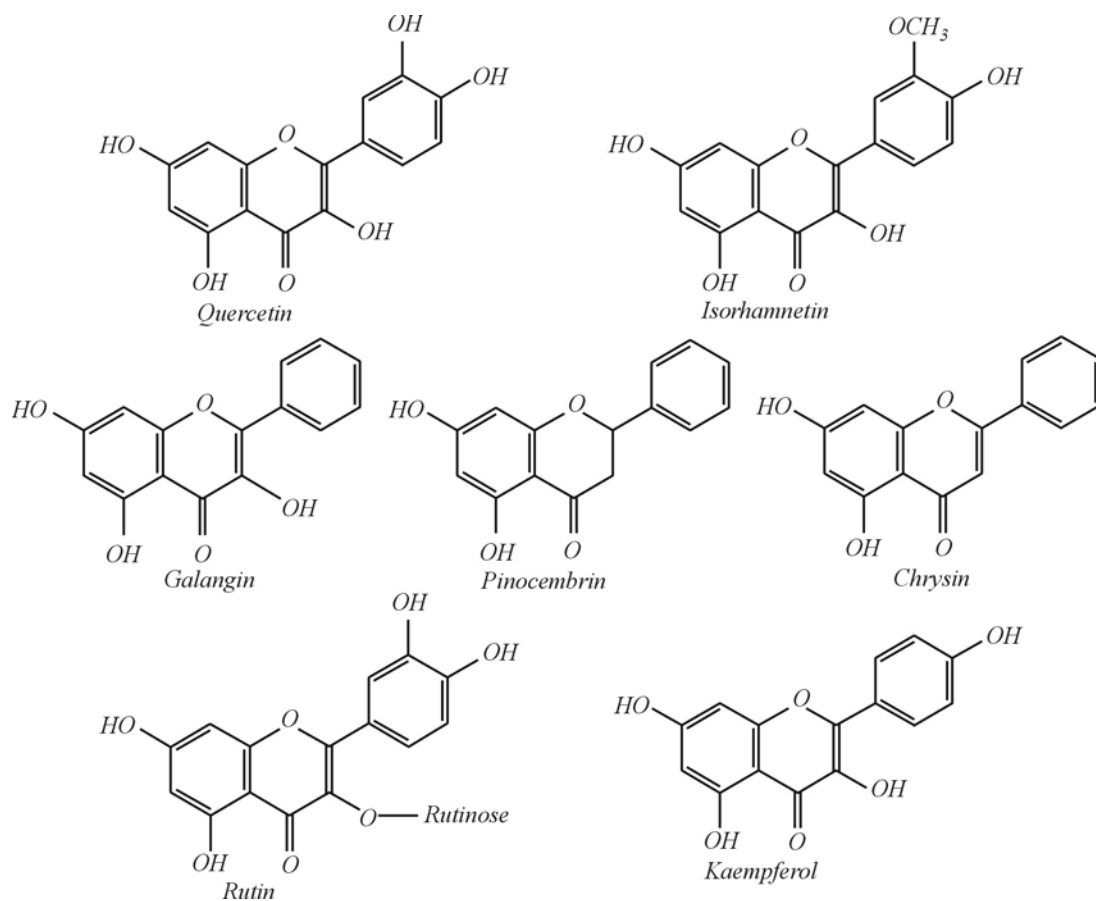


Fig. 4. Structure of flavonoids found in the honey samples

These compounds were found also in various honeys by other researchers [4, 10, 20]. Michalkiewicz et al. [21] analysed lime-tree and heather honeys, also from Poland. The flavonoids rutin and kaempferol were found in our honeys, while quercetin was not found in these kinds of honey; also, the composition of phenolic acid was slightly different. Gallic, *p*-coumaric, vanillic and syringic acids were not determined in our samples. Michal-

kiewicz et al. [21] used a different extraction column (Oasis HLB). Since some peaks in our chromatograms were not identified due to the lack of reference compounds, possibly several compounds identified by Michalkiewicz et al. can be present also in our samples.

As previously mentioned, honey is a unique product, and its composition highly depends on the floral source, geographic origin, climatic conditions, even bee

species. Also, essential are the sample preparation [11, 20] and analysis methods used for the separation of analysed compounds [22].

The flavonoid quercetin was determined in many honeys [5, 10, 20], however, in our case, this compound was found only in one out of four samples. A significant content of quercetin (54.0–286.2 µg/100 g) is suggested as a possible floral marker of rapeseed and sunflower honeys [20], but it was not found in our sample of rape honey.

Ellagic acid is regarded as a floral marker of heather honey [20]. Unfortunately, due to the lack of reference compound, we cannot confirm this finding.

The origin of phenolic compounds in honey is not very clear. Several compounds attributed to propolis were found in the samples (caffeic and ferulic acids, kaempferol, pinocembrin, chrysin). It is considered that some compounds appear in honey directly from the pollen of corresponding plants. However, rutin, which is responsible for the antioxidant activity of buckwheat [23], was not found in our sample of buckwheat honey. Neither was this flavonoid found in buckwheat honey tested by Gheldof et al. [4]. Another possible explanation could be that the identification of honey source was wrong. Usually, beekeepers like to name honey according to the plants that bloomed before its collection, because a precise determination of the botanical source of honey is difficult and requires an expert and special equipment [24].

Conclusions

The total content of phenolic compounds in the honey samples varied from 71.7 to 202.6 µg/g. The content of phenolic compounds was highest in darker (buckwheat and heather) honey samples. The intensity of honey colour correlated with the total content of phenolic compounds. The range of total content of flavonoids was five times lower than the total content of phenolic compounds and varied from 13.5 to 44.5 µg/g. Phenolic acids prevailed in the samples. Caffeic, ferulic and benzoic acids, rutin, kaempferol and pinocembrin were identified in most of the test samples; however, the content of individual compounds was different.

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KIEKYBINIS IR KOKYBINIS MEDAUS FENOLINIŲ JUNGINIŲ NUSTATYMAS

S a n t r a u k a

Pastaraisiais metais ypač tiriamos biologiškai aktyvios natūralios kilmės medžiagos. Šio darbo tikslas – atlikti kiekybinę ir kokybinę medaus fenolio junginių analizę. Buvo tirtas rapsų, liepų, grikių ir viršių medus, surinktas Lenkijoje. Fenolio junginiai išekstrahuoti kietafazės ekstrakcijos būdu. Folin-Ciocalteu metodu nustatytas bendras fenolio junginių kiekis; taip pat nustatytas bendras flavonoidų kiekis. Kokybinė fenolio junginių analizė atlikta panaudojant efektyviają skysčių chromatografiją.

Bendras fenolio junginių kiekis tirtų rūšių meduje kito nuo 71,7 iki 202,6 µg/g. Didžiausias šių junginių kiekis nustatytas tamsiame meduje – grikių ir viršių. Bendras flavonoidų kiekis buvo mažesnis (13,5–44,5 µg/g). Fenolio junginių ir flavonoidų kiekio santykis grikių, viršių, liepų ir rapsų meduje buvo atitinkamai 4,8, 4,5, 4,7 ir 5,3. Galima daryti išvadą, kad didžiausią fenolinių junginių dalį tirtame meduje sudarė ne flavonoidai, o kiti junginiai. Chromatografinė analizė taip pat parodė, kad mėginiuose vyravo fenolio rūgštys. Mėginiuose buvo identifikuotos benzkarboksirūgštis, kavos ir ferulo rūgštys, rutinas, kvercetas, kamferolis. Kai kurios chromatogramų smailės nebuvo identifikuotos.