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Botanical Origin Causes Changes in Nutritional Profile and Antioxidant Activity of Fermented Products Obtained from Honey

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Supporting Information

ABSTRACT: Honey as rich source of enzymatic and nonenzymatic antioxidants serves as health-promoting nutrient in the human body. Here, we present the first time a comparative study of nutritional profiles (e.g., acidities, sugar, organic acid profile, total polyphenolic, flavonoid content) for different unifloral, multifloral honeys and their fermented products, in correlation with their antioxidant activity. Additionally, an optimized method for HPLC separation of organic acids from honey was established. The total phenolic content of honey samples varied widely among the honey types compared to fermented products. High amounts of total flavonoids were quantified in heather honey, followed by raspberry, multifloral, black locust, and linden honey. A positive correlation between the content of polyphenols, flavonoids, and antioxidant activity was observed in honey samples. After fermentation, the flavonoid content of dark honey fermented products decreased significantly. Black locust and linden honeys are more suitable for fermentation because the decrease in antioxidant substances is less pronounced.

KEYWORDS: *honey, honey-must, fermentation, organic acids, polyphenols, flavonoids, antioxidant activity*

INTRODUCTION

Antioxidants scavenge free radicals (e.g., superoxide anion, hydrogen peroxide, and hydroxyl radical) with negative effects in the human body.[1] In recent decades, there is an increasing interest in natural food products that may contain phytochemicals possessing antioxidant activity. Knowing that nutrition is very important for fighting against free radicals, most of the antioxidant active compounds in the human diet are of plant origin.[1−3]

There are numerous scientific studies demonstrating honey as a rich source of enzymatic or nonenzymatic antioxidants, including glucose oxidase, phenolic acids, flavonoids, organic and amino acids, proteins, and carotenoids.[4−6] In addition, the health-promoting activity of honey was proven by reducing the occurrence risk for heart diseases, reducing inflammatory processes,[7] improving the activity of the immune system,[8] and treatment of gastric ulcers and gastritis.[9,10] Chemical composition, especially the antioxidant capacity of honey, depends mostly on the floral source where bees forage for nectar and pollen as well as on geographical and environmental factors.[11−13] Plant nectar is the main source of antioxidants in honey.[14,15] Thus, different types of unifloral honeys may possess different amounts of biologically active compounds[16−19] and exhibit different antioxidant properties.

Since ancient times, humans use beverage products obtained from fermented honey, such as mead and vinegar in their diet.[20,21] Mead is an alcoholic beverage produced by fermenting a solution of honey and water using different yeast strains.[22] Mead has positive effects on metabolism in general and particularly on digestion, possesses physiological benefits, and reduces the risk of chronic disease beyond basic nutritional functions.[23]

Vinegar is produced in two different ways: (1) slow method, where acetic acid bacteria (AAB) grow on the surface of the liquid containing the raw material, and (2) quick process in closed recipients, where oxygenation is ensured by agitation.[24,25] Generally, honey vinegar is produced by the quick process.[26] Increasing interest was given in the past years to vinegar as a food product, its chemical and nutritional properties being determined by production method as well as raw material used for fermentation.[25]

Studies on biochemical transformations of organic acids in honey during fermentation and fermentation conditions (correct pH and temperature, specific yeast breeds, or growth biocatalysts) are discussed in the literature.[27] According to the fact that different honey types are characterized by distinctive organoleptic properties, due to the aromatic compounds present in their composition, using different honey types to produce honey vinegars is a challenging idea.

The main objective of the study was the determination of changes occurring during the fermentation process in the main nutrient compounds and functional products for different types of honey. Furthermore, we performed an optimization of a HPLC-DAD method to quantify the organic acids present in honey and products obtained from fermentation. As a part of this study on the functional properties of different honey types, phenolic and flavonoid content, antioxidative effects, scavenging abilities, and antioxidant power were evaluated and possible correlations were established.

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Table 1. Validation Parameters for Organic Acids Obtained Using Improved HPLC Detection Method[a]

	method sensitivity			recovery studies			method precision		
acid	t_R (min)	LOD (mg/kg)	LOQ (mg/kg)	added amount level 1 (mg/kg)	added amount level 2 (mg/kg)	recovery (%)	t_R (RSD%)	intraday (RSD%)	interday (RSD%)
oxalic[b]	4.57	0.50	1.62	2.2	6.6	98	0.2	0.3	0.6
gluconic[b]	4.76	5.64	15.12	15.8	47.4	100	0.3	0.8	1.2
tartaric[b]	5.30	5.52	15.44	16.2	48.6	101	0.1	0.5	1.1
formic[b]	5.57	1.45	4.25	4.8	14.4	95	0.2	0.9	1.3
malic[b]	6.69	1.21	3.20	3.8	11.4	100	0.5	0.6	1.3
ascorbic[c]	7.59	2.05	5.86	6.5	19.5	99	0.2	0.8	2.7
acetic[b]	8.34	8.32	18.25	18.8	56.4	105	0.6	0.8	1.6
maleic[b]	10.78	4.25	12.42	13.2	39.6	94	1.2	0.4	1.1
citric[b]	11.86	1.50	4.60	5.0	15.0	97	0.3	0.2	0.8
succinic[b]	13.49	2.12	5.84	6.2	18.6	98	0.5	0.9	1.8

[a] t_R, retention time; LOD, limit of detection; LOQ, limit of quantification; RSD%, percent of relative standard deviation; intraday, repeatability; interday, reproducibility. [b] Detection at 202 nm. [c] Detection at 242 nm.

MATERIALS AND METHODS

Honey-Must Preparation and Fermentation. Fermented products were obtained from five unifloral honeys, namely black locust (*Robinia pseudoacacia* L.), linden (*Tilia* sp.), heather (*Calluna vulgaris* L. Hull), raspberry (*Rubus idaeus* L.), canola (*Brassica* sp.), and one multifloral. Three different batches of each unifloral and multifloral honeys were thoroughly mixed, and for each honey type, a single pool was used for the specific analyses and fermentation experiments to reduce between-samples variation. Honey samples raw material for fermentation were fluid (black locust), partially crystallized (linden 10%, raspberry 60%, and multifloral 40%), totally crystallized (canola), or showed special thixotropic structure as with heather honey. The botanical origin of honey was verified by physicochemical and melissopalynological analysis (Supporting Information Table S1). Characteristics and quality were confirmed in accordance with the limits established by the Council Directive 2001/110/EC.[28]

The honey-must was prepared by diluting honey, 800 g in 800 mL of distilled water at 40 °C, in glass jars, stopped with fermentation trap tubes. *Saccharomyces cerevisiae* (baker's yeast) was inoculated to the must (5 g/tank) for speeding the fermentation process.[29] The fermentation took place at room temperature during 60 days. Additional samples, to observe the fermentation process, were taken at 30 and 40 days, for pH and acidity measurements. All fermentation experiments were conducted in duplicate.

Analytical Methods. pH and acidity were determined by automatic titration using Titrolyne Easy and Titrolyne Alpha10plus equipment (SCHOTT Instruments GmbH, Mainz, Germany) following a method described previously.[30] Alcohol content was determined in fermented products according to official methods.[31]

To determine organic acids, a previous method[32] was modified and adapted to honey; using a Shimadzu instrument equipped with two pumps, system controller, auto sampler, degasser, column oven, and photo diode array detector. Chromatographic separation of 10 organic acids was carried out with a 15 mM phosphate solution ($Na_2HPO_4{\cdot}2H_2O$) buffered at pH 2.70 with concentrated sulfuric acid (mobile phase A), acetonitrile (mobile phase B), and a difunctionally bonded C18 stationary phase column (Atlantis dC18; 250 mm × 4.6 mm; particle size 5 μm; Milford, MA, USA), using isocratic elution with 100% A for 5 min and a gradient program afterward (5–10 min, 0–10% B; 10–20 min 10% B linear; 20–23 min, 10–0% B; followed by 22 min washing and re-equilibration). Flow rate of separation was 0.75 mL/min, and the column was thermostated at 40 °C. Injection volume was set at 10 μL. Each standard and sample was analyzed in triplicate. An ALLTECH Vacuum Manifold system (Alltech Associates, Inc., Deerfield, IL, USA) was used for sample preparation following the method described for minority organic acids from honey.[33]

Organic acids, except ascorbic acid (242 nm), were detected at 202 nm. Signals were registered in a chromatography data system using LC-Solution software (Shimadzu, Kyoto, Japan). Peaks in the chromatograms were identified by comparison with retention time, elution order, and UV–VIS spectra of pure compounds and spiked samples. External standard method was used for quantification of identified compounds.

Adapting the original method,[32] we aimed to develop a simple, sensitive, and robust method for separation of a maximum number of organic acids from honey samples or honey-derived products in the shortest analysis time, using simple sample preparation (dilution of honey in ultrapure water, purification on SPE-Strata-X-A33u polymeric strong anion column (Phenomenex, Torrance, CA, USA), using a vacuum manifold system, before HPLC injection). Single injection of each organic acid (0.25 mg/mL) was performed for retention time and intensity of signal determination. Estimations of expected results were used to prepare higher concentrations of standard solutions. Six additional working solutions were prepared by successive dilutions and injected for linearity tests. Internal validation was realized by assessing method sensitivity, precision, and recovery rate. Sensitivity was measured by establishing limit of detection (LOD) and limit of quantification (LOQ) of each compound, calculated as the concentration corresponding to the signal of each standard plus 3 times (for LOD) and 10 times (for LOQ) the standard deviations of the replicates ($N = 6$). Precision was evaluated through successive replicate determination, and recovery was determined by addition of known standard concentrations to the samples at two concentration levels (lower level meaning concentrations above limit of quantification and higher level meaning three times higher) and replicated three times. Mean values of replicates were compared with theoretical concentration to calculate the average recovery (Table 1).

Sugar profile was determined following a HPLC-RID method described by Bogdanov et al.[30] on a Shimadzu instrument with refractive index detector, amino modified column Alltima Amino (100 A, 5 μm, 250 mm × 4.6 mm), using a mixture of acetonitrile and water as mobile phase (75:25 v/v). Injection volume was 20 μL, column pressure 6.3 MPa, and 1 mL/min the flow rate of the mobile phase.

Sugar standards (glucose, fructose, sucrose, turanose, maltose, trehalose, isomaltose, and erlose) were injected separately and in mixture in the range of 0–50% concentration (depending on the amount present in honey) for calibration curve and linearity range test.

Concentration of total phenolics in diluted honey (50% w/v with ultrapure water) and fermented honey products were determined using the original method[34] adapted for honey. Honey and fermented product solutions (0.5 mL) were mixed with 0.2 N Folin–Ciocalteu reagent and 2 mL of Na_2CO_3 solution (75 g/L). The reaction mixture was left in darkness for 2 h, and the absorbencies were measured at 760 nm toward a blank (methanol, Folin reagent and sodium carbonate) using a calibration curve of gallic acid (GAE) in the range of 0–0.1 mg/mL and the UV-1700 PharmaSpec UV–VIS spectrophotometer (Shimadzu, Japan). Results were expressed as mgGAE/kg honey.

Aluminum chloride was used for quantification of flavone/flavonol entities, as a part of total flavonoid content in food or plant samples.[35] Original method[3] for quantification of total flavonoids adapted for honey, using quercetin in the range of 0−0.1 mg/mL as standard for calibration curve, was used in this study. Briefly, 1 mL of the sample was mixed with 0.3 mL of $NaNO_3$ (5%), 0.3 mL of $AlCl_3$ (10%), and 2 mL of NaOH (1 M). The absorbance was read toward a blank at 510 nm using the Synergy HT multidetection microplate reader (BioTek Instruments Inc.Vermont, Winooski, VT, USA) using a calibration curve of quercetin (QE) and expressed as mgQE/kg honey.

Antioxidant activity was measured by two different methods using electron transfer assays. In the presence of an antioxidant, the purple color of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) decayed; the decreasing intensity was directly proportional to the amount of antioxidants. Then 0.3 mL of each sample was mixed with 2.8 mL of DPPH methanolic solution (0.03 mg/mL) and kept in darkness for 15 min. Absorbencies of blank (methanol and DPPH solution) and samples were measured at 517 nm toward methanol, using the Synergy HT multidetection microplate reader. The radical scavenging activity (RSA) was calculated as percentage of inhibition for the DPPH radical.

Ferric Reducing Antioxidant Power (FRAP) original assay,[36] measured the reducing capacity of natural antioxidants that reacted with 2,4,6-tripyridyl-*s*-triazine complex (Fe^{3+}-TPTZ), transforming the complex to its reduced form (Fe^{2+}-TPTZ). Briefly, 10 μL sample solutions and 300 μL of freshly prepared FRAP reagent (10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$, and 0.3 M acetate buffer, pH 3.6) were placed in 96-well plates, including the blank (10 μL of ultra pure water and 300 μL of FRAP) and standards (0.1−1 mmol/L of $FeSO_4$). The absorbance at 593 nm was read out in the microplate reader after 10 min incubation at 37 °C. Results were expressed as FRAP value (μmol Fe(II)) of the sample solution.

Statistical Analysis. Data analysis was performed using STATISTICA 8.0 (StatSoft, Tulsa, Oklahoma, USA) to test for significant differences between several honey and fermented product samples. The Shapiro−Wilk test was used to test for normality of the data set. However, none of the tested characteristics of honey and fermented products showed the required normal distribution ($P < 0.05$). Neither square root, log, nor reciprocal transformation changed this deviation, thus Kruskal−Wallis one-way analysis of variance by ranks (nonparametric Kruskal−Wallis test) was used to test for significant differences.

Spearman rank correlation coefficients (r) were used to determine the relationships between antioxidant activity, polyphenol, and flavonoid content. This approach required pooling of all honey and fermented product samples.

RESULTS AND DISCUSSION

Evolution of pH, Free and Lactonic Acidity during Honey-Must Fermentation. High variability was found in the evaluated parameters of honey types and fermented products. Free acidity rose in the first period of fermentation due to the changes in acetic, gluconic, malic, and succinic acid production.

Monitoring pH and acidity after honey-must preparation and during fermentation are important issues to prevent premature fermentation arrest and incomplete sugar breakdown. After 30 days of fermentation, honey fermented samples were analyzed for pH, free and lactonic acidity. These analyses were repeated at 40 and 60 days of fermentation. As expected, the pH of the products decreased as the fermentation process occurred due to the changes in the organic acids spectrum. This was beneficial because it stopped the development of disadvantageous microflora.[27,37] Thereafter, pH remained almost unchanged until the end of fermentation (Supporting Information Figure S1), with different values according to different initial pH of honey solutions.

Free acidity reflects the amount of the substance (active acid molecules) and can react with other surrounding molecules. This parameter increased significantly in the first 30 days of fermentation (53−105%), more after 45 days (62−120%), but thereafter, a slight decrease was observed (Supporting Information Figure S2A). Changes in organic acids content during fermentation, mainly formation of acetic and succinic acid are the major causes of increasing free acidity.[27] Lactonic acidity presents the highest values after 30 days of fermentation (14−19% increase) (Supporting Information Figure S2B). Subsequently, at 45 days of fermentation, a decrease in lactonic acidity was observed, with consequences on the fermentation process that was slowed down. Increasing free acidity in the first week of fermentation due to the production of acetic and succinic acids, causing the decrease of pH, was observed in other studies.[27,37]

Method Validation and Organic Acids Determination. Organic acids are very important contributors to honey properties. Despite being in small quantities (< 0.5%), organic acids contribute to antibacterial and antioxidant activities.[4] The original method[32] describes a protocol to characterize organic acids from wine samples. Optimization helped to improve separation of organic acids present in honey and honey derived products in order to obtain proper resolution considering: wavelength detection, used solvents, elution program, and working conditions for column and sample preparation. Considering the organic acid profile present in honey and its importance in the antioxidant properties, additionally, gluconic, ascorbic, and maleic acids were included in the mixture of standards used in the optimization of the method. Tested concentrations of phosphate buffer used as mobile phase were 5, 10, and 15 mM. Furthermore, different values for pH were also tested (2.5, 2.7, 3.0, and 3.5). Finally, the best separation of 10 organic acids (no overlapping, well-resolved chromatograms) was obtained during a 14 min gradient program (see Materials and Methods) using 15 mM phosphate buffer (2.7 pH) as mobile phase A, acetonitrile as mobile phase B, and a flow rate of 0.75 mL/min (Figure 1). Method sensitivity was measured by limit of detection (LOD) and limit of quantification (LOQ). Recovery studies determined the

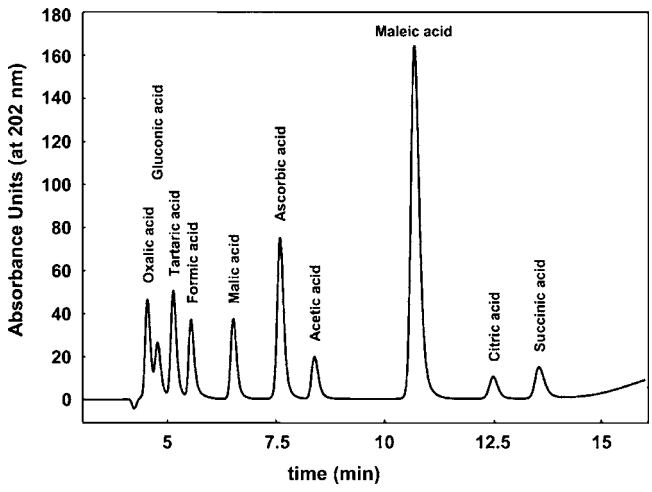

Figure 1. HPLC-DAD chromatogram for mixture of organic acids standard solution, separated with optimized method (concentration 0.25 mg/mL). Organic acids were detected at 202 nm, except for ascorbic acid (242 nm).

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accuracy of the method. A honey sample, prepared as described before, was analyzed with or without the addition of different amounts of organic acids (Table 1). The recovery rate was situated between 94 and 105% (Table 1). Repeatability and reproducibility were also assessed to evaluate the precision of the method. Two different standard working solutions (0.5 and 25 g/L) were evaluated in three different days for intra- and interday repetitions. Relative standard deviation (RSD%) of retention times (t_R) and pick area showed a small variation for intra- and interday analysis (1.2% for t_R and 2.7% for pick area, Table 1).

According to the intensity of absorbance of each organic acid present in the mixture, LOD ranged from 0.50 to 8.32 mg/kg and LOQ values ranged between 1.62 and 18.25 mg/kg for all 10 separated organic acids. Using HPLC-DAD analysis in approximately 14 min,[33] five organic acids were determined compared to our improved method that separates 10 organic acids in the same period. LOD and LOQ values obtained by our improved method are comparable with other HPLC determinations,[38] who used two or four coupled columns in the chromatographic system or used capillary zone electrophoresis.[39]

HPLC-DAD analyses of organic acids were determined in honey samples raw material and after 60 days of fermentation. In the first weeks of fermentation, the microorganisms synthesized acetic and succinic acid, and the amount found in fermented products was much higher than in initial honey samples (Table 2). In a previous study,[40] much more acetic acid was synthesized if the honey-must had a low pH. This was the case in our products obtained from black locust and raspberry honey, which had the lowest pH of the honey samples, but the highest accumulation of acetic acid after fermentation (4.05 and 5.71 g/L), compared to honey with higher pH values (4.36 linden honey) (Table 2) and the lowest synthesis of acetic acid (3.93 g/L). The increase of tartaric acid after 60 days of fermentation was about 0.4−2.7 times. Malic acid was detected (in the limit of the detection method) in linden, raspberry, and canola honey, and all fermented products except raspberry honey fermented product (Table 2). Small quantities of maleic acid were quantified in all fermented products, ranging between 3.22 and 11.61 mg/kg (Table 2). This acid was not detectable in any honey raw material. Additionally, in none of the honey or fermented product samples was formic acid detectable, and maybe the detectable amount lay below the detection limits of the method. Gluconic acid increment was measured in fermented products compared with honey raw material (from 58.82−66.38 g/kg to 56.44−61.26 g/kg) due to the decomposition of glucose, lactone formation, and oxidation to the corresponding organic acid. Oxalic, ascorbic, and citric acid content did not change significantly during fermentation process (Table 2). Studies, using a HPLC system with an UV-DAD detector for acid detection, showed similar results for oxalic, gluconic, and citric acids in heather honey,[38] while for fermented products,[27] comparable results for acetic, succinic, citric, and tartaric acids were achieved. This verifies the usability of the improved method to control quality of honeys and fermented products.

Sugar Spectrum in Honey and Honey-Fermented Products. Sugar spectrum of honey samples was similar to those obtained from different types of unifloral honey.[41,42] Glucose and fructose are the main sugars in honey and fermented products (Table 3). The spectrum also showed small quantities of turanose, maltose, trehalose, and isomaltose.

Table 2. Content of Nine Different Organic Acids in Six Honeys and Their Respective Fermented Products[a]

	oxalic acid (mg/kg) mean ± SD	gluconic acid (g/kg) mean ± SD	tartaric acid (mg/kg) mean ± SD	malic acid (mg/kg) mean ± SD	ascorbic acid (mg/kg) mean ± SD	acetic acid (g/kg) mean ± SD	maleic acid (mg/kg) mean ± SD	citric acid (mg/kg) mean ± SD	succinic acid (mg/kg) mean ± SD
BLH	180.67 ± 4.11 ^{c}	59.31 ± 1.96 $^{a-d}$	67.51 ± 1.46 ^{a,b}	nd ^{a}	1.23 ± 0,35 ^{a,b}	0.09 ± 0.01 ^{a}	nd ^{a}	115.02 ± 6.75 $^{a-c}$	nd ^{a}
BLH-FP	138.52 ± 3.56 ^{a,b}	65.36 ± 2.61 ^{c,d}	111.57 ± 9.76 $^{a-d}$	120.25 ± 2.17 $^{a-c}$	1.37 ± 0.19 $^{a-c}$	4.05 ± 0.09 $^{c-e}$	8.26 ± 1.19 ^{b}	107.08 ± 7.92 ^{a,b}	607.58 ± 13.35 ^{a,b}
LH	158.25 ± 2.55 $^{a-c}$	57.81 ± 1.70 ^{a,b}	86.17 ± 3.71 $^{a-c}$	15.83 ± 2.85 ^{a,b}	2.90 ± 0.16 $^{a-d}$	0.57 ± 0.04 $^{a-e}$	nd ^{a}	442.66 ± 22.73 ^{e}	nd ^{a}
LH-FP	127.46 ± 3.51 ^{a}	66.38 ± 2.42 ^{d}	97.05 ± 3.99 $^{a-d}$	117.99 ± 4.09 $^{a-c}$	3.27 ± 0.26 $^{a-d}$	3.93 ± 0.15 $^{b-e}$	5.86 ± 0.26 ^{a,b}	179.12 ± 5.92 $^{a-e}$	1061.53 ± 40.38 ^{b}
RH	182.35 ± 3.99 ^{c}	56.44 ± 4.36 ^{a,b}	84.31 ± 5.19 $^{a-c}$	195.03 ± 3.53 ^{b,c}	6.95 ± 0.13 ^{c,d}	0.09 ± 0.02 ^{a,b}	nd ^{a}	296.15 ± 5.03 ^{d,e}	nd ^{a}
RH-FP	143.82 ± 3.60 $^{a-c}$	63.62 ± 1.90 $^{b-d}$	136.08 ± 3.24 ^{c,d}	nd ^{a}	8.08 ± 0,15 ^{d}	5.71 ± 0.19 ^{e}	3.22 ± 0.21 ^{a,b}	122.16 ± 3.03 $^{a-d}$	462.88 ± 13.27 ^{a,b}
CH	162.25 ± 4.74 $^{a-c}$	58.17 ± 2.03 $^{a-c}$	97.41 ± 2.68 $^{a-d}$	165.49 ± 5.29 $^{a-c}$	4.45 ± 0.36 $^{b-d}$	0.29 ± 0.01 $^{a-d}$	nd ^{a}	292.44 ± 11.04 $^{c-e}$	nd ^{a}
CH-FP	133.10 ± 4.22 ^{a,b}	62.84 ± 1.85 $^{a-d}$	121.89 ± 1.44 $^{b-d}$	167.54 ± 4.34 $^{a-c}$	4.86 ± 0.09 $^{b-d}$	4.50 ± 0.35 ^{d,e}	6.83 ± 0.37 ^{a,b}	169.11 ± 9.10 $^{a-d}$	561.69 ± 7.48 ^{a,b}
HH	180.71 ± 6.60 ^{c}	61.26 ± 4.94 $^{a-d}$	362.54 ± 30.53 ^{d}	nd ^{a}	0.72 ± 0,25 ^{a}	0.11 ± 0.01 $^{a-c}$	nd ^{a}	298.97 ± 12.50 ^{d,e}	nd ^{a}
HH-FP	134.89 ± 4.45 ^{a,b}	58.82 ± 1.06 $^{a-c}$	477.80 ± 15.79 ^{d}	241.48 ± 4.79 ^{b,c}	0.97 ± 0.17 ^{a}	4.08 ± 0.08 ^{d,e}	11.61 ± 0.89 ^{b}	210.90 ± 5.68 $^{a-e}$	1013.17 ± 18.50 ^{a,b}
MH	170.57 ± 2.10 ^{b,c}	56.49 ± 3.01 ^{a}	59.74 ± 2.12 ^{a}	nd ^{a}	1.16 ± 0,09 ^{a,b}	0.22 ± 0.02 $^{a-d}$	nd ^{a}	262.58 ± 14.24 $^{b-e}$	nd ^{a}
MH-FP	150.51 ± 2.90 $^{a-c}$	61.48 ± 0.83 $^{a-d}$	163.33 ± 3.48 ^{c,d}	259.05 ± 11.45 ^{c}	2.25 ± 0.13 $^{a-d}$	3.15 ± 0.17 $^{a-e}$	6.52 ± 0.36 ^{a,b}	93.02 ± 5.69 ^{a}	1614.16 ± 31.46 ^{b}

[a]Each product is characterized by the mean and SD for the specific acid. Multiple comparison tests (2-tailed) and Kruskal−Wallis tests (H (df = 11, N = 72) = 49.98−70.59, P < 0.001) were used to test for significant differences between honeys and fermented products. SD, standard deviation; nd, not detected; BLH, black locust honey; LH, linden honey; RH, raspberry honey; CH, canola honey; HH, heather honey; MH, multifloral honey; FP, fermented product. Values in the same column with different superscripts (a−e) are significantly different.

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Table 3. Content of Seven Different and Total Sugar in Honeys and Their Respective Fermented Products[a]

	fructose (%) mean ± SD	glucose (%) mean ± SD	turanose (%) mean ± SD	maltose (%) mean ± SD	trehalose (%) mean ± SD	isomaltose (%) mean ± SD	erlose (%) mean ± SD	total sugar (%) mean ± SD
BLH	41.76 ± 0.67 ^{b,c}	31.72 ± 0.49 $^{b-d}$	1.65 ± 0.14 $^{a-d}$	1.72 ± 0.04 ^{e}	0.61 ± 0.02 $^{a-c}$	0.42 ± 0.03 $^{a-e}$	0.58 ± 0.03 ^{c}	78.45 ± 1.05 ^{d,e}
BLH-FP	46.09 ± 0.53 ^{c}	27.18 ± 1.36 $^{a-c}$	1.99 ± 0.06 ^{d}	0.75 ± 0.05 ^{a,b}	0.76 ± 0.06 ^{c,d}	0.45 ± 0.05 $^{a-e}$	0.12 ± 0.03 $^{a-c}$	77.34 ± 1.55 $^{c-e}$
LH	35.79 ± 0.61 ^{a}	30.83 ± 0.71 $^{b-d}$	1.65 ± 0.06 $^{a-d}$	1.22 ± 0.10 $^{c-e}$	0.76 ± 0.07 ^{c,d}	0.46 ± 0.06 $^{a-e}$	0.0002 ± 0.0 ^{a,b}	70.70 ± 0.92 $^{a-d}$
LH-FP	39.49 ± 0.51 $^{a-c}$	23.63 ± 0.43 ^{a,b}	1.85 ± 0.10 $^{b-d}$	0.87 ± 0.06 $^{a-d}$	0.90 ± 0.09 ^{d}	0.48 ± 0.03 $^{b-e}$	nd ^{a}	67.22 ± 0.77 $^{a-c}$
RH	38.49 ± 0.39 ^{a,b}	34.91 ± 0.26 ^{c,d}	1.28 ± 0.03 ^{a,b}	0.87 ± 0.05 $^{a-d}$	0.49 ± 0.04 ^{a,b}	0.33 ± 0.03 ^{a,b}	0.23 ± 0.05 ^{b,c}	76.60 ± 0.45 $^{b-e}$
RH-FP	42.30 ± 0.47 ^{b,c}	27.44 ± 0.61 $^{a-c}$	1.64 ± 0.04 $^{a-d}$	1.11 ± 0.05 $^{b-e}$	0.68 ± 0.05 $^{a-d}$	0.39 ± 0.04 $^{a-d}$	nd ^{a}	73.55 ± 1.02 $^{a-e}$
CH	39.02 ± 0.83 $^{a-c}$	36.08 ± 0.72 ^{c,d}	1.43 ± 0.02 $^{a-c}$	0.89 ± 0.07 $^{a-e}$	0.53 ± 0.04 ^{a,b}	0.37 ± 0.04 $^{a-c}$	0.17 ± 0.05 $^{a-c}$	78.48 ± 1.14 ^{d,e}
CH-FP	40.59 ± 0.43 ^{b,c}	28.49 ± 0.33 $^{a-d}$	1.62 ± 0.07 $^{a-d}$	0.58 ± 0.06 ^{a}	0.62 ± 0.06 $^{a-d}$	0.35 ± 0.07 $^{a-c}$	nd ^{a}	72.26 ± 0.25 $^{a-d}$
HH	39.54 ± 0.57 $^{a-c}$	28.99 ± 0.92 $^{a-d}$	2.12 ± 0.12 ^{d}	1.57 ± 0.04 ^{d,e}	0.67 ± 0.05 $^{a-d}$	0.98 ± 0.08 ^{e}	nd ^{a}	73.88 ± 1.32 $^{a-e}$
HH-FP	33.17 ± 0.69 ^{a}	14.40 ± 0.51 ^{a}	1.92 ± 0.04 ^{c,d}	1.10 ± 0.06 $^{b-e}$	0.65 ± 0.05 $^{a-d}$	0.90 ± 0.04 ^{d,e}	nd ^{a}	52.14 ± 0.95 ^{a}
MH	38.85 ± 0.35 $^{a-c}$	39.17 ± 0.60 ^{d}	0.95 ± 0.12 ^{a}	1.01 ± 0.10 $^{a-e}$	0.39 ± 0.06 ^{a}	0.18 ± 0.03 ^{a}	0.09 ± 0.05 $^{a-c}$	80.63 ± 0.86 ^{e}
MH-FP	38.38 ± 0.79 ^{a,b}	24.28 ± 0.59 ^{a,b}	2.00 ± 0.08 ^{d}	0.75 ± 0.11 $^{a-c}$	0.72 ± 0.05 $^{b-d}$	0.62 ± 0.04 $^{c-e}$	nd ^{a}	66.75 ± 0.71 ^{a,b}

[a]Each product is characterized by the mean and SD for the specific sugar. Multiple comparison tests (2-tailed) and Kruskal–Wallis tests (H (df = 11, N = 72) = 60.82–68.97, $P < 0.001$) were used to test for significant differences between honeys and fermented products. SD, standard deviation; nd, not detected; BLH, black locust honey; LH, linden honey; RH, raspberry honey; CH, canola honey; HH, heather honey; MH, multifloral honey; FP, fermented product. Values in the same column with different superscripts (a–e) are significantly different.

Erlose was quantified in small amounts in honey samples but was under the limit of detection in the fermented products. Small decrease in amount of sugar was observed after 60 days of fermentation. Multifloral and heather honey presented the most important decrease in sugars, mainly for glucose (Table 3), this sugar being the precursor for gluconic acid formation in the fermentation process.[43]

Spectrophotometric Determination of Total Phenolic Content. All honeys are rich sources for secondary metabolites with antioxidant activity, higher amounts of polyphenols being determined in darker unifloral honeys and also in multifloral honeys.[44,45]

Total phenolic content of honey samples used in this study varied widely among the honey types, between 148.47 and 435.99 mgGAE/kg. Less-colored honeys (black locust and raspberry) showed the lowest phenolic content (Figure 2A). The highest amount of polyphenols was determined in heather honey (435.99 mgGAE/kg); the amount of total phenolic content was approximately 3-fold higher than in raspberry honey (148.47 mgGAE/kg). Previous reports showed similar amounts for black locust, linden, raspberry, canola, and multifloral honeys from Europe.[46,47]

In the fermentation process, polyphenols are subjected to different biochemical modifications through polymerization processes and complexation with proteins.[48] The total phenolic content in fermented products obtained from different types of honey ranged between 194.87 and 315.89 mgGAE/kg. Heather and canola honey fermented products exhibited lower quantities of total polyphenols (down to 38%), whereas black locust, linden, raspberry, and multifloral presented an increase in polyphenol content (19–23%) (Figure 2A). Recent publications[49,50] also revealed higher quantities of phenolics, organic acids, or other types of metabolites after the fermentation process from other matrices (e.g., barly, *Allium cepa*) using different fermentation conditions.

Spectrophotometric Determination of Flavonoid Content. High amount of total flavonoids was quantified in heather honey (43.17 mgQE/kg), followed by raspberry and multifloral honey (15.94 and 11.95 mgQE/kg). Lower amounts were quantified in black locust and linden honey (4.61–8.90 mgQE/kg honey). The fermentation products obtained from these types of honey showed a lower flavonoid content than the unfermented honey samples (Figure 2B). In the remaining analyzed honeys (canola, raspberry, multifloral, and heather) with higher quantities of flavonoids (8.83–43.17 mgQE/kg), the flavonoid content decreased after fermentation. Products obtained from heather honey presented the highest decrease of flavonoid content (approximately 42.35%), followed by products obtained from raspberry and multifloral honey (15.08 and 9.18%) (Figure 2B). In general, the flavonoid content is decreasing in the fermentation process.[51] Some flavonoids might be condensed into polymers and not react anymore with specific reagents used in spectrophotometric determinations. To explain this different behavior of honey types in the fermentation process, individual phenolic and flavonoid pattern have to be determined using already developed HPLC methods.[45,52]

Antioxidant Activity. *DPPH Scavenging Activity.* The six honey samples used in the study were tested to find any relationship between radical scavenging activity (RSA) and its origin. Results, summarized in Figure 2D, showed that all samples are active and inhibit the DPPH radical, but in a wide range, according to their botanical origin. Baltrušaitytė and co-

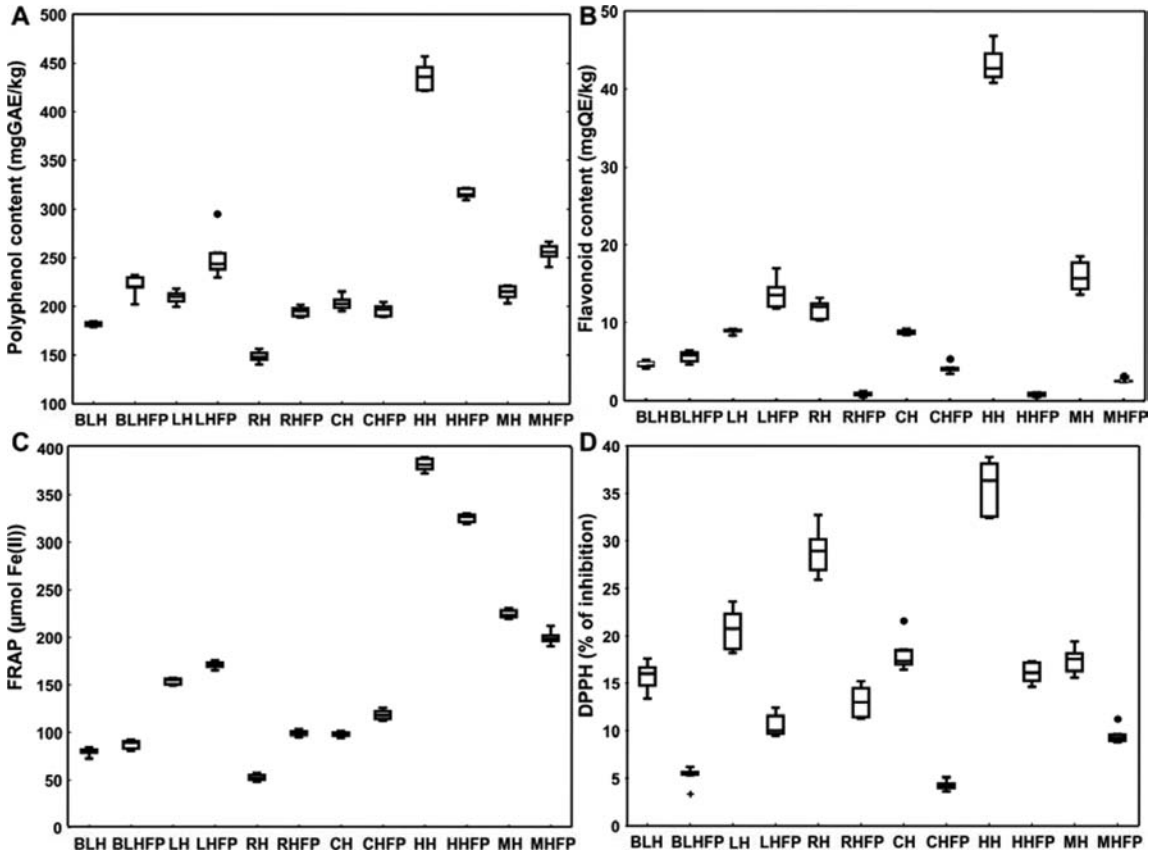

Figure 2. Content of different substances closely related to the antioxidative activity of honey and fermented products. (A) Polyphenols (N = 72), (B) flavonoids (N = 72), (C) FRAP (N = 72), and (D) DPPH values (N = 72). Each box-whisker plot represents the content of substance (median ± 25−75% variability and nonoutlier range; filled circles, outlier; cross, extremes; BLH, black locust honey; LH, linden honey; RH, raspberry honey; CH, canola honey; HH, heather honey; MH, multifloral honey; FP, fermented product).

workers[53] reported that the floral origin also influences the DPPH activity in bee bread and not only honey. The phenolic content of honey was shown to be responsible for its antioxidant activity.[6,47] This relationship was verified for heather and raspberry honey (highest phenolic and flavonoid content for the first and high flavonoid content for the second with RSA of 35.77% and 28.9%, respectively). The RSA for the remaining honey samples varied from 15.7% to 20.7% but not in direct correlation to their amount of total phenolic or total flavonoid content.

Fermented products obtained from the six honey types exhibit lower RSA than the sample raw materials. The product obtained from heather honey present the highest RSA value (16.1%), followed by raspberry and linden (13.1 and 10.5%). The other honey products present lower values for radical scavenging activity.

Spearman rank correlation of flavonoid content and DPPH activity of honeys and fermented products revealed a positive correlation for honey and negative correlation for the fermented products (P < 0.05), maybe reflecting that the antioxidative activity of honey is provided by the flavonoids (Table 4).

Antioxidant Power (FRAP Method). The FRAP assay showed large differences in honey and fermented products, and the least active were unifloral honeys. Heather honey and multifloral honey showed again high antioxidant content (Figure 2C). Similar results for unifloral and multifloral honeys were obtained in other studies.[46,54]

Table 4. Correlation of Antioxidant Activity (FRAP and DPPH), Polyphenol and Flavonoid Content of All Honey (N = 6) and Fermented Products (N = 6)[a]

	FRAP	DPPH	polyphenols	flavonoids
FRAP		**0.506**	**0.752**	−0.313
DPPH	0.233		**0.470**	**−0.565**
polyphenols	**0.933**	0.267		−0.087
flavonoids	**0.621**	**0.625**	**0.572**	

[a]The lower half matrix illustrates correlation coefficient values (r) for the honey samples and the upper half matrix contains correlation coefficients for the corresponding fermented products. Significant r values are marked in bold (Spearman rank correlation, N = 36, P < 0.05).

As already mentioned for the DPPH scavenging activity, the antioxidant power (FRAP assay) also correlates with the flavonoid content of the different honeys (P < 0.05) but not anymore with the fermented products (P > 0.05) (Table 4). This phenomenon might be explained by the fact that some flavonoids were destroyed during the fermentation process, as much fewer flavonoids were detected in fermented products (Spearman rank correlation for polyphenols and flavonoids; $P_{\text{before fermentation}}$ < 0.05, $P_{\text{after fermentation}}$ > 0.05). Those results might indicate flavonoids as the origin of the antioxidant activity in honey.

However, there might be some additional factors contributing to the antioxidant activity of honey and fermented

products, as the polyphenolic content of both also correlated significantly ($P < 0.05$) with the FRAP activity (Table 4).

The lack of validated assays to measure the antioxidant capacity makes it difficult to compare the results when analyzing different food matrices. Different reviews are published discussing the chemistry of antioxidant assays[55] or the advantages/disadvantages of each method.[56] Hence, choosing the correct method depends on the food matrices due to the nature of biological antioxidants present in the sample to be analyzed (enzymatic, nonenzymatic, biological, or just dietary).

To obtain high quality products containing nutrients with antioxidant activity, we recommend using black locust and linden honeys because their flavonoid profile did not change significantly by fermentation.

■ ASSOCIATED CONTENT

Supporting Information

Pollen spectra of analyzed honey samples. Changes of pH during fermentation process. Changes of free acidity (A) and lactonic acidity (B) during fermentation process. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AAB, acetic acid bacteria; HPLC-DAD, high performance liquid chromatography, diode array detection; HPLC-RID, high performance liquid chromatography, refractive index detection; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; GAE, gallic acid equivalents; QE, quercetin equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; RSA, radical scavenging activity; FRAP, Ferric Reducing Antioxidant Power; TPTZ, 2,4,6-tripyridyl-*s*-triazine

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