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FREE PHENOLIC ACIDS, FLAVONOIDS AND ABSCISIC ACID RELATED to HPLC SUGAR PROFILE IN ACACIA HONEY

Otilia Bobis, L. Al. Mărghitaş, Victorița Bonta, D. Dezmirean, O. Maghear

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca
Mănăstur str. 3, Cluj-Napoca, E-mail: otilia63@yahoo.com

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Abstract. A high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) method was adapted to investigate the polyphenol composition (free phenolic acids, flavonoids and abscisic acid) in honey samples belonging to black locust type.

The saccharide profile of black locust honey was also registered, using a HPLC method with UV detection, according to International Honey Commision analysis methods.

The phenolic acids found in this type of honey were: para-hidroxybenzoic, vanillic, para-coumaric, ferulic, trans-cinnamic acids,as well as vanillin, pinobanksin, apigenin, kaempherol, pinocembrin, crysina and acacetin. Abscisic acid was also identified in black locust honey**.**

Monosaccharides fructose and glucose were the most abundant sugars (mean 42.39±2.73 g% and 31.93±2.6 g% respectively), while oligosaccharides maltose, sucrose and trehalose, represent 6.43 % from the total saccharides present in honey samples.

Multivariate statistical techniques were used to investigate whether polyphenolic and sugar profiles could be used as a basis for identification of floral sources of honey.

INTRODUCTION

The composition of honey depends on the floral source as well as on some external factors such as: season, environmental factors, physiological stage of the bees and honey processing.

Even thow honey main constituents are sugars and water, there are some small quantity components in the composition of honey that are responsible for honey properties. The components of honey that have antioxidant properties are phenolic acids and flavonoids (Ferreres et al., 1992; Andrade et al., 1997), enzymes like catalase and glucose-oxidase, ascorbic acid (White, 1975), organic acids, amino-acids and proteins .

Several studies made on honey show that European honeys have a rich phenolic profile, consisting of benzoic, cinnamic acids and flavonoid aglycones (Ferreres et al., 1992; Andrade et al., 1997; Ferreres et al., 1994; Martos et al., 2000, Tomas-Barberan et al., 2001).

Fructose and glucose, the major sugars present in honey (Mărghitaş 2002; Da Costa & col. 2000; Costa & col. 1999; Doner 1977; Siddiqui 1970) can be determined titrimetrically or spectrophotometrically (Gonnet 1973), but the content of minor carbohydrates in honey can be determined accurately by liquid chromatography (Swallow & Low 1990; Godall & col. 1995; Weston and Brocklebanc 1999).

Our research team has made studies on components that might have antioxidant power (total phenolisc and total flavonoids)(Bobiş si col., 2005, 2006, 2007), but not on individual phenolic acids and flavonoids, and the literature indicates that no previous studies have been done on Romanian honeys in this respect. Also HPLC method was used previously to evaluate the sugar profile in Romania honey (Bobiş şi col.,2006; Bonta şi col., 2007).

The main objective of this study was to evaluate and quantify the composition of black locust honey from Romania in respect of phenolic and flavonoid content, free phenolic acids and flavonoids in correlation with the sugar profile by HPLC from the same type of honey.

MATERIAL AND METHOD

Honey samples

Black locust honey samples of different locations from Transilvania were obtained from individual beekeepers, associations (raw honey) and commercial honey (processed).

Chemicals and standards

The protocol for obtaining the phenolic extracts from honey followed the scheme showed in figure 1.

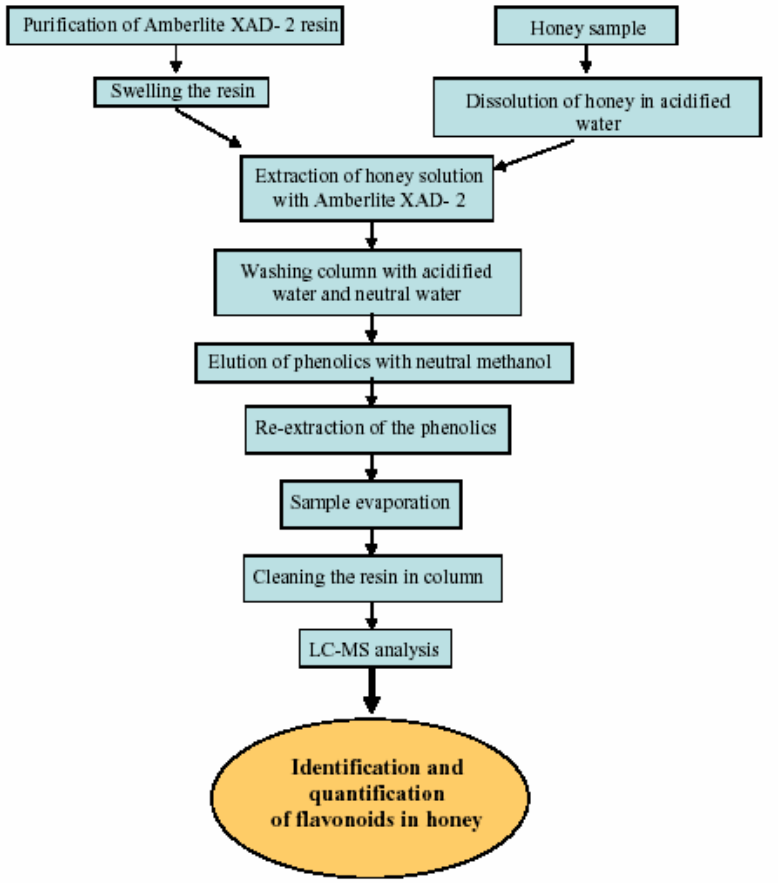

Fig.1. Extraction and analysis of flabonoids and phenolic acids from honey

The solvents for the column chromatography were deionized water, analytical grade HCl and methanol. The solvents used for re-extraction of polyphenols were water and ethyl acetate. The solvents for HPLC analysis of phenolic acids were deionized water, methanol and acetic acid. For sugar analysis by HPLC methanol and ultra pure water was used also.

Phenolic acids, flavonoids and sugar standards were purchased from Sigma Chemical Company.

Procedure

Botanical origin of the honey samples was verified by pollen analysis in accordance with the Harmonised Methods of the International Honey Commission.

The original method for phenolic acid determination was carried out as described by Martos and col. (1997) and Yao (2002). A solution of methanol and deionised water was

added to Amberlite resin in a flask and left overnight to ensure the complete swelling of the resin.

Liquefied honey, was mixed with acidified water, adjusted to pH 2 with concentrated HCl for 30 minutes until completely dissolved, The resulting honey solution was mixed with swelled Amberlite and stirred slowly on a magnetic stirrer for 60 min. The resulting slurry was packed in a glass column and the sugars were washed from the column with acidified water, followed by rinsing with deionised water to remove all the polar constituents of honey.

The phenolic compounds adsorbed on the column were eluted with neutralized methanol. This extract was concentrated to dryness on a rotary evaporator. Next, the residue was redissolved in deionised water and extracted with ethyl acetate 3 times, in a separation funnel. The erhyl acetate extracts were combined and evaporated to dryness. LC-MS grade methanol was used for redisolving the extracts for chromatographic analysis.

A 10% honey solution in ultra pure water was prepared from each sample and filtered through a Millipore syringe filter for HPLC sugar analysis.

Calibration curves of available standards were made for quantifying the phenolic acids, flavonoids and individual sugars.

The LC-MS system for polyphenol analysis (Agilent system 1100 LC-MSD) consist of: DAD detector with variable wavelength, 1100 6-portautoinjector valve, Phenomenex C18 (ODS, Octadecyl) precolumn, Phenomenex Luna C18 (2) 100 Å column, maintained at 35 ºC

MS Equipmen (Agilent system 1100) consist in : MS Agilent 1100 (LC-MSD) detector, mass spectral selected ion recording (SIR) was applied, based on m/z values (molecular weight), controlled by Agilent v.A.09.03 software.

Separation method use water/acetic acid (solvent A) and methanol (solvent B) as mobile phases, in 1 ml/min flow rate.

HPLC sugar analysis were carried out in a system equipped with a LC-10AD pump, DGU-14A degasser, CTO-10AS VP column oven, SIL-10AV VP auto injector, SCL-10A VP system controller and a RID-10A refractive index detector. Separation of the sugars was made on a Alltech (Altima amino 100a 5 µm , containing amino modified silicagel), and acetonitril/water mixture (75/25 v/v) a mobile phase, in 1.3 ml/min flow rate..

Statistical analysis of data was performed by one-way analysis of variance.

RESULTS AND DISCUSIONS

Polyphenol composition of honey samples

In black locust investigated honey samples our studies revealed: para-hidroxybenzoic, vanillic, para-coumaric, ferulic, trans-cinnamic acids,as well as vanillin, pinobanksin, apigenin, kaempherol, pinocembrin, crysina and acacetin. Abscisic acid was also identified in Acacia honey**.**

Individual phenolic acids and flavonoids were identified according to retention time of the standards, shape of UV spectra and MS fragmentation ($M-H^-$).

From the 13 compounds identified in investigated black locust honey samples, 6 of them were present in all the samples (100% ferulic acid, abscisic acid, pinobanksine, pinocembrine, crysine and acacetine), 4 compounds were present in 50% of the samples (p-hydroxibenzoic acid, t-cinnamic acid, kaempherol and apigenine) and 3 compounds were present in 25% of the samples (vanillic acid, p-coumaric acid and vanilline). The same results obtained Tomas-Barberan and col. (2001) in analysis of phenolic compounds profile: not all the compounds were present

in all samples from the same type and semnificative differences between quantified amount of the same compound.

In figure 2 it is presented a chromatogram registered at 280 nm in LC-MS, from a black locust honey sample.

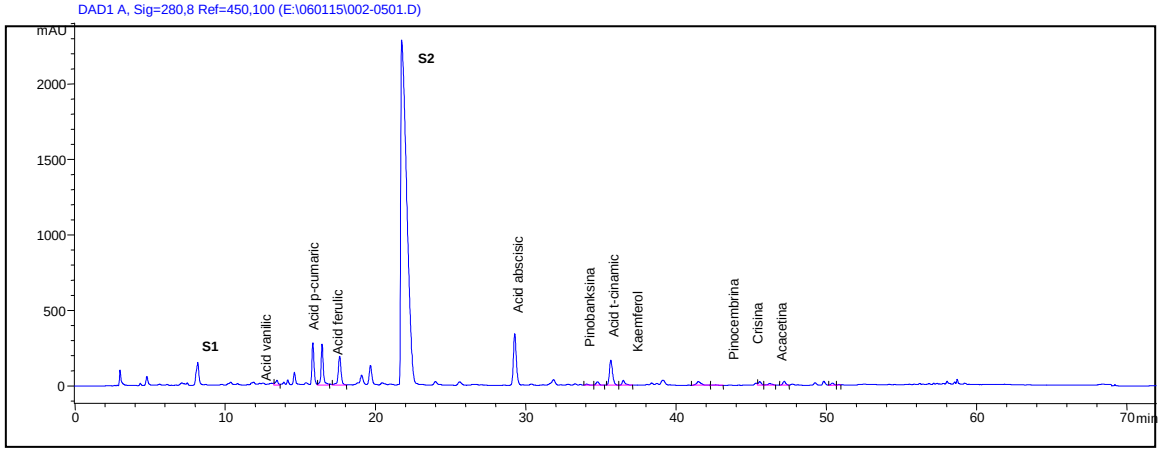

Fig.2. Black locust honey chromatogram and the identified polyphenol compounds

As it can be seen from the chromatogram, abscisic acid was quantified in the highest amount in the samples (mean of 16.16 mg/kg honey). Abscisic acid is not a phenolic acid, but it has similat behaviour in UV (presenting two absorbtion maxima at 238 and 260 nm). The plant hormone abscisic acid (ABA) is the major player in mediating the adaptation of the plant to stress.

Ferulic acid was quantified also in high amounts (3.23 mg/kg), pinobanksin (1,38 mg/kg), and pinocembrin, crysin and acacetin, in amounts below 1 mg/kg honey.

In figure 3 it is presented the distribution of common identified phenolic acids and flavonoids in honey samples.

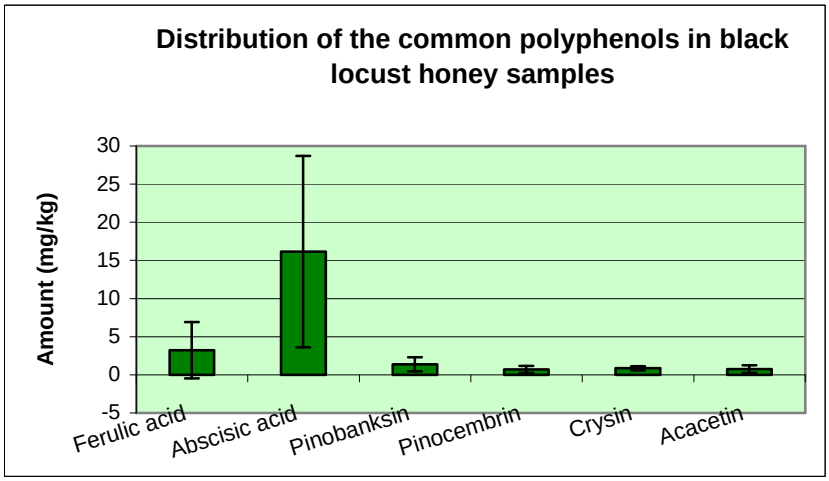

Fig.3. Common phenolic acids and flavonoids distribution in black locust honey

Comparing the data obtained from black locust honey and other types of honey (lime tree, raspberry, sunflower, multifloral, honeydew), we noticed that there are two compounds found only in black locust honey. These compounds are: **ferulic acid** and

acacetin. We conclude that these two could have, together with other specific tests, role of markers for this type of honey.

One way ANOVA Testul showed statistic signigicance (***) between the means of the common phenolic acids and flavonoids identified and quantified in honey samples.

Saccharide composition of honey samples

With the HPLC method used for determining the profile of sugars from honey, we could identify and quantify 5 sugars.

In figure 4 it is presented a chromatogram obtained at HPLC determination of sugars in analysed honey samples.

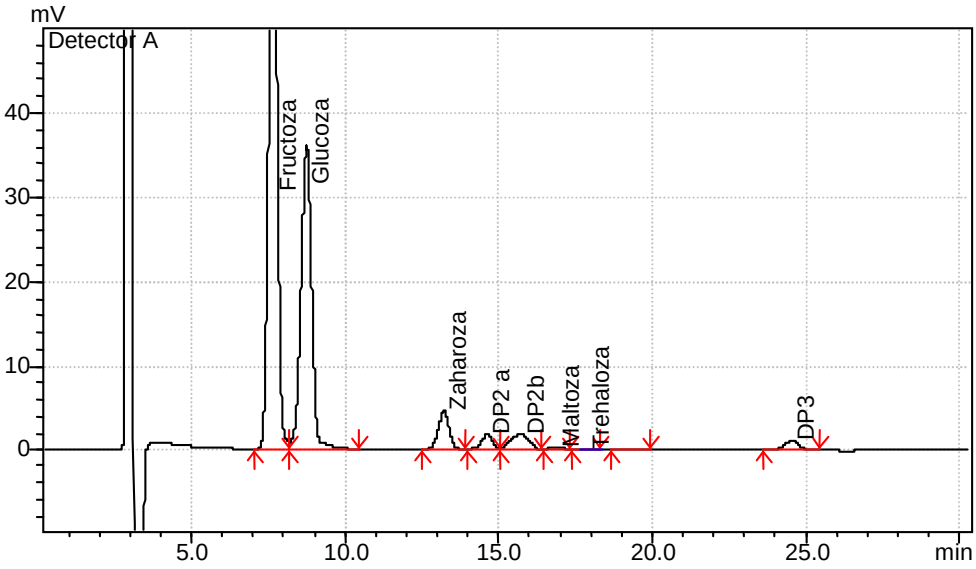

Fig. 4. Saccharide profile in *Acacia* honey

Fructose (42.39±2.73 g%) and glucose (31.93±2.6g%) are the most abundant monosaccharides present in all honey samples.

Disaccharides maltose (2.94±0,98 g%) and sucrose (2.16±2.63 g%) are the most abundant from this class of sugars. Trehalose was also quantified in amount of 0.91±0.65 g%

Figure 5 presents the distribution of this sugars between all the samples, and the concentrations quantified.

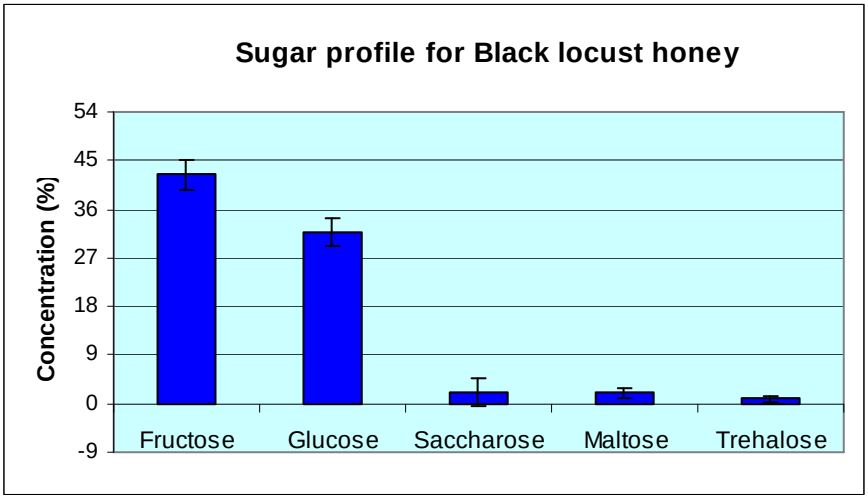

Fig.5. Distribution of sugars (mean ± SD) in black locust honey samples

CONCLUSIONS

1. The HPLC-MS method used for polyphenol analysis, could separate the free phenolic acids and flavonoids from honey and calculate the concentration of the identified compounds
2. 13 polyphenols from the class of phenolic acids and flavonoids were identified and quantified for the first time from Romania black lockust honey
3. Ferulic acid and acacetin were identified and quantified only in this type of honey and they can be used as possible markers for origin authentification
4. The percentage of fructose is higher than the percentage of glucose, the ratio between those two (1.32), being an important factor in crystallisation process
5. All honey samples studied, presented in different concentrations a pattern of 5 sugars: fructose, glucose, maltose, sucrose and trehalose

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