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Towards better quality criteria of European honeydew honey: phenolic profile and antioxidant capacity

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Abstract

Concerning the particular nutritive value of honeydew honey compared to blossom honey, and small number of studies defining botanical origin of honeydew honey, comprehensive analysis of phenolic profile of 64 honeydew honey samples of specific botanical origin was performed. Two advanced techniques of liquid chromatography hyphenated with mass spectrometry were used for identification of a total of 52 compounds and quantification of 25 of them. Pattern recognition analysis applied on data on phenolic compounds content confirmed that quercetin, naringenin, caffeoylquinic acid, hydroxyphenylacetic acid, apigenin and genistein, could be considered as potential markers of botanical origin of honeydew honey. Spectroscopic and electrochemical approaches were applied for the evaluation of the antioxidant capacity. *Quercus* sps. samples, *Quercus frainetto* and *Quercus ilex,* showed high biological activity and specific chemical composition. Additionally, cyclic voltammetry profiles were used for characterization and natural clustering of honeydew honey for the first time.

Keywords: Honeydew honey; Phenolic profile; UHPLC-LTQ OrbiTrap MS; UHPLC–DAD–MS/MS; Pattern recognition methods; Antioxidant activity

1. Introduction

Honeydew honey mainly derives from secretions of living parts of plants or excretions of plant-sucking insects on plants (Directive 2014/63/EU of the European Parliament and of the Council amending Council Directive 2001/110/EC relating to honey). It is rather known known for conifers, i.e. fir, pine and spruce and for deciduous trees, such as oak, chestnut, birch, willow and linden (Primorac et al., 2009). Honeydew honey is generally dark, strongly flavoured, less acidic and less sweet when compared to blossom honey (Pita-Calvo & Vázquez, 2017).

The number of consumers that appreciate flavour and nutritive properties of honeydew honey are increasing. Growing market for this honey type significantly contributes to the increase of its' price. However, although having particularly different nutritional value, being attributed with higher

antibacterial and antioxidant properties, when compared to blossom honey (Pita-Calvo & Vázquez, 2018), this specific type of honey is still less studied and less known to consumers. In order to avoid adulteration and frauds, with regards to their botanical and geographical authentication, standardized methods for the routine quality control of honeydew honeys with regard to their botanical and geographical authentication are required (Pita-Calvo & Vázquez, 2018).

Honeydew honey authenticity, however, has some difficulties when considered the compliance of declaration of botanical and geographical origin. Melissopalynological analysis, based on the identification and quantification of the percentage of pollen by microscopic examination, has traditionally been accepted to ascertain honey blossom source (Aronne & Micco, 2010). On the other hand, honeydew secreted by sap-sucking insects will contain no pollen other than air-borne pollen grains that ended up trapped and which will not necessarily be from the plant that was the source of the secretion. However, honeydew honeys, like all honeys, contain significant portion of pollen, deriving from small amounts of inevitable nectars of nectariferous flowers as well as gathered pollen of non-nectariferous plants. They may also provide significant contribution to the identification of geographical origin as well as to the confirmation of the period of production. However, due to some limitations of identification of honey sources by melissopalynological assessment, and some misleading conclusions that sometimes may be obtained by it, other methods have been suggested for more accurate identification of the honey botanical sources. Phenolic compounds, sugars, amino acids and proteins are among markers which are considered as useful to characterize various botanical types of honey (Pita-Calvo et al., 2017).

Additionally, several physicochemical parameters have been suggested as complementary information for characterization of honey. Honey physicochemical composition and its' flavour may vary with the botanical source, geographical origin, beekeeping practices as well as environmental and climatic variations. It has been given attention that physicochemical analysis could be very useful for

differentiation between honeydew honey and blossom honey (Manzanares, García, Galdón, Rodríguez, & Romero, 2011).

Compounds that naturally occur in honey, such as phenolic compounds (flavonoids and phenolic acids), some enzymes (e.g. glucose oxidase, catalase), ascorbic acid, carotenoid-like substances, organic acids, proteins and amino acids, significantly contribute to its antioxidant capacity (Pita-Calvo et al., 2017) and honey quality. A great number of phenolic compounds in honey as well as their confirmed correlation with antioxidant activity indicate their importance in the assessment of authenticity (Pita-Calvo et al., 2017). Detection of phenolic compounds and estimation of total antioxidant capacity could be performed by spectroscopic and subsequent electrochemical methods (Lugonja, Stankovic, Spasic, Roglic, Manojlovic, & Vrvic, 2013). Nowadays, electrochemical methods found wide application in the field of analytical chemistry due to their rapidity and simplicity. Advantages of these methods are wider linear range, higher sensitivity and easier manipulation steps.

The aim of this work is comprehensive assessment of phenolic composition and antioxidant activity of 64 honeydew honey samples of different botanical origin. According to our knowledge, previously there has been no previously published article that comprises focused information about such a high number of different, specific honeydew honey types. Characterization of phenolic acids, their derivatives and flavonoid aglycones and glycosides was performed by ultra-high-performance liquid chromatography coupled with hybrid mass spectrometer which combines the Linear Trap Quadrupole and OrbiTrap MS mass analyser (UHPLC-LTQ OrbiTrap MS technique). Quantification was performed by ultra-high-performance liquid chromatography with a diode array detector and a triple-quadrupole mass spectrometer (UHPLC-DAD-MS/MS). Two different approaches, spectroscopic and electrochemical, were combined and applied for the evaluation of the antioxidant capacity of honeydew honey samples for the first time. In addition, phenolic composition and antioxidant capacity were used in authentication of botanical origin of the analysed honey samples. Namely, by studying the

literature we realized that differentiation and classification of honeydew honey are difficult to perform by means of only one class of compounds, not combined with several other groups of parameters. Taking into account thus far observed significance of phenolic compounds for differentiation of honeys in relation to their origin, we expect that it should be possible that only phenolic profile and antioxidant capacity could be used for authenticity assessment of honeydew honey. The data from this study could contribute to the project of the International Honey Commission that is working on a data bank for quality criteria of the most important European unifloral honeys (Bogdanov & Martin, 2002).

2. Materials and Methods

2.1. Chemicals and materials

Acetonitrile and acetic acid (both MS grade), methanol (HPLC grade), sodium carbonate, potassium chloride, hydrochloric acid and Folin–Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). The SPE cartridges used for extraction and concentration of samples were Strata C18–E (500 mg/3 mL) obtained from Phenomenex (Torrance, CA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and phenolic compounds standards (protocatechuic acid - PrA, aesculin, 5-*O*-caffeoylquinic acid – CQA, *p*-hydroxybenzoic acid - HBA, gentisic acid, *p*-hydroxyphenylacetic acid - HPA, vanillic acid - VA, aesculetin, caffeic acid - CA, rutin - Rut, *p*-coumaric acid - CouA, quercetin 3-*O*-glucoside, sinapic acid - SA, naringin - Nar, ferulic acid - FA, quercetin 3-*O*-rhamnoside – Que-3 rha, coniferyl aldehyde, quercetin - Que, eriodictyol - Erio, luteolin - Lut, naringenin - Narn, apigenin - Api, genistein - Gen, kaempferol - Kfrl, pinobanksin , isorhamnetin, chrysoeriol, genkwanin - Genkw, chrysin - Chry, pinocembrin - Pin, kaempferide - Kfrd, galangin - Gal, and acacetin - Aca) were supplied by Sigma–Aldrich (Steinheim, Germany).

Ultrapure water (ThermoFisher TKA MicroPure water purification system, 0.055 μS/cm) was used to prepare standard solutions and blanks. Syringe filters (13 mm, PTFE membrane 0.45 μm) were

purchased from Supelco (Bellefonte, PA). Filter paper (Whatman No. 1) was supplied by Merck (Darmstadt, Germany).

2.2. Honeydew honey samples

A total of 64 honeydew honey samples comprising Silver fir (*Abies alba* Mill.) - 22, Evergreen oak (*Quercus ilix* L.) - 15, Hungarian oak (*Quercus frainetto* Ten.) - 4, Montpellier maple (*Acer monspessulanum* L.) - 6, conifers – 17 samples, were collected from different regions of Croatia. Small number of samples of Hungarian oak and Montpellier maple honeydew honeys is a consequence of the fact that the rather small areas are covered by these specific plants. Furthermore, the occurrence of honeydew honeys deriving from their respective honeydews are relative rare and does not happen every year. Furthermore, it is very hard to obtain honeydew honey with the significant prevalence of these plants.

Honey sampling was carried out straight at the filling facilities of the primary beekeepers. Once collected, samples were filled into glass jars and sealed with metal lids. Honey samples had been kept in dark at 4–8 °C until delivered in laboratory. In order to comprehend the origin of the honeys, samples were subjected to comprehensive melissopalynological and sensory assessment. Immediately before the analysis, the samples were homogenized by mechanical mixing in the original packaging.

2.3. Determination of physicochemical properties

Physicochemical parameters were analysed by means of the Harmonised Methods of the International Honey Commission (IHC) (Bogdanov, 2009).

Moisture was determined by an Abbe-type refractometer (Digital Refractometer, Atago Co., Ltd., Tokyo, Japan). All measurements were performed at 20 °C and the refractive index of the honey sample was correlated using Chataway Charts. Electrical conductivity was determined with a conductivity meter (Jenway Conductivity Meter 4310; Stone, UK) using a 20% (w/v; dry matter basis) honey solution in deionised water. The pH value was measured in 10% aqueous solution by a pH meter

(WTW Inolab). Free acidity was determined using the titrimetric method: acid components were neutralised with a standard solution of sodium hydroxide (C = 0.05 mol/dm^3) in aqueous honey solution (10 g in 75 mL distilled water). Lactone acidity was obtained by adding excess of standard solution of sodium hydroxide (C = 0.05 mol/dm^3) to a solution of honey and titrating excess of sodium hydroxide with a standard solution of hydrochloric acid (C = 0.05 mol/dm^3). Specific rotation was evaluated using an Atago Polax-2L polarimeter.

2.4. Extraction of phenolic compounds from honeydew honey samples

2.4.1. Preparation of extracts for LC-MS analysis

The method used for extraction and isolation of phenolic compounds from the honeydew honeys was previously described by Gašić et al., 2015. Prior the UHPLC-LTQ OrbiTrap MS and UHPLC–DAD-MS/MS analysis the extracts were filtered through a 0.45 μm PTFE membrane filter.

2.4.2. Sample preparation for determination of total phenolic content and radical-scavenging activity

Samples were prepared according to the slightly modified method proposed by Meda et al., 2005. Each honey sample (2.5 g) was mixed with 10 mL ultrapure water, homogenized in ultrasonic bath for 15 min at room temperature, transferred to 25 mL volumetric flask and filled with ultrapure water. The solution was then filtered through 0.45 μm PTFE membrane filter and analysed for determination of total phenolic content and radical-scavenging activity.

2.5. UHPLC-LTQ OrbiTrap MS qualitative analysis of phenolic compounds

Chromatographic separations of compounds of interest were performed on Syncronis C18 column (100 × 2.1 mm, 1.7 μm particle size) using an UHPLC system consisting of a quaternary Accela 600 pump and Accela autosampler (ThermoFisher Scientific, Bremen, Germany). The mobile phase consisted of (A) water containing 0.1% acetic acid and (B) 100% acetonitrile. The gradient program at flow rate of 0.300 mL/min was as follows: 0.0–1.0 min 5% B, 1.0–16.0 min from 5% to

95% (B), 16.0–16.1 min from 95% to 5% (B), then 5% (B) for 4 min. The injection volume for all samples was 5 μL.

The UHPLC system was coupled to a linear ion trap-OrbiTrap hybrid mass spectrometer (LTQ OrbiTrap MS) equipped with heated electrospray ionization probe (HESI-II, ThermoFisher Scientific, Bremen, Germany). The mass spectrometer was operated in negative ion mode. Settings of dynamic exclusion and other ion source parameters were as previously described by Gašić et al., 2015. The data-dependent MS^2 events were always performed on the most intense ions detected in the full scan MS. The ions of interest were isolated in the ion trap and activated with 35% collision energy levels (CEL).

Phenolic compounds were identified according to their spectral characteristics: mass spectra, accurate mass, characteristic fragmentation and characteristic retention time. Xcalibur software 2.1 (Thermo Fisher, Bremen, Germany) was used for instrument control, data acquisition and data analysis of accurate mass spectrometry data. ChemDraw 12.0, molecule editor program, was used for drawing structures and calculating the exact monoisotopic masses of compounds of interest. Full scan analysis was employed to detect the monoisotopic mass of unknown compounds, while the fragmentation pathway was obtained by MS^4.

2.6. UHPLC–DAD-MS/MS quantitative analysis of phenolic compounds

A 1000 mg/L stock solution of a mixture of phenolic standards was prepared in methanol. Dilution of the stock solution with mobile phase yielded working solutions at concentrations of 0.025, 0.050, 0.100, 0.250, 0.500, 0.750 and 1.000 mg/L. Calibration curves were obtained by plotting the peak areas of the standards against their concentration. Calibration curves revealed good linearity, with R^2 values exceeding 0.99 (peak areas *vs* concentration).

The separation and quantification of the phenolic compounds in the studied honeydew honey samples were performed using a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector that was connected to TSQ Quantum Access Max triple-quadrupole mass spectrometer

(ThermoFisher Scientific, Basel, Switzerland). The specification of the analytical column, mobile phases, gradient elution program, as well as other settings UHPLC system were the same as in the previous section about qualitative analysis of phenolic compounds.

The parameters of a TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with heated electrospray ionization (HESI-II) were as previously described by Gašić et al., 2015. The mass spectrometry data were acquired in negative ion mode, in the *m/z* range from 100 to 1000. The selected reaction monitoring (SRM) mode of the instrument was used for the quantification of the targeted compounds in the tested samples (Gašić et al., 2015).

The phenolic compounds were identified by direct comparison with commercial standards. The total amounts of each compound were evaluated by calculation of the peak areas and were expressed as mg/kg.

Limits of quantification (LOQ) were calculated using standard deviations of the responses (SD) and the slopes of the calibration curves (S) according to the formulas: LOQ = 10 (SD/S). The values of standard deviations and slopes were obtained from the calibration curves created in the MS Excel.

2.7. Determination of the total phenolic content (TPC)

The TPC was spectrophotometrically determined with the Folin-Ciocalteu method reported by Singleton et al., 1965. Briefly, 0.3 mL of the sample extracts and 6 mL of deionized water were mixed with 0.5 mL of Folin-Ciocalteu reagent and solution was incubated 6 min at room temperature. A total of 3 mL of 20% sodium carbonate was added. After 30 min at 40 ^{o}C, absorbance was measured at 765 nm. Calibration curve was built with gallic acid in the range of concentration 20-100 μg/mL. The results were expressed as mg gallic acid equivalents (GAE) per gram of honey.

2.8. Determination of radical-scavenging activity (RSA)

The RSA of the extracts of honey samples was evaluated by a modified method of Wang et al., 2011. Briefly, 0.1 mL of the sample extract was mixed with 3 mL of methanol solution of DPPH (71

mM). The mixture was left in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. The radical-scavenging activity (RSA) was calculated as the percentage of DPPH discoloration. The assays were carried out in duplicate and the results were expressed as mean values.

2.9. Cyclic voltammetry

Cyclic voltammetry measurements were performed using an electrochemical system CH Instruments, Model CHI 760b with corresponding software. Antioxidant activity of honeydew honey was determined by a procedure developed by Lugonja, Stanković, Spasić, Roglić, Manojlović, & Vrvić, 2013 in infant formula with breast milk and lately applied for propolis samples (Ristivojević, Trifković, Stanković, Radoičić, Manojlović, & Milojković-Opsenica, 2017).

3.0. Statistical analysis

Descriptive statistics and Kruskal-Wallis one-way analysis of variance by ranks test have been performed by means of a demo version of NCSS statistical software (Hintze, 2001).

Principal component analysis (PCA) has been performed by means of PLS ToolBox, v.6.2.1, for MATLAB 7.12.0 (R2011a). All data were pre-treated (mean-centred and scaled to the unit standard deviation) before any statistical operations in order to prevent highly abundant components to dominate in the final result over the components present in much smaller quantities. The number of components was chosen according to Kaiser Criterion and only factors with eigenvalues greater than 1 were retained.

3. Results and discussion

Given the significant number of three botanical types of honeydew honey: silver fir (*Abies alba* Mill.), evergreen oak (*Quercus ilex* L.), and conifers, characterization based on phenolic compounds profiles and antioxidant capacity, was performed. Although not having a fully representative set of

samples of Hungarian oak (Quercus frainetto L.) and Montpellier maple (Acer monspessulanum L.) due to the small areas covered by these botanical species, as well as inconsistent production yielding only occasionally, these rather rare honeydew honeys were also characterized. Nonetheless, it came to our attention that more exhaustive further studies should be carried out in order to fully comprehend these peculiar honeys.

3.1. Physicochemical data

Excluding the classical approach of diagnosis of botanical origin of samples, as time-consuming method that additionally requires personnel specialized in carrying out sensory analysis (Bogdanov, 2009) and taking into consideration melissopalynological challenges of identification of honeydew honey botanical origin, with regards to their botanical source, the final confirmation of origin of analysed samples was performed by means of physicochemical parameters. Table S1 (Supplementary material) summarizes parameters of descriptive statistics (mean, median, standard deviation and range) obtained from physicochemical analysis (moisture (ω), free acidity (FA), lactone acidity (LA), total acidity (TA), electrical conductivity (EC), pH value and specific rotation (SR)) of the honeydew honey samples of five different botanical origins.

Electrical conductivity, specific rotation, ash content and pH value were in common use as the main parameters widely used to distinguish blossom from honeydew honeys (Pita-Calvo et al., 2017). According to European legislation (EU Directive 2014/63) honeydew honey must have EC values ≥ 0.8 mS/cm. The mean value of EC for analysed samples varied between 0.91 and 1.29 mS/cm, confirming its originality as honeydew honeys, with highest values observed for Evergreen oak honeydew honey (Table S1). Honeydew honeys usually contain somewhat lower fructose content and contain melezitose or erlose which, together with glucose, give a positive net specific rotation (Pita-Calvo et al., 2017). All analysed honeydew samples were dextrorotatory (Table S1). Honeydew honeys are usually showing a higher mean pH value and acidity when compared to blossom honey (Pita-Calvo et al., 2017). Mean

pH value of analysed samples ranged between 4.1 and 4.7 that were higher compared to some blossom honeys, *e.g.* acacia, sunflower (Lazarević, Andrić, Trifković, Tešić, & Milojković-Opsenica, 2012; Vela, de Lorenzo, & Perez, 2007). Additionally, the free acidity of samples was below 50 mili-equivalents/kg (EU Directive 2014/63) with exception of Hungarian oak showing the mean value of 78.6 mili-equivalents/kg (Table S1), also showing high value of lactone and total acidity (Table S1). The moisture content in all samples indicated a proper degree of maturity, in compliance with the international requirements with levels of humidity lower than 20% (EU Directive 2014/63) (Table S1).

Physicochemical parameters confirmed that samples were authentic with respect to honey production and originated from secretion produced by certain trees and other plants or from excretions of plant-sucking insects.

3.2. UHPLC-Orbitrap MS profiles

Phenolic compounds profiles of honeydew honey obtained by UHPLC-Orbitrap MS analysis using the non-targeted metabolomics approach, revealed the presence of a significant number of phenolic compounds (Table 1). LC-MS analysis resulted in the identification of 52 compounds belonging to three structurally distinct groups: phenolic acids and their derivatives (19 compounds), flavonoids aglycones (23 compounds) and flavonoid glycosides (10 compounds). Thirty three of them were confirmed using available analytical standards, while the others were identified by exact mass search of their $[M-H]^-$ deprotonated molecule and their MS^4 fragmentation patterns compared with the previously reported chromatographic and MS data. Selected base peak chromatograms of phenolic compounds identified in some of honeydew honey samples were shown in Figure 1. During the identification of flavonoid glycosides, some already published rules of mass fragmentation, such as the determination of the nature of interglycosidic linkage (Ferreres, Llorach, & Gil-Izquierdo, 2004) and the characterization of glycosylation position (Cuyckens & Claeys, 2005) were used. Table 1 shows peak numbers, assigned compound names, molecular formulas, calculated and exact masses ($[M-H]^-$,

m/z), mass accuracy errors, the retention times (t_R, min) and major MS^2, MS^3, and MS^4 fragment ions used for identification of phenolic compounds in the absence of standard. Phenolic acids and their derivatives were identified in all of the examined honeydew honey samples (Table S2, Supplementary material). In addition to simple phenolic acids (derivatives of hydroxybenzoic and hydroxycinnamic acids), several glycosides have been identified. Thus, for example, two derivatives of caffeic acid hexoside (341 *m/z*, compounds **4** and **7**) were identified at 4.74 and 5.10 min, respectively (Table 1). Both of them produced an MS^2 base peak fragment at 179 *m/z*, corresponding to the mass generated by loss of hexose unit (162 Da) and secondary MS^2 peaks at 161 and 135 *m/z*, corresponding to the $[\text{caffeic acid}-H-H_2O]^-$ and $[\text{caffeic acid}-H-CO_2]^-$, respectively. As expected, in this case, the MS^3 base peak was at 135 *m/z*. Next to found phenolic acid glycosides, several other compounds were identified and confirmed using standards: aesculin and its glycoside (aesculin 6-*O*-glucoside), coniferyl aldehyde and 5-*O*-caffeoylquinic acid. Benzyl caffeate (269 *m/z*, compound **46**), which is known to originate from propolis (Yamauchi, Kato, Oida, Kanaeda, & Ueno, 1992) was identified in five samples at 11.30 min. It produced MS^2 base peaks at 178 *m/z* (generated by loosing of benzyl group) and 134 *m/z* (resulting in further loss of CO_2 molecule).

Most of the identified flavonoid aglycones are originated from propolis (Bankova et al., 2016). During the identification of flavonoid aglycones, MS data obtained from an available literature describing specific retro-Diels-Alder (RDA) fragmentation, were used (Yang, Ye, Qiao, Wang, Bo, & Guo, 2012; Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001). A similar flavonoid profile of some honeydew honey samples has already been described in literature (Halouzka, Tarkowski, & Zeljković, 2016; Majtan et al., 2013). Compound **36** at 9.66 min and 315 *m/z* was identified in all samples. By examining the empirical formula of this compound, it was concluded that it may be an *O*-methylated flavonol. It produced MS^2 base peak at 300 *m/z*, most probably corresponding to $[M-H-CH_3]^-$ fragment and secondary MS^2 peak at 181 *m/z*. In the MS^3 spectra of this compound we

can notice the absence of fragment at 151 *m/z*, which indicates that the flavonoid core was *O*-methyl substituted on the B ring. Also, fragment 165 *m/z* is missing, which can be formed by *O*-methyl substitution of the A ring of flavonoids (Kečkeš, Gašić, Ćirković Veličković, Milojković-Opsenica, Natić, & Tešić, 2013). The most interesting fragment in the MS^3 spectra is 166 *m/z*, indicating that A ring of flavonoids is most likely substituted with two hydroxyls and one methoxy group (Figure S1, fragmentation is shown on 8-methoxy kaempferol, as an example). We can assume that the secondary MS^2 fragment at 181 *m/z* was formed by the RDA fragmentation of flavonoids and that's actually $[^{1,3}A]^-$ fragment, while 166 *m/z* is probably $[^{1,3}A–CH_3]^-$ fragment. Based on all these findings, this compound was marked as methoxy kaempferol.

For the purpose of identifying flavonoid glycosides (the structures of aglycone and glycosidic parts, as well as interglycosidic linkage between two sugars) some mass fragmentation data, already described in the literature, has been used (Kachlicki, Piasecka, Stobiecki, & Marczak, 2016). Numerous derivatives of flavonoid glycosides were identified in honeydew honey extracts; rhamnosides (loss of 146 Da), hexosides (loss of 162 Da), pentosylhexosides (loss of 294 Da), rhamnosylhexoside (loss of 308 Da) and dihexosides (loss of 324 Da). For example, in MS^2 spectra of compound **15** at 5.83 min and 625 *m/z*, it can be observed base peaks fragment at 301 and 300 *m/z* (corresponding to deprotonated quercetin) and additional three fragment ions resulting from loss of 180, 162, and 120 Da. This specific fragmentation shows presence of 1→2 interglycosidic linkage between two sugars at 3-*O*-position of quercetin. In MS^3 spectrum of this compound, base peak at 271 *m/z* was noticed (generated by loss of 29 Da, HCO), while MS^4 base peak was at 243 *m/z*, resulting from loss of CO molecule (28 Da). Further fragmentation of 300 *m/z* confirmed presence of quercetin as aglycone, due to the observed RDA fragments at 179 and 151 *m/z*. Based on MS fragmentation this compound was identified as quercetin 3-*O*-(2"-hexosyl)hexoside (Figure S2).

Presence of particular phenolic compounds in the tested honeydew honey samples is reported in Table S2. Certain phenolic compound was considered present in honeydew honey of particular botanical origin if it was found in more than 80% samples of one specific species. Forty one compounds were identified in all samples. It is important to notice that in the samples of Evergreen oak, all 52 compounds were found. However, some of the compounds were found only in one or two botanical origins. Quercetin 3-*O*-(2"-hexosyl)hexoside (compound **15**) was identified only in samples of Evergreen oak and Montpellier maple. Similarly, luteolin 7-*O*-rhamnoside (compound **27**), kaempferol 7-*O*-rhamnoside (compound **29**), tectochrysin (compound **32**), and dimethyl quercetin (compound **41**) were identified only in the samples of Evergreen oak, while pinobanksin 3-*O*-propionate (compound **52**) was found in samples of Hungarian and Evergreen oak, *Quercus* sps.

3.3. Quantification of specific compounds

UHPLC-DAD-MS/MS analysis was used for quantification of 25 targeted compounds selected based on their abundance in the analysed honeydew honey samples (Table 2). Qualitative analysis indicate the presence of phenolic compounds listed in Table 1, however certain compounds identified according to their spectral characteristics were not quantified due to the low content (below the limit of quantification). Although the investigated samples contained mainly the same phenolic compounds, the amount of individual compounds could indicate the differences among the botanical types of honey and possibly used as a marker of origin.

Among the quantified compounds, phenolic acids such as HPA, HBA, CA, VA, CouA, FA and PrA, were present in the highest amount, followed by flavonoids Que, Chry and Pin. The content of HPA, Api and Gen were higher in silver fir and conifers compared to *Quercus* sps. and Montpellier maple. Similarly, CQA, CouA, and FA were more represented in silver fir, conifers and Hungarian oak in relation to Evergreen oak and Montpellier maple. Qurcetin and Narn were present in higher quantity

in *Quercus* spp. compared to other investigated botanical types, while PrA was present in two to seven times higher amount in Hungarian oak honeydew honey (Table 2).

The results reported from other authors indicated large variability in phenolic content of honeydew honey (Karabagias, Vavoura, Nikolaou, Badeka, Kontakos, & Kontominas, 2014; Oroian & Sorina, 2017; Silici, Sarioglu, & Karaman, 2013). Additionally, in majority of articles the authors label samples only as honeydew honey, without defining the specific botanical origin of the same. However, in those with marked origins the species were not matched with those from our study. For that reason, it was hard to compare our results with those reported previously. Only Kuś, Jerković, Marijanović, & Tuberoso, 2017 reported the content of several phenolic compounds in five samples of silver fir honeydew honey with the results similar to those obtained in our study. It should be also emphasized that there is no report that comprises such a large number of quantified phenolic compounds. Moreover, this is a first report on phenolic profile of Evergreen and Hungarian oak and Montpellier maple honeydew honey.

3.4. Antioxidant activity of honeydew samples

Many spectroscopic methods have been developed for assessing the antioxidant activity of phenolic compounds. UV/Vis spectroscopy is highly suitable for its investigation due to the presence of π conjugated aromatic rings substituted with OH-groups. These methods are focused either on the ability of phenolic compounds to inhibit free radical formation or scavenge free radicals. Phenolic compounds, however, often show inconsistent antioxidant capacity depending on the applied assay, due to the different reaction systems and the presence of multiple oxidants (Granato et al, 2018). All mentioned problems are overcome by electrochemical oxidation processes. Application of cyclic voltammetry (CV) in the analysis of the phenolic compounds is a result of electronic delocalization on the aromatic nucleus, which could be easily oxidized at inert electrodes (Sochor et al., 2013). The advantage of CV over the spectroscopic methods is that it does not require the labor-intensive

characterization of the antioxidant activity of each component against a specific radical oxygen species, which is the basis for methodologies measuring the total antioxidant capacity (Ristivojević, Trifković, Stanković, Radoičić, Manojlović, & Milojković-Opsenica, 2017). It is simple and rapid method and could be used directly in biological and crude samples.

In that sense, different approaches, two *in vitro* spectroscopic screening methods (total phenolic content and antioxidant activity measurement) and electrochemical method were applied for the evaluation of the antioxidant capacity of honeydew honey samples. The results of TPC, RSA and CV of the honeydew honey samples are presented in Table 2.

The mean value of TPC ranged from 0.57 to 1.60 mg GAE/g of honey (Table 2). Hungarian oak honeys showed the highest mean value of TPC (1.60 mg GAE/g), high value was also marked for evergreen oak (1.19 mg GAE/g), while silver fir and conifers showed similar, but lower values (0.61 and 0.57 mg GAE/g of honey, respectively). Report concerning common oak (*Qurcus robur* L.) indicate high phenolic content and high antioxidant capacity associated with it (Can, Yildiz, Sahin, Turumtay, Silici, & Kolayli, 2015). Namely, contrary to majority of other botanical types of honeydew honey that are produced from a secretion of plant sucking insects, oak honey may also be obtained by collecting of sugary substances that seep out of oak under stress conditions (Gonzales-Paramas et al., 2007). Previously reported results indicate a high variability of TPC values for honeydew honeys, however they mostly contain higher amounts of phenolic compounds compared to blossom honeys (Pita-Calvo et al., 2017; Kachlicki, Piasecka, Stobiecki, & Marczak, 2016; Can et al., 2015; Alves, Ramos, Gonçalves, Bernardo, & Mendes, 2013).

The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl was found to range from 12.20 to 48.89% (Table 2). Some differences between the mean values of antioxidant activity for samples of specific botanical origin are noted, with a trend identic to those marked in TPC. RSA mean values for silver fir and conifers were the lowest and similar (13.14% and 12.20%, respectively), while the highest

value was found for the Hungarian oak honey (48.89%). Generally, species from *Quercus* family showed higher biological activity compared to other investigated botanical origins, similar to the previously reported results for other oak tree honey samples (Can et al., 2015; Gonzales-Paramas et al., 2007). *Quercus* sps. samples were darker in colour compared to other botanical types of honeydew honey, which was also confirmed by other authors (Can et al., 2015; Gonzales-Paramas et al., 2007). Dark-coloured honeys have higher antioxidant activity (Pita-Calvo et al., 2017; Alves et al., 2013).

The correlation between the TPC and RSA resulted in a linear model $RSA = 0.209(\pm 0.049) + 0.032(\pm 0.002)TPC$ with statistical parameters: r = 0.8598, s = 0.18, t = 13.04 ($t_{cr(64)}$ = 1.99), F = 170.12. A significant correlation between RSA and TPC pointed out the significant contribution of flavonoids and phenolic acids, among all active phytochemicals, in antioxidant potential of the honeydew honey. Similar conclusion was demonstrated by other authors (Pita-Calvo et al., 2017).

Cyclic voltammograms of the selected honeydew honeys are shown in Figure 2. The area under curves (charges of the anodic wave) found in all voltammograms were used for estimation of antioxidant capacity (Table 2). In the voltammograms of honeydew samples up to four oxidation peaks were observed in three different regions: around -0.1 – 0.1 V (1); around 0.3 – 0.6 V (2) and around 0.9 V (3). Peaks at highest potential can be attributed to the high presence of HPA and CouA. Middle potential regions can be attributed to the presence of ferulic acid and sinapic acid and their synergetic effect in some of the samples. Low intensity peaks at lowest potential can be attributed to the presence of rutin, which provides oxidation behaviour at this potential (Simić, Manojlović, Šegan, & Todorović, 2007). Additionally, CV indicated the existence of several oxidation processes which vary with the botanical origin of honeydew honey and consequently different antioxidant capacity of the samples. As for RSA test, the highest values were obtained for Hungarian oak, *i.e. Quercus* sps. samples, while the smallest were marked in the case of silver fir and conifers. Estimated results were also compared with

those obtained for the RSA values. The comparison of the electrochemical approach with RSA clearly indicated that CV values fit well with RSA test (Table 2). Cyclic voltammetry data showed a very good positive correlation with the antioxidant capacity ($RSA = -1.5(\pm 1.2) + 2.04(\pm 0.06)CV$, r = 0.9499, s = 4.43, t = 33.75 ($t_{cr(64)}$ = 1.99), F = 1139.15) determined spectrophotometrically.

Both, total phenolic content and antioxidant activity indicated the existing of different chemical profiles of honeydew honey samples according to their botanical origin and pointed out on possibility of authenticity assessment with respect to description, *i.e.* botanical origin. Additionally, it was confirmed that CV as highly attractive, convenient, and sensitive method could be used in the analysis of bioactive compounds as well as the determination of antioxidant capacity. It could be a method of choice for determination of biological activity of honeydew honey in order to exceed the problems connected with spectroscopic methods (Halouzka, Tarkowski, & Zeljković, 2016).

3.5. Botanical origin of honeydew honey

Descriptive statistics provided information regarding general phenolic composition and indicate some specific compounds and parameters that can differentiate the samples of various botanical origins. However, the mostly influential factors that would enable decisive characterization of the botanical origin of honeydew honey were not marked. In that sense, Kruskal-Wallis test and Principal Component Analysis (PCA) were applied to differentiate groups of samples according to their botanical origin.

Kruskal-Wallis test was used to compare the medians and variances of 25 quantified phenolic compounds and three quality control parameters (TPC, RSA and CV) for five varieties of honey (Silver fir, Evergreen oak, Hungarian oak, Montpellier maple and conifers). Based on the results presented in Table S3 (Supplementary material), parameters: TPC, RSA and CV, and PrA, HPA, VA, CouA, Nar, FA, Lut, Que, Api, Gen and Kfrl, whose $\chi 2$ values exceed critical one. *i.e.* p values were below 0.05, can be selected as the most discriminating factors. According to multiple-comparison Z-value test,

honeydew honeys of different botanical origins which showed mutual significant difference were denoted in parentheses (Table S3). The results indicated that two physicochemical parameters (EC and SR) that are, among all others, the only parameters which discriminate honeydew honey from blossom honey, also the parameters which could be used for the assessment of botanical origin of honeydew honey. Additionally, acidity parameters indicated different proportion and profiles of organic acids and amino acids according to botanical origin of the analysed samples. Ten phenolic compounds and antioxidant capacity were also marked as factors that could be used for the classification and differentiation of five botanical origins of honeydew honey samples. Majority of parameters indicated differences between silver fir and conifers compared to *Quercus* sps. samples (Table S3).

In order to get the insight into data structure and to confirm whether phenolic profile, together with antioxidant activity, on one side, and the intensities of current obtained from cyclic voltammograms, could differentiate the honeydew honeys according to their botanical origin, the data were subjected to pattern recognition analysis.

PCA performed on data of 25 phenolic compounds and three quality control parameters, resulted in a five-component model which explains 68.45% of the total data variance. The principal components (PC's) accounted for approximately similar data variances (from PC1 to PC5 – 24.17%, 16.61%, 12.51%, 9.09%, 6.07%, respectively). Concerning the botanical origin of the investigated honeydew honey samples the most informative PC's were PC2 and PC3. Score plot (Figure 3a) revealed natural clustering of *Quercus* sps., Evergreen oak and Hungarian oak, distinguished from each other and from Silver fir and conifers samples. Silver fir samples formed a compact natural cluster on the opposite part of score plot compared to *Quercus* species, but however overlapped with conifers samples which were widespread on big part of the plot indicating high variability in chemical profile. This was expected since conifers samples contained significant percent of silver fir exudates among other conifer botanical species (spruce, etc.). Samples of Montpellier maple were placed between groups of *Quercus*

species and conifers with silver fir and similar to conifers show heterogeneous phenolic composition. Grouping tendency of *Quercus* sps samples was imposed with total phenolic content and antioxidant activity and additionally with Que, Narn, Que-3-rha, Rut, Kfrl and Kfrd (Figure 3b). Differentiation of Hungarian oak was particularly imposed by high value of three quality control parameters indicating specific nutritive value of this rare honey type. Hungarian oak tree is distributed throughout southeast Europe and Asia Minor, while the western border is in Požega valley, Kutjevo, Croatia. This honeydew honey is specifically produced only there. Sweet sap with foam formed at the places where the green acorns were discarded, is gathered directly by the bees and processed into this dark honey. The specific production of the sap obviously results in unique phenolic content and antioxidant activity. The most influential parameters discriminating silver fir and conifers samples from other botanical species were Api, CQA, Gen and HPA. It must be emphasize that HPA could be considered as marker of silver fir honeydew honey due to its significantly higher content in silver fir compared to other botanical origins (also confirmed by Kruskal-Wallis Multiple Comparison Z-test). Its high content in conifers is due to the high percent of silver fir in this specific type of honeydew honey.

All samples were grouped alongside the PC2 and in that sense the variables with the highest influence on this PC determined markers of certain botanical origin (Figure S3, Supplementary material). Phenolic compounds positively correlated with PC2 assessed *Quercus* sps, while those with the highest negative influence estimated Silver fir and conifers. Although accounting for the highest variability among the data, PC1 could not differentiate among investigated botanical origins due to the similar, positive influence of the majority of the variables on it (Figure S3). Additionally, two groups of parameters with the highest positive (VA, Aca, Genkw and HBA), *i.e.* negative (Chry, Pin and Gal) influence on PC3 determined conifers and maybe suggested the markers of some particular source of origin included in this specific type of honeydew honey (Figures 3b and S3).

Due to the fact that honey samples present complex natural mixtures that contain vast number of compounds, differentiation and classification are difficult to perform with distinct separation among groups, particularly observing only one class of compounds, such as phenolic compounds, not combining several groups of parameters. Conventional quality parameters mainly gave a substantially better classification results when apply alone, while all other analytical parameters should combine to evaluate better results (Manzanares, García, Galdón, Rodríguez, & Romero, 2011; Habib, Meqbali, Kamal, Souka, & Ibrahim, 2014). The obtained results are rare example of successful botanical discrimination of honeydew honey based only on phenolic content. Fingerprint analysis proved to be a good source of information for an assay of authenticity and quality of food (Gašić et al, 2015).

Recently, cyclic voltammetry profiles together with multivariate chemometric technique were successfully used for characterization of propolis (Ristivojević et al, 2017). A PCA was applied on data of 64 honeydew honey samples and 1400 data point generated from oxidation current in order to evaluate different types of honeydew honey. First five PCs described 86.43% of variability (PC1 58.63 and PC2 18.89%) among data. PC2 clearly distinguished the Hungarian honey samples from all others, while PC1 differentiate Evergreen oak samples from Silver fir and conifers which form overlapped natural clusters (Figure 4a). Samples of Montpellier maple were dissipated over broader area of score plot and could not be distinguished from other species.

Oxidation potentials at 0.670 and 0.096 are the major factor which leads to the grouping tendency of Hungarian oak samples, since it shows a highest negative impact on PC1, while positive potential at 0.385 differentiate all other samples. PC1 was negatively contributed by peak at 0.980 at represented the main potential for grouping of Evergreen oak and group of silver fir and conifers samples (Figure 4b).

Therefore, PCA showed that data on phenolic compounds content and CV profile are able to provide enough information to allow the natural clustering and distinction of honeydew honeys originating from different botanical origins.

4. Conclusion

According to our best knowledge, so far there has been no published articles that comprises so detailed information about the phenolic composition and antioxidant capacity of honeydew honey of particular botanical origin (Hungarian and Evergreen oak, Montpellier maple, and Silver fir).

The physicochemical characteristics of all honeydew honey samples analysed in this study can be considered to be within the parameters expected for honeydew honeys in general and constitute a quality product within national and international parameters. Additionally, these parameters indicated that samples were authentic with respect to honey production and were obtained from secretion produced by certain trees and other plants or from excretions of plant-sucking insects. UHPLC–LTQ OrbiTrap MS and UHPLC-DAD-MS/MS analysis provided information about a numerous phenolic compounds specific for a particular botanical origin of honeydew honeys. Also, among numerous antioxidant constituents, flavonoids and phenolic acids could be identified as components that are mainly responsible for the antioxidant activity in honeydew honey samples. The study reports that various species of oak (evergreen oak and Hungarian oak) showed high phenolic content and high antioxidant capacity associated with it. Results from this study could be used for more precise definition of quality parameters, as well as confirmation of authenticity in terms of botanical origin. The results confirm the possibility of authenticity assessment of honeydew honey based only on phenolic profile and antioxidant capacity, and additionally the advantages of application of cyclic voltammetry for determination of biological activity of honeydew honey over the spectroscopic methods. It must be emphasized that the conclusions revealed for Hungarian oak and Montpellier

maple are considered as preliminary due to relatively lesser number of samples and further studies should surely be applied.

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Human and animal rights and informed consent

The article does not contain any studies with animals or human performed by any of the authors.

Figure caption

Figure 1. Base peak chromatograms of phenolic compounds identified in: A) Evergreen oak; B) Hungarian oak; and C) Montpellier maple honeydew honey samples.

Figure 2. Cyclic voltammograms of honeydew honey samples with separated potential regions.

Figure 3. Principal component analysis applied on data on phenolic compounds content, a) score plot, b) loading plot.

Figure 4. Principal component analysis applied on data of cyclic voltammetry profile, a) score plot, b) loading plot.

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Table 1. High resolution MS data and negative ion MS^2, MS^3, and MS^4 fragmentation of phenolic compounds identified in honeydew honeys.

No	t_R, min	Compound name	Molecular formula, $[M–H]^-$	Calculated mass, $[M–H]^-$	Exact mass, $[M–H]^-$	Δ mDa	MS^2 Fragments, (% Base Peak)	MS^3 Fragments, (% Base Peak)	MS^4 Fragments, (% Base Peak)
1	4.20	**Dihydroxybenzoic acid hexoside**[*]	$C_{13}H_{15}O_9^-$	315.07216	315.07083	1.33	**153**(100), 152(50), 109(15), 108(10)	**109**(100)	**84**(100), 81(60)
2	4.44	**Protocatechuic acid**[a,*]	$C_7H_5O_4^-$	153.01933	153.01848	0.85	**109**(100)	**81**(100), 68(25), 65(15)	–
3	4.51	**Hydroxybenzoic acid hexoside I**[*]	$C_{13}H_{15}O_8^-$	299.07724	299.07584	1.40	**137**(100)	**93**(10)	–
4	4.74	**Caffeic acid hexoside I**[*]	$C_{15}H_{17}O_9^-$	341.08781	341.08521	2.60	**179**(100), 161(35), 135(10)	**135**(100)	135(10), 117(15), **107**(100), 91(35)
5	4.91	**Aesculetin 6-*O*-glucoside (Aesculin)**[a,*]	$C_{15}H_{15}O_9^-$	339.07216	339.07034	1.82	**177**(100)	177(5), 149(10), **133**(100), 105(10), 89(5)	**89**(100)
6	4.92	**Hydroxybenzoic acid hexoside II**[*]	$C_{13}H_{15}O_8^-$	299.07724	299.07626	0.98	**137**(100)	**93**(10)	–
7	5.10	**Caffeic acid hexoside II**[*]	$C_{15}H_{17}O_9^-$	341.08781	341.08615	1.66	**179**(100), 161(35), 135(10)	**135**(100)	135(10), 117(15), **107**(100), 91(35)
8	5.26	**5-*O*-Caffeoylquinic acid**[a,*]	$C_{16}H_{17}O_9^-$	353.08781	353.08566	2.15	**191**(100), 179(5)	173(75), **127**(100), 111(40), 93(60), 85(90)	109(30), 99(60), **85**(100)
9	5.41	***p*-Hydroxybenzoic acid**[a,*]	$C_7H_5O_3^-$	137.02442	137.02359	0.83	109(10), **93**(100)	**93**(100)	–
10	5.48	**Gentisic acid**[a,*]	$C_7H_5O_4^-$	153.01933	153.01837	0.96	**109**(100), 107(10)	95(10), **81**(100), 68(75)	–
11	5.62	***p*-Hydroxyphenylacetic acid**[a,*]	$C_8H_7O_3^-$	151.04007	151.03914	0.93	**136**(100), 95(5)	108(25), **92**(100)	**108**(100)
12	5.63	**Vanillic acid**[a,*]	$C_8H_7O_4^-$	167.03498	167.03398	1.00	153(10), 152(80), 124(10), **123**(100), 108(20)	**108**(100)	123(30), 80(35), **78**(100)
13	5.77	**Aesculetin**[a,*]	$C_9H_5O_4^-$	177.01933	177.01877	0.56	147(10), **135**(100), 133(60), 131(30), 105(10)	151(10), 107(10), 94(10), **91**(100), 71(10)	–
14	5.80	**Caffeic acid**[a,*]	$C_9H_7O_4^-$	179.03498	179.03389	1.09	**135**(100), 117(10), 91(20), 59(15)	**107**(100), 59(50)	–
15	5.83	**Quercetin 3-*O*-(2"-hexosyl)hexoside**	$C_{27}H_{29}O_{17}^-$	625.14102	625.13940	1.62	505(20), 463(20), 445(30), 301(90), **300**(100)	**271**(100), 255(50), 179(5), 151(5)	271(10), 253(10), **243**(100), 227(50), 215(20)
16	6.13	**Methoxy kaempferol 3-*O*-(2"-hexosyl)hexoside**[*]	$C_{28}H_{31}O_{17}^-$	639.15667	639.15479	1.88	624(20), 459(90), 444(30), **315**(100), 300(60)	**300**(100)	**271**(100), 255(55), 166(15)
17	6.39	**Kaempferol 7-*O*-(6"-hexosyl)hexoside**[*]	$C_{27}H_{29}O_{16}^-$	609.14611	609.14380	2.31	**285**(100)	**257**(100), 241(50), 229(40), 213(30), 151(70)	255(10), 239(30), **229**(100), 163(40)
18	6.44	**Quercetin 3-*O*-(6"-rhamnosyl)glucoside (Rutin)**[a,*]	$C_{27}H_{29}O_{16}^-$	609.14611	609.14343	2.68	343(5), **301**(100), 300(30), 271(10), 255(5)	273(25), 257(20), **179**(100), 151(75)	**151**(100)
19	6.45	**Kaempferol 3-*O*-(2"-rhamnosyl)hexoside**	$C_{27}H_{29}O_{15}^-$	593.15119	593.14948	1.71	429(50), 327(20), 285(80), **284**(100), 255(10)	**255**(100), 227(10)	**227**(100), 211(60)

20	6.68	***p*-Coumaric acid**[a,*]	$C_9H_7O_3^-$	163.04007	163.03917	0.90	**119**(100)	119(60), 101(20), 93(25), **91**(100), 72(10)	–
21	6.71	**Quercetin 3-*O*-glucoside**[a,*]	$C_{21}H_{19}O_{12}^-$	463.08820	463.08627	1.93	**301**(100), 300(30)	273(25), 257(20), **179**(100), 151(75)	**151**(100)
22	7.02	**Sinapic acid**[a,*]	$C_{11}H_{11}O_5^-$	223.06120	223.05992	1.28	**208**(100), 179(30), 164(20)	193(10), **164**(100), 149(15), 135(5)	**149**(100), 135(35)
23	7.03	**Naringenin 7-*O*-(2"-rhamosyl)glucoside (Naringin)**[a,*]	$C_{27}H_{31}O_{14}^-$	579.17190	579.17230	0.64	**459**(100), 357(5), 313(25), 271(45), 235(10)	441(30), **357**(100), 339(30), 271(55), 235(85)	**339**(100), 169(20), 151(50), 125(20)
24	7.04	**Ferulic acid**[a,*]	$C_{10}H_9O_4^-$	193.05063	193.04973	0.90	178(70), **149**(100), 134(50)	**134**(100)	**106**(100)
25	7.17	**Quercetin 3-*O*-rhamnoside**[a,*]	$C_{21}H_{19}O_{11}^-$	447.09329	447.09357	0.63	**301**(100), 300(35), 284(20)	273(25), 257(20), **179**(100), 151(75)	**151**(100)
26	7.90	**Coniferyl aldehyde**[a,*]	$C_{10}H_9O_3^-$	177.05572	177.05460	1.12	163(10), **162**(100)	**134**(100), 133(40), 120(20), 106(30)	**106**(100), 65(80)
27	7.98	**Luteolin 7-*O*-rhamnoside**	$C_{21}H_{19}O_{10}^-$	431.09837	431.09744	0.93	286(10), **285**(100)	257(30), **241**(100), 217(75), 199(85), 175(95)	241(5), 226(15), 213(30), **198**(100), 185(20)
28	8.24	**Quercetin**[a]	$C_{15}H_9O_7^-$	301.03538	301.03384	1.54	271(50), 255(20), **179**(100), 151(80), 107(5)	**151**(100)	**107**(100), 83(10)
29	8.40	**Kaempferol 7-*O*-rhamnoside**	$C_{21}H_{19}O_{10}^-$	431.09837	431.09671	1.66	286(10), **285**(100), 284(20), 257(5), 151(5)	257(40), 241(30), 213(10), **151**(100), 107(10)	**107**(100), 83(10), 65(5)
30	8.59	**Eriodictyol**[a,*]	$C_{15}H_{11}O_6^-$	287.05611	287.05582	1.01	269(10), 253(20), 241(20), 199(10), **151**(100)	1**07**(100), 83(10), 65(10)	–
31	8.69	**Luteolin**[a,*]	$C_{15}H_9O_6^-$	285.04046	285.03909	1.37	257(40), **241**(100), 217(50), 199(70), 175(70)	255(50), **227**(100), 211(75), 197(35), 183(85)	–
32	9.33	**Tectochrysin**	$C_{16}H_{11}O_4^-$	267.06628	267.06500	1.28	**252**(100), 224(20)	**224**(100), 180(5)	195(5), **180**(100)
33	9.44	**Naringenin**[a,*]	$C_{15}H_{11}O_5^-$	271.06120	271.05994	1.26	177(10), **151**(100)	**107**(100)	**65**(100)
34	9.52	**Apigenin**[a,*]	$C_{15}H_9O_5^-$	269.04554	269.04462	0.92	225(5), 177(15), **151**(100)	**65**(100)	–
35	9.53	**Genistein**[a,*]	$C_{15}H_9O_5^-$	269.04554	269.04590	1.34	**225**(100), 201(30), 183(20), 181(10), 151(40)	197(50), 183(50), **181**(100), 169(20), 157(10)	–
36	9.66	**Methoxy kaempferol**[*]	$C_{16}H_{11}O_7^-$	315.05103	315.04959	1.44	301(20), **300**(100), 181(5)	**272**(100), 256(60), 216(20), 202(10), 166(35)	**244**(100), 216(30), 166(50), 137(40), 110(10)
37	9.70	**Kaempferol**[a,*]	$C_{15}H_9O_6^-$	285.04046	285.03885	1.61	**255**(100), 227(10)	**211**(100), 195(5), 167(15)	211(40), **137**(100)
38	9.74	**Pinobanksin**[a,*]	$C_{15}H_{11}O_5^-$	271.06120	271.05883	2.37	**253**(100), 243(10), 225(25), 215(15), 197(20)	225(90), 205(50), **197**(100), 185(15), 181(20)	179(65), **169**(100), 151(85)
39	9.87	**Isorhamnetin**[a,*]	$C_{16}H_{11}O_7^-$	315.05103	315.04910	1.93	301(20), **300**(100)	283(30), **271**(100),	**243**(100), 227(70), 216(10),

								255(50), 227(52), 151(90)	199(20)
40	9.94	**Chrysoeriol**[a,*]	$C_{16}H_{11}O_6^-$	299.05611	299.05493	1.18	285(10), **284**(100)	**255**(100), 227(100)	255(10), **237**(100), 211(90), 187(10)
41	10.19	**Dimethyl quercetin**	$C_{17}H_{13}O_7^-$	329.06668	329.06500	1.68	315(10), **314**(100), 299(5)	299(75), **285**(100), 271(80), 257(15), 243(25)	**270**(100)
42	10.65	**Rhamnetin**[*]	$C_{16}H_{11}O_7^-$	315.05103	315.04916	1.87	300(40), 207(10), 193(40), **165**(100), 121(10)	150(50), **121**(100), 97(60), 91(15), 65(20)	**91**(100)
43	10.67	**Rhamnocitrin**	$C_{16}H_{11}O_6^-$	299.05611	299.05447	1.64	**284**(100)	**256**(100)	**239**(100), 227(60), 212(70), 200(80), 188(90)
44	10.75	**Genkwanin**[a,*]	$C_{16}H_{11}O_5^-$	283.06119	283.05994	1.25	**268**(100), 239(80), 211(20)	**239**(100), 211(20)	239(10), **211**(100), 196(70)
45	11.02	**Tricin**	$C_{17}H_{13}O_7^-$	329.06668	329.06488	1.80	315(10), **314**(100)	**299**(100), 285(5), 271(10)	**271**(100), 255(5), 243(5)
46	11.30	**Benzyl caffeate**	$C_{16}H_{13}O_4^-$	269.08193	269.08063	1.30	225(10), 179(10), 178(70), 161(10), **134**(100)	121(90), 111(40), **106**(100)	–
47	11.61	**Chrysin**[a,*]	$C_{15}H_9O_4^-$	253.05063	253.04953	1.10	253(30), **209**(100), 181(20), 165(15), 151(15)	**181**(100), 165(30), 153(20), 141(10)	171(10), **153**(100), 152(10), 139(50)
48	11.70	**Pinocembrin**[a,*]	$C_{15}H_{11}O_4^-$	255.06628	255.06508	1.20	**213**(100), 187(15), 151(30), 145(10), 107(5)	**185**(100), 169(20), 145(20)	185(10), 157(15), **143**(100), 141(50), 117(15)
49	11.82	**Kaempferide**[a,*]	$C_{16}H_{11}O_6^-$	299.05611	299.05484	1.27	**284**(100), 271(20), 255(5), 240(5), 165(20)	255(25), 240(25), 227(20), 164(40), **151**(100)	**107**(100), 83(10), 65(20)
50	11.84	**Galangin**[a,*]	$C_{15}H_9O_5^-$	269.04554	269.04425	1.29	241(40), 227(80), **213**(100), 197(90), 169(50)	211(10), 198(20), 185(40), **169**(100), 143(25)	–
51	12.20	**Acacetin**[a,*]	$C_{16}H_{11}O_5^-$	283.06119	283.05994	1.25	**268**(100)	**239**(100), 211(20)	239(10), **211**(100), 196(70)
52	12.72	**Pinobanksin 3-*O*-propionate**	$C_{18}H_{15}O_6^-$	327.08741	327.08551	1.90	271(5), **253**(100)	225(10), **209**(100), 181(20), 165(15), 151(10)	**181**(100), 180(80), 165(60), 153(30), 141(10)

[a] Confirmed using standards, the other compounds were identified according high resolution mass spectrometry (HRMS) and MS^n; No - peak numbers (corresponding to Figure 1 and Table S2), t_R – retention time; Δ mDa – mean mass accuracy errors.

[*] Compounds identified in all samples

Table 2. Parameters of descriptive statistics for the content of the individual phenolic compounds (mg/kg), total phenolic content (mg GAE/g), radical scavenging activity (%) and cyclic voltammetry activity (%) in the honeydew honey

Botanical origin	Parameter	Phenolic compounds[a,b]													
		PrA	CQA	HBA	HPA	CA	VA	Rut	CouA	Nar	FA	SA	Que-3-rha	Erio	Lut

Abies alba Mill. (n = 22)	Mean	0.175	0.021	0.348	3.514	0.211	0.181	0.002	0.760	0.021	0.455	0.010	0.002	0.016	0.016
	Median	0.185	0.015	0.314	3.933	0.223	0.183	0.007	0.710	0.024	0.325	0.009	0.001	0.018	0.011
	St dev	0.100	0.020	0.200	3.000	0.100	0.070	0.005	0.500	0.020	0.400	0.010	0.005	0.006	0.030
	Min	0.004	0.004	0.003	0.003	0.005	0.003	0.002	0.004	0.004	0.004	0.006	0.002	0.004	0.002
	Max	0.373	0.077	0.682	6.869	0.390	0.293	0.015	2.111	0.047	1.337	0.034	0.014	0.022	0.111
Conifers (n = 17)	Mean	0.117	0.032	0.410	2.429	0.247	0.277	0.002	0.902	0.033	0.601	0.011	0.005	0.020	0.037
	Median	0.130	0.019	0.381	2.180	0.229	0.222	0.007	0.852	0.028	0.625	0.011	0.003	0.018	0.025
	St dev	0.080	0.030	0.200	3.000	0.100	0.200	0.004	0.500	0.020	0.300	0.020	0.006	0.020	0.040
	Min	0.004	0.008	0.251	0.154	0.110	0.125	0.002	0.284	0.017	0.122	0.006	0.002	0.004	0.002
	Max	0.251	0.082	0.643	7.292	0.428	0.739	0.015	2.076	0.071	1.169	0.037	0.021	0.058	0.134
Quercus ilex L. (n = 15)	Mean	0.051	0.013	0.448	0.131	0.210	0.171	0.006	0.581	0.018	0.328	0.015	0.006	0.021	0.024
	Median	0.066	0.011	0.422	0.125	0.191	0.167	0.006	0.470	0.017	0.256	0.016	0.004	0.017	0.013
	St dev	0.050	0.020	0.100	0.050	0.100	0.060	0.006	0.500	0.020	0.400	0.020	0.006	0.009	0.040
	Min	0.004	0.004	0.317	0.054	0.148	0.097	0.003	0.284	0.004	0.123	0.006	0.006	0.016	0.002
	Max	0.119	0.045	0.584	0.222	0.550	0.286	0.016	2.341	0.057	1.591	0.036	0.014	0.042	0.160
Quercus frainetto Ten. (n = 4)	Mean	0.299	0.026	0.412	0.254	0.273	0.250	0.007	0.900	0.041	0.590	0.006	0.008	0.019	0.006
	Median	0.354	0.018	0.405	0.260	0.280	0.253	0.009	0.848	0.039	0.557	0.006	0.009	0.019	0.007
	St dev	0.300	0.030	0.080	0.100	0.200	0.030	0.005	0.300	0.020	0.400	0.000	0.007	0.003	0.005
	Min	0.004	0.006	0.323	0.131	0.116	0.212	0.004	0.647	0.028	0.252	0.006	0.006	0.016	0.002
	Max	0.488	0.062	0.515	0.366	0.420	0.281	0.010	1.255	0.059	0.991	0.006	0.014	0.022	0.010
Acer monspessulanum L. (n = 6)	Mean	0.041	0.013	0.361	0.529	0.165	0.227	0.004	0.582	0.026	0.335	0.008	0.005	0.013	0.050
	Median	0.024	0.013	0.294	0.230	0.158	0.159	0.004	0.585	0.018	0.327	0.005	0.006	0.018	0.030
	St dev	0.050	0.009	0.300	0.700	0.040	0.300	0.005	0.200	0.030	0.200	0.020	0.006	0.011	0.060
	Min	0.004	0.004	0.162	0.065	0.128	0.094	0.002	0.308	0.004	0.175	0.006	0.006	0.004	0.005
	Max	0.119	0.022	0.802	1.700	0.208	0.672	0.010	0.908	0.079	0.475	0.025	0.014	0.024	0.138

Table 2. Continuation

Botanical origin	**Parameter**	**Phenolic compounds[a,b]**											**TPC**	**RSA**	**CV[c]**
		Que	**Narn**	**Api**	**Gen**	**Kfrl**	**Chry**	**Pin**	**Aca**	**Genkw**	**Gal**	**Kfrd**			
Abies alba Mill. (n = 22)	Mean	0.412	0.028	0.068	0.029	0.058	0.241	0.321	0.011	0.014	0.086	0.008	0.61	13.14	25.42
	Median	0.358	0.025	0.050	0.016	0.061	0.231	0.320	0.010	0.013	0.075	0.001	0.58	10.88	20.59
	St dev	0.400	0.030	0.060	0.040	0.040	0.200	0.300	0.006	0.007	0.080	0.006	0.17	4.62	9.07
	Min	0.008	0.002	0.003	0.003	0.020	0.002	0.002	0.006	0.004	0.003	0.002	0.43	8.63	17.06
	Max	1.414	0.065	0.175	0.139	0.119	0.439	0.956	0.025	0.029	0.312	0.016	1.14	23.32	50.29
Conifers	Mean	0.575	0.039	0.111	0.036	0.098	0.231	0.300	0.011	0.015	0.096	0.008	0.57	12.20	22.45

(n = 17)	Median	0.426	0.031	0.077	0.022	0.073	0.208	0.185	0.010	0.012	0.061	0.009	0.57	11.15	20.29
	St dev	0.500	0.030	0.090	0.040	0.070	0.200	0.300	0.010	0.020	0.100	0.004	0.14	4.23	10.01
	Min	0.009	0.011	0.026	0.003	0.027	0.041	0.035	0.006	0.004	0.012	0.002	0.29	8.64	9.71
	Max	1.779	0.085	0.338	0.106	0.250	0.630	1.104	0.045	0.058	0.357	0.016	0.79	25.72	50.59
Quercus ilex L. (n = 15)	Mean	1.103	0.069	0.032	0.008	0.155	0.272	0.298	0.007	0.011	0.100	0.011	1.19	26.58	52.82
	Median	0.960	0.035	0.200	0.003	0.134	0.240	0.226	0.009	0.012	0.069	0.010	1.28	26.51	53.68
	St dev	0.700	0.090	0.040	0.020	0.200	0.200	0.300	0.008	0.008	0.100	0.005	0.25	5.20	10.34
	Min	0.410	0.010	0.009	0.003	0.060	0.058	0.066	0.006	0.004	0.020	0.002	0.79	17.92	35.00
	Max	3.194	0.282	0.127	0.043	0.595	0.667	1.161	0.020	0.023	0.414	0.019	1.57	35.32	67.94
Quercus frainetto Ten. (n = 4)	Mean	1.093	0.078	0.039	0.003	0.103	0.198	0.267	0.014	0.016	0.107	0.011	1.60	48.89	94.12
	Median	1.219	0.090	0.036	0.003	0.106	0.175	0.270	0.016	0.019	0.121	0.014	1.60	48.89	94.12
	St dev	0.600	0.050	0.025	0.000	0.045	0.200	0.200	0.006	0.007	0.062	0.009	0.20	3.83	8.32
	Min	0.280	0.016	0.012	0.003	0.046	0.089	0.076	0.005	0.006	0.020	0.002	1.46	46.18	88.24
	Max	1.656	0.116	0.070	0.003	0.155	0.353	0.452	0.019	0.021	0.166	0.017	1.74	51.59	100.00
Acer monspessulanum L. (n = 6)	Mean	0.576	0.027	0.051	0.004	0.091	0.237	0.258	0.015	0.017	0.085	0.008	0.91	23.43	50.47
	Median	0.667	0.020	0.041	0.003	0.085	0.234	0.240	0.013	0.016	0.074	0.009	1.04	22.98	44.12
	St dev	0.308	0.030	0.030	0.003	0.060	0.100	0.200	0.008	0.008	0.057	0.005	0.29	4.65	18.27
	Min	0.092	0.005	0.030	0.003	0.020	0.106	0.101	0.006	0.007	0.033	0.002	0.44	18.88	35.88
	Max	0.908	0.070	0.098	0.010	0.181	0.405	0.554	0.026	0.031	0.183	0.014	1.15	30.96	82.35

[a] Phenolic compounds: PrA – Protocatechuic acid; CQA – 5-O-Caffeoylquinic acid; HBA – *p*-Hydroxybenzoic acid; HPA – *p*-Hydroxyphenylacetic acid; CA – Caffeic acid; VA – Vanillic acid; Rut – Rutin; CouA – *p*-Coumaric acid; Nar – Naringin; FA – Ferulic acid; SA – Sinapic acid; Que–3 rha – Quercetin 3-O-rhamnoside; Erio – Eriodictyol; Lut – Luteolin; Que – Quercetin; Narn – Naringenin; Api – Apigenin; Gen – Genistein; Kfrl – Kaempferol; Chry – Chrysin; Pin – Pinocembrin; Aca – Acacetin; Genkw – Genkwanin; Gal – Galangin; Kfrd – Kaempferide.

[b] LOQ values were: 0.002 mg/kg for the Rut, Que-3-rha, Lut, Narn, Chry, Pin, and Kfrd; 0.003 mg/kg for HBA, HPA, VA, CouA, FA, Erio, Api, Gen, Genkw, and Gal; 0.004 mg/kg for PrA, CQA, CA, and Nar; 0.005 mg/kg for SA, Que, Kfrl, and Aca.

[c] Sample with highest value was labelled as 100%.

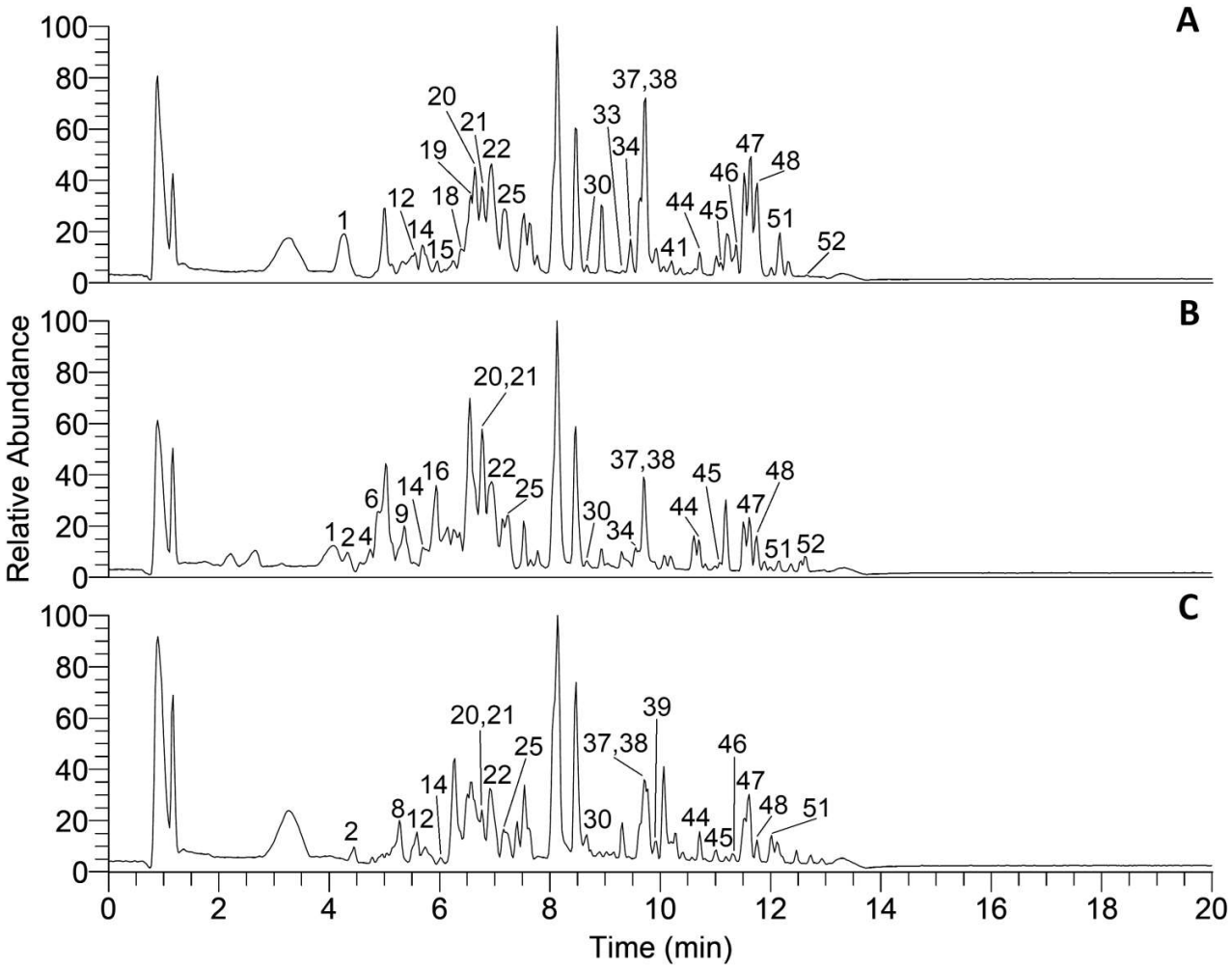


Figure 1.

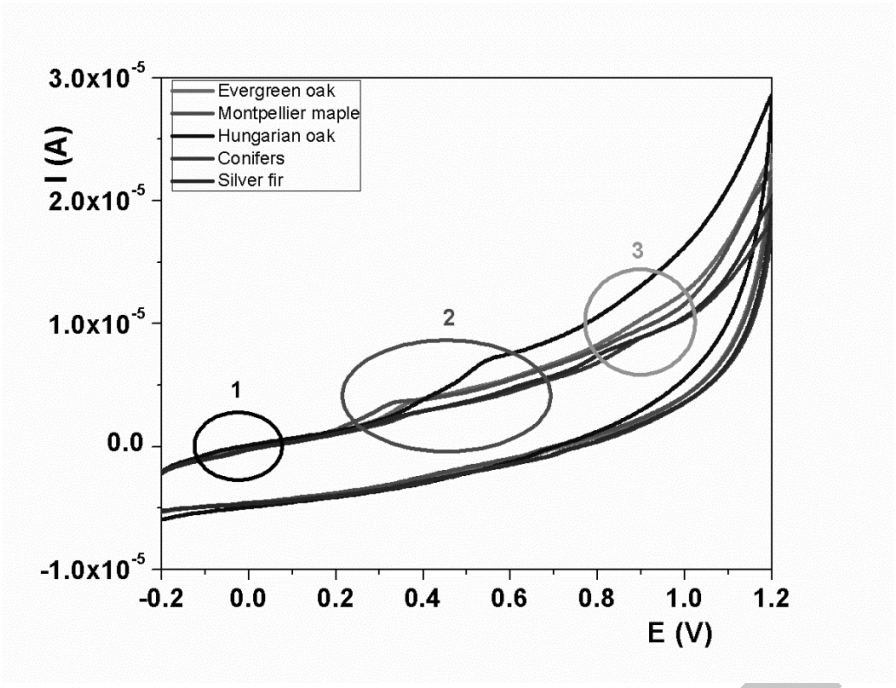
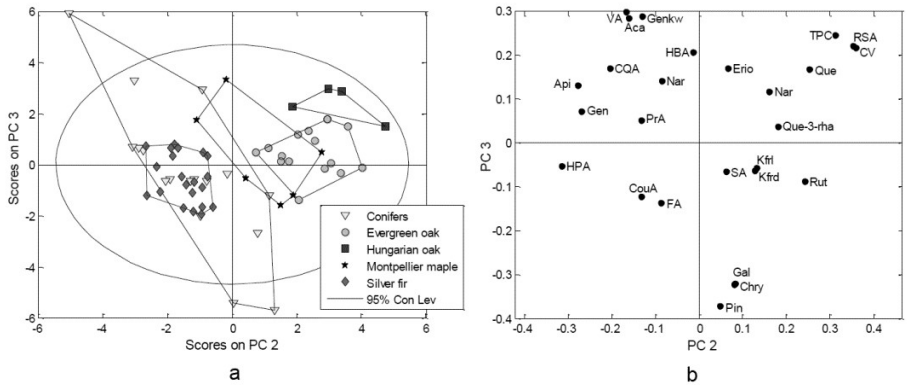


Figure 2.



a b

Figure 3.

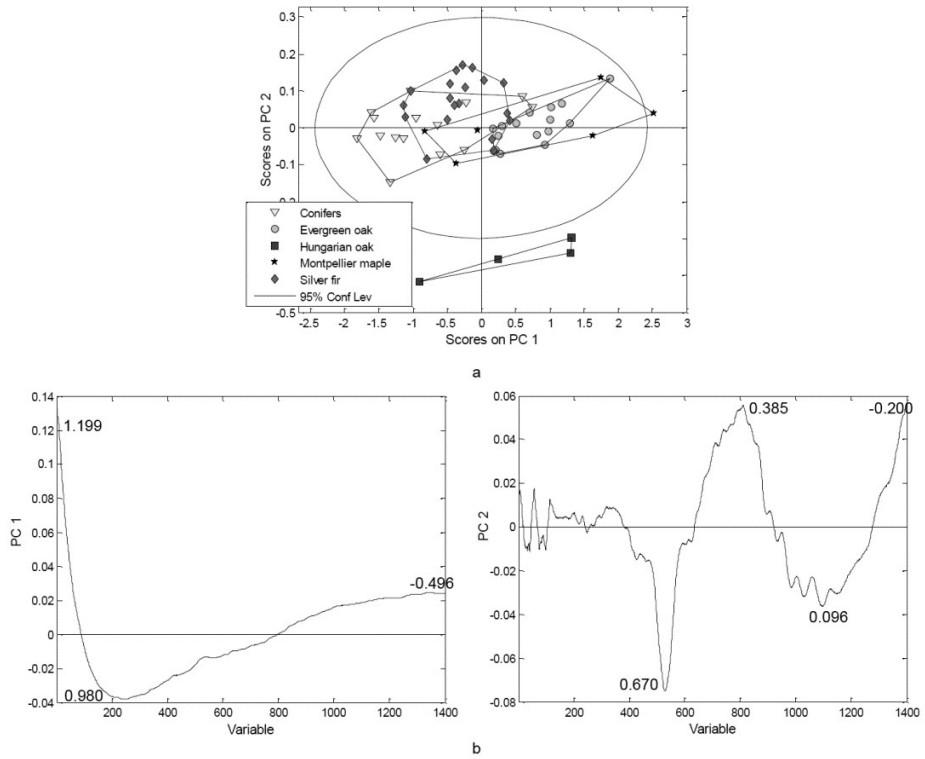


Figure 4.