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Analysis of flavonoids in honey by HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry

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Abstract The analysis of flavonoids in unifloral honeys by high-performance liquid chromatography (HPLC) coupled with coulometric electrode array detection (CEAD) is described. The compounds were extracted by a nonionic polymeric resin (Amberlite XAD-2) and then separated on a reversed phase column using gradient elution. Quercetin, naringenin, hesperetin, luteolin, kaempferol, isorhamnetin, and galangin were detected in a coulometric electrode array detection system between +300 and +800 mV against palladium reference electrodes, and their presence was additionally confirmed by HPLC coupled with electrospray ionization mass spectrometry. The method was applied to analysis of 19 honeys of different varieties and origin. The limits of detection and quantitation ranged between 1.6 and 8.3 μg/kg and 3.9 and 27.4 μg/kg, respectively. The recoveries were above 96% in fluid and above 89% in creamy honeys. Some of these honeys (melon, pumpkin, cherry blossom, dandelion, maple, and pine tree honey) were investigated for their flavonoid content and profile for the first time. Differences between honeys were observed both in flavonoid concentrations and in the flavonoid profiles. The flavonoid concentrations ranged from 0.015 to 3.4 mg/kg honey. Galangin, kaempferol, quercetin, isorhamnetin, and luteolin were detected in all investigated honeys, whereas hesperetin occurred only in lemon and orange honeys and naringenin in lemon, orange, rhododendron, rosemary, and cherry blossom honeys.

Introduction

A wide range of minor constituents like phenolic acids, flavonoids, certain enzymes, carotenoid-like substances, vitamins, organic acids, and Maillard products are present in honey. The composition is rather variable and primarily depends on the floral source. Among other parameters, flavonoid as well as flavonoid glucoside profiles or the presence of a single flavonoid can contribute to identify unifloral honeys. A great number of such honeys are on the market and have to be checked regarding their quality, authenticity, and labeling in order to prevent consumer deception. Therefore, there is a necessity for analytical methods which enable reliable evaluation of unifloral honeys.

So far, different methods have been developed for determination of flavonoids in honey. Due to the complex matrix of honey and the low concentration of these compounds, numerous authors have proposed a sample cleanup including a preconcentration step. The cleanup depends on the question whether flavonoid aglycones or glycosides should be analyzed. In the first case, the honey sample is dissolved in acidified water. Then, the flavonoid aglycones can be extracted with ethylacetate [1, 2], by solid phase extraction on reversed phase materials [3–5] or on nonpolar resins with [6–13] or without [14–16] an

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additional extraction step with ethyl ether or 2-butanol [17] or further purification on a Sephadex column [18]. With the nonionic resins, Amberlite XAD-2 and XAD-4 sugars, acids, pigments, and disturbing compounds can be more effectively eliminated than on reversed-phase cartridges. Additionally, the aglycones, being less polar than glycosides, are effectively bound on the resins. Only in some papers no extraction step was applied, and the flavonoids were determined by analyzing the compounds after dissolution of the honey in pure or acidified water [19, 20]. As a consequence of the high concentration of polar and ionic compounds, contamination of the analytical column has to be expected if numerous honey samples are analyzed. For analysis of flavonoid glycosides, the sample was dissolved in pure water, and the glycosides were pre-concentrated on a C18-SPE-cartridge and eluted with methanol [21, 22]. For determination of glycosides, the extraction on reversed phase material is more appropriate than the extraction on nonpolar resins.

Due to the polarity of the compounds, it is not surprising that GC with flame ionization [17] or mass spectrometric detection [23, 24] was applied only occasionally. Capillary electrophoresis with UV [25–27] or mass spectrometric detection [28] has the advantage of high separation efficiency and shorter analysis time in comparison to high-performance liquid chromatography (HPLC). Nevertheless, it was difficult to separate all flavonoids in one run, and it was necessary to wash the capillary after each analysis in order to attain a better reproducibility [25]. Still, these methods are well suited for qualitative analysis of flavonoids in honey and propolis [25, 26, 28, 29].

The method of choice for qualitative and quantitative analysis of flavonoids is HPLC coupled with UV [1, 3, 6, 7, 9–18, 30–38], electrochemical [20], or mass spectrometric detectors [2, 4, 5, 19, 21, 22, 39–41]. Isocratic elution of phenolic compounds in honey extracts by HPLC/UV suffered from co-elution of compounds, and as a result of the lack of selectivity, complex chromatograms were obtained. Therefore, gradient elution was proposed. Nevertheless, UV detection is not sensitive enough to determine trace amounts of some flavonoids. Using ultra-performance liquid chromatography coupled with time of flight mass spectrometry (UPLC-Q/TOF-MS), more phenolic compounds could be identified compared to diode array detection [2]. HPLC-MS^n on an ion trap mass spectrometer is a promising technique in the determination of the floral origin of unifloral honeys, especially when the flavonoid glycoside profiles should be investigated [21]. HPLC-electrospray ionization (ESI/MS) has proved as a valuable method for qualitative and quantitative assay of some flavonoids in propolis [42].

Liang et al. have shown [20] that electrochemical detection of phenolic compounds in citrus honey is six to 14 times more sensitive than diode array detection. Inoue et al. [43] investigated the radical scavenging activity caused by phenolic compounds with HPLC coupled with coulometric electrode array detection (CEAD) without using the high selectivity of this method for determination of single phenolic compounds. Already in 1993, Jörg et al. [44] have demonstrated that HPLC/CEAD is—due to its selectivity and sensitivity—well suited for determination of phenolic acid esters in different honeys with detection limits between 0.1 and 1 μg/kg.

The objective of the present study was to compare SPE extraction of flavonoids in honey with accelerated solvent extraction (ASE) and to develop a method based on HPLC/CEAD for qualitative and quantitative determination of flavonoids in different honeys. Additionally, to verify the presence of the flavonoids, these compounds should be identified by HPLC/ESI-MS. Some not so well known unifloral honeys like those of melon, maple, pumpkin, cherry blossom, dandelion, and one nonfloral honey (pine tree) should be investigated with this method.

Materials and methods

Collection of honey samples

Honey samples (Table 1) of various floral sources were collected in different countries from 2006 to 2008.

Chemicals and solutions

Galangin, hesperetin (purity ≥95%), isorhamnetin (purity ≥95%), kaempferol (purity ≥96%), luteolin (purity ≥99%), myricetin (purity ≥96%), and quercetin dihydrate (purity ≥99%) were purchased from Sigma-Aldrich (Buchs, Switzerland). Methanol, HiPerSolvChromanorm gradient grade, was from VWR (Vienna, Austria); ethyl acetate (Rotisolve® HPLC grade) and acetic acid (Rotipuran 100% p.a.) were from Carl Roth (Karlsruhe, Germany). Ethanol (p.a.) was purchased from AustrAlco (Öster. HandelsgesmbH, Spillern, Austria); hydrochloric acid (37% p.a.), sodium hydroxide (p.a.), potassium dihydrogenphosphate (p.a.), and ortho-phosphoric acid (85% p.a.) were from Merck (Darmstadt, Germany); and Amberlite XAD-2 was obtained from Supelco (Vienna, Austria). Bi-distilled water was used in all experiments.

Stock solutions Four milligrams of each flavonoid was dissolved in 10 ml of methanol by sonication. The solutions were stored in dark flasks at 4 °C.

Standard mixture Fifty microliters of each stock solution were pipetted into a 20-ml flask and filled up with methanol, yielding 1 mg/L of each compound. This

Table 1 Investigated honeys

Honey	Color	Consistence	Year	Provenience
Acacia	Golden yellow, clear	Liquid	2006	Burgenland, Austria
Buckwheat	Golden brown	Creamy	2006	Lower Austria, Austria
Maple	Cream-colored	Solid	2008	Lower Austria, Austria
Phacelia	Reddish brown, clear	Low viscosity	2006	Burgenland, Austria
Pumpkin	Yellow	Creamy	2006	Lower Austria, Austria
Raspberry	Yellow	Creamy	2008	Lower Austria, Austria
Orange (organic)	Ivory-colored	Creamy	2006	Italy[b]
Cherry blossom	Red brown clear	Liquid	2007	Cervia, Emilia Romagna, Italy
Dandelion	Yellowish brown	Creamy	2007	Cervia, Emilia Romagna, Italy
Melon	Light yellow	Creamy	2007	Cervia, Emilia Romagna, Italy
Rhododendron	Yellowish	Creamy	2007	Cervia, Emilia Romagna, Italy
Rosemary	Dark yellowish	Creamy	2007	Cervia, Emilia Romagna, Italy
Citrus blossom	Golden yellow	Creamy	2007	Zafferana Etnea, Sicily
Orange blossom	Yellow	Creamy	2007	Zafferana Etnea, Sicily
Lavender	Golden brown, unclear	Liquid	2007	Croatia[b]
Sage	Brown	Liquid	[a]	Croatia[b]
Thyme	Brown	Liquid	[a]	Crete, Greece
Pine tree	Brown unclear	Liquid	2007	Greece[b]
Rape seed	Dark brown	Creamy	2007	Mecklenburg-Vorpommern, Germany

[a] Date could not be confirmed

[b] Geographical origin not known

solution was diluted with mobile phase A to the corresponding concentrations and stored at 4 °C.

Phosphate buffer (0.2 M) Potassium dihydrogenphosphate, 27.22 g, was dissolved in bi-distilled water, 2 ml of ortho-phosphoric acid was added, and the solution was adjusted to 1 L with bi-distilled water.

Pre-conditioning of Amberlite XAD-2 resin

The dry resin was suspended in methanol, and the suspension was stirred with a magnetic stirrer for 1 min. After 15 min, methanol was decanted and displaced by bi-distilled water and stirred again for 1 min. Ten minutes before using the resin, water was removed, and acidified water (pH 2 adjusted with 1 M hydrochloric acid) was added to the resin.

Sample preparation, equipment, and chromatographic conditions

One hundred grams of each honey were homogenized by manual stirring and sonication.

Sample preparation with nonpolar resin Five grams of honey were dissolved in 25 ml of acidified water (adjusted to pH 2 with 1 M hydrochloric acid) under constant stirring.

The solution was filtered over cotton wool, and 10 g of preconditioned Amberlite XAD-2 resin were added to the solution, and the resulting slurry was filled into a glass column (25×1.5 cm). The column was washed with 40 ml of acidified water (pH 2) and then with 60 ml of bi-distilled water. The flavonoids were eluted with 50 ml methanol, and the solvent was evaporated to dryness at 40 °C using rotary evaporator. Then, the residue was ultra-sonicated in 1 ml of methanol and 1 ml of 0.02 M phosphate buffer, and the solution was filtered through a 13-mm PTFE 0.45-μm syringe filter (Alltech, Deerfield, IL, USA). This solution was injected into the HPLC/CEAD as well as into the HPLC/ESI-MS system.

Sample preparation based on accelerated solvent extraction (ASE) Five grams of honey (orange, organic) unspiked or spiked with 50%, 100%, 150%, and 200% of the estimated concentration of each flavonoid were mixed with 10 g diatomaceous earth (Mueller-Scherr, Linz, Austria) and placed into a 34-ml extraction cell adapted with a cellulose filter. This cell was placed into a Dionex ASE 100 instrument (Dionex, Vienna, Austria). The flavonoids were extracted with ethylacetate or ethanol/water (80:20, *v:v*) under the following operating conditions: extraction temperature, 100 °C; extraction time, 5 min; flush volume, 50%; purge time, 90 s; and number of cycles, 2. The obtained extract was evaporated to dryness at 40 °C using

rotary evaporator. The residue was dissolved in 1 ml of methanol. Then, 1 ml of 0.02 M phosphate buffer was added, and the solution was sonicated for 5 min. One milliliter of the solution was filtered through a 13 mm PTFE 0.45 μm filter, transferred into a vial, and this solution was injected into the HPLC-CEAD as well as into the HPLC/ESI-MS system.

HPLC/CEAD The HPLC system consisted of a Merck Hitachi pump L-6200, an AS-2000A auto-sampler (Merck Hitachi, Tokyo, Japan), a Phenomenex C18 pre-column (4×3 mm; particle size, 5 μm; Phenomenex, Torrance, CA, USA), a Nucleodur Sphinx RP analytical column (150×4.6 mm; particle size, 5 μm Macherey-Nagel, Düren, Germany), a thermostat (Merck Hitachi), and a CEAD (ESA, Chelmsford, MA, USA) equipped with eight flow-through cells. The potentials of six working electrodes were set at +300, +400, +500, +650, +750, and +800 mV against palladium reference electrodes. Due to the high background current, the last two cells were set at +100 and +200 mV.

Fifteen microliters of the standard solutions or the sample extracts were injected into the HPLC/CEAD system. Chromatographic separation was achieved at a flow rate of 1 ml/min at 20 °C using gradient elution. Mobile phase A consisted of methanol/0.02 M phosphate buffer (20:80, *v:v*) adjusted to pH 3.2 with 1 M sodium hydroxide; mobile phase B was made of methanol/0.02 M phosphate buffer (80:20, *v:v*) adjusted to pH 3.2 with 1 M sodium hydroxide. The following gradient was used: linear increase from 0% to 40% B within 40 min, isocratic elution at 40% B from 40 to 65 min, linear increase from 40% to 70% B from 65 to 70 min, isocratic period at 70% B from 70 to 85 min, linear increase to 80% B within further 15 min, decrease to 0% B from 100 to 102 min, and re-equilibration at 0% B from 102 to 120 min. The software for data acquisition and evaluation was Coul Array Win (ESA).

HPLC/ESI-MS The HPLC 1100 series system (Agilent Technology, Palo Alto, CA, USA) consisted of a G1312A binary pump, a G1322A mobile phase vacuum degassing unit, a G1313A autosampler, a Nucleodur Sphinx RP analytical column (150×4.6 mm; particle size, 5 μm; Machery-Nagel, Düren, Germany) protected by a Phenomenex C18, precolumn (4×3 mm; particle size, 5 μm; Phenomenex, Torrance, CA, USA), and an ion trap mass spectrometric (MS) detector (HCT plus, Bruker Daltonics, Bremen, Germany) equipped with an ESI source. Hystar 3.1, software for chromatography and hyphenated techniques, was used for data acquisition. The compounds were separated at 20 °C at a flow rate of 1 ml/min using the gradient elution.

Fifteen microliters of the standard solutions or the sample extracts were injected into the HPLC/ESI-MS system. Mobile phase C consisted of 0.5% acetic acid in methanol/water (20:80, *v:v*); mobile phase D contained 0.5% acetic acid in methanol/water (80:20, *v:v*). Chromatographic separation was achieved using gradient elution: linear increase from 20% to 40% D within 30 min, isocratic elution at 40% D from 30 to 55 min, linear increase to 60% D within 10 min, linear increase from 60% to 65% D from 65 to 75 min, isocratic elution at 65% D for 10 min, decrease to 20% D within 5 min, and re-equilibration at 20% D from 90 to 110 min.

The flow was splitted (1:4) so that only a part of the eluate reached the ionization source. ESI was performed with nitrogen as nebulizing gas (10 L/min), 40 psi nebulizing pressure, at 300 °C drying gas temperature. The capillary voltage was set at −4.5 or +4.5 kV. The total ion chromatograms (TIC) in the range of 100 to 400 *m/z* of the standard solutions and the honey extracts were measured, and the extracted ion chromatograms (EIC) of the deprotonated $[M-H]^-$ and protonated molecular ions $[M+H]^+$ of the flavonoids (myricetin, 317.2 and 319.2; quercetin, 301.2 and 303.2; naringenin, 271.3 and 273.3; luteolin, 285.2 and 287.2; hesperetin, 301.2 and 303.2; kaempferol, 285.2 and 287.2; isorhamnetin, 315.3 and 317.3; and galangin, 269.2 and 271.2) were collected and compared. Data were then integrated using the software DataAnalysis™ version 3.3 (Bruker Daltonics).

Determination of flavonoids

Qualitative analysis The retention times and the peak areas of sample and standard compounds of the HPLC/CEAD chromatograms were measured. The peak areas were plotted against the potentials of the working electrodes and resulted in peak shaped current/voltage curves (Fig. 1). These voltammograms served as identification criterion for flavonoids and for determination of the optimal detection potential (Table 2).

HPLC/ESI-MS measurements were performed to verify the identification of the major peaks. Positive and negative ESI-MS spectra were recorded, and the extracted ion chromatograms of the samples were compared with those of the standard mixture.

Quantitative analysis The contents of the flavonoids in honeys were determined by HPLC/CEAD at the optimal detection potentials on the basis of external calibration curves and using the standard addition method. Each sample was worked up in triplicate and injected once. For quantitative analysis and recovery determination by standard addition method, the concentration of each flavonoid

Fig. 1 Current voltage curves of galangin and hesperetin
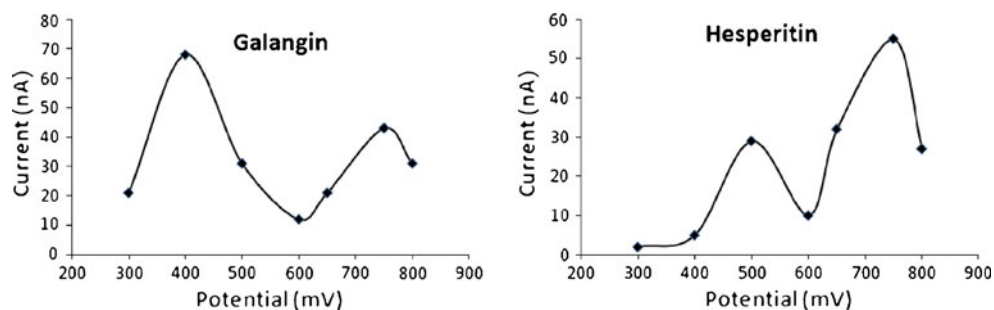
in the unspiked sample was estimated by the external calibration curve, and then 50%, 100%, 150%, and 200% of the experimentally determined concentration of each flavonoid were added to aliquots (5 g) of the honey samples. The spiked samples were processed as the unspiked. The analyte concentrations in the sample extracts were determined by plotting the peak areas versus the added concentrations of the corresponding flavonoid, linear regression, and division of the y-intersection (d) by the slope (k) of the regression line $y=kx+d$.

Recoveries ($N=5$) were determined for a creamy (orange, organic) and a fluid honey (sage) by dividing the slope of the standard addition regression line of each flavonoid by the slope of the external calibration function recorded during the same analysis run and multiplying by 100.

Validation of the HPLC/CEAD method

Standard calibration curves of each flavonoid at the optimal detection potentials were generated between 0.01 and 20 mg/L by plotting the peak area versus concentration of the compound, and the correlation coefficients were evaluated. The limits of detection in standard solutions were determined by dilution of the 0.01-mg/L standard until a signal to noise ratio of 3 was reached. The limit of quantitation (S/$N=10$) was determined by inserting the

tenfold noise into the calibration curve of the corresponding flavonoid. The intra-day reproducibility of the chromatographic method was evaluated by fivefold injection of the standard mixture containing 1 mg/L of each flavonoid in 1 day and measuring the peak areas. The inter-day reproducibility was assessed on the basis of five injections of the standard mixture containing 1 mg/L of each flavonoid over a period of 5 days. For determination of the inter- and intra-day reproducibility in sample solutions, one honey sample extract (orange, organic) was injected five times in 1 day and five times each day during a period of 5 days. The limits of detection and quantitation in orange (organic) and sage honey (Table 2) were determined by inserting the three- and tenfold noise in the calibration curve of the flavonoid.

Results and discussion

Sample preparation

The extraction of the flavonoids with Amberlite XAD-2 resin used by Martos et al. [10] was modified in the following way. The amount of honey (100 g) for sample preparation could be reduced due to the sensitivity and selectivity of the HPLC/CEAD method. Only 5 g of the honey were extracted, and an additional extraction with

Table 2 Characteristic parameters of the HPLC/CEAD method

Compound	Optimal detection potential (channel; mV)	Recovery of fluid honey (%)	Recovery of creamy honey (%)	Limit of detection (S/$N=3$; µg/kg)	Limit of quantitation (S/$N=10$; µg/kg)	CV (%) intraday ($n=5$)	CV (%) interday ($n=5$)
Myricetin	+300	nd	nd	8.2	22.3	nd	nd
Quercetin	+300	98.0±2.2	98.2±5.3	4.5	13.2	4.9	4.8
Naringenin	+800	nd	93.1±7.8	4.7	27.4	5.8	9.6
Luteolin	+300	100±6.1	89.2±7.07	4.5	13.1	4.0	6.1
Hesperetin	+750	nd	95.4±9.5	3.7	8.9	4.0	8.4
Kaempferol	+300	96.2±4.5	94.6±8.2	6.1	18.4	3.4	4.3
Isorhamnetin	+300	99.6±3.5	89.9±4.1	8.3	21.7	4.6	9.9
Galangin	+400	96.8±2.2	90.3±7.6	1.6	3.9	2.6	4.9

Creamy honey orange (organic), fluid honey lavender, nd not detected

diethyl ether before injection as used for the HPLC/UV method [10] was not necessary. The recoveries (N=5) of the flavonoids were in the range from 96% to 100% for fluid and between 89% and 98% for creamy honeys (Table 2). Additionally, ASE of the orange (organic) honey with different solvents (ethanol, methanol, ethyl acetate, and water) was investigated. With ethyl acetate and ethanol/ water (80:20, v:v) as solvents recoveries between 82% to 99% and 84% to 100%, respectively, were obtained. Based on these data, ASE could be an alternative to extraction with nonpolar resins, but it is more cost intensive.

Separation, detection of flavonoids, and validation of the method

Due to the different polarity of the phenolic compounds in honeys, an isocratic elution with relatively low content of organic solvent, which would be optimal for electrochemical detection, is impossible. Hence, different gradient programs were tested in order to achieve not only an adequate separation of myricetin, quercetin, naringenin, luteolin, hesperetin, kaempferol, isorhamnetin, and galangin but also a good repeatability if a great number of samples have to be investigated. Therefore, a relatively long analysis method with appropriate gradient and washing steps was developed. The current/voltage curves of the flavonoid standards were established. This is shown in Fig. 1 for galangin and hesperetin. The optimum detection potentials for determination of flavonoids are located in the maxima of the current/voltage curves. At these potentials, the calibration curves between 0.01 and 2 mg/L of the investigated flavonoids were linear and showed correlation coefficients (R^2) between 0.9991 (quercetin) and 0.9997 (hesperetin). The standard addition curves had similar correlation coefficients, e.g., 0.9995 for hesperetin in orange (organic) honey. The limits of detection and quantitation, the reproducibility of the method, and the recoveries are summarized in Table 2.

Qualitative and quantitative analysis of honeys

Eighteen samples labeled as unifloral honeys (Table 1) and one nonfloral honey (pine tree) were analyzed and evaluated. In Fig. 2, the chromatogram of a creamy (orange, organic) honey is illustrated. For identification of the compounds, the shape of the current/voltage curves was compared with those of the standard substances in addition to the retention time. Additionally, HPLC/ESI-MS chromatograms in positive and negative ionization mode yielding the characteristic protonated $[M+H]^+$ and the deprotonated molecular ions $[M-H]^-$ were recorded. MS as independent detection mode served for confirmation of CEAD data. The qualitative data of so far not investigated honeys are presented in Table 3. In this way,

quercetin, naringenin, luteolin, hesperetin, kaempferol, iso-rhamnetin, and galangin could be identified. To the best of our knowledge, the flavonoid profiles of melon, pumpkin, cherry blossom, dandelion, maple, and pine tree honeys were determined for the first time.

As can be seen in Fig. 2, hesperetin is overlapped by another compound at low potentials (+300, +400, and +500 mV). Due to the coulometric detection mode, this compound is fully oxidized at these potentials so that hesperetin could be quantified without any problem at the second maximum (+750 mV) of the current voltage curve. So, with exception of myricetin (LOD, 8.2 µg/kg), all investigated flavonoids could be quantified in at least some of the investigated honeys (Fig. 3). The flavonoid contents of the unifloral honeys were different. In principle, one can distinguish between honeys of low total flavonoid content like citrus (0.66 to 1.77 mg/kg), lavender (0.91 mg/kg), thyme (1.04 mg/kg), sage (1.15 mg/kg), acacia (1.6 mg/kg), raspberry (2.28 mg/kg), and rhododendron (2.54 mg/kg) honeys and those with a high content like cherry blossom (6.9 mg/kg), melon (5.78 mg/kg), pumpkin seed (4.93 mg/ kg), rosemary (4.72 mg/kg), dandelion (4.35 mg/kg), phacelia (3.85 mg/kg), and buckwheat (3.26 mg/kg) honeys. While quercetin, luteolin, kaempferol, isorhamnetin, and galangin were present in all honeys, hesperetin was found only in the three citrus honeys from Italy (continent and Sicily) in concentrations between 0.16 and 0.30 mg/kg. It could be confirmed that this substance is characteristic for those honeys [8, 12, 16, 26]. Hesperetin was accompanied by low concentrations (0.03–0.27 mg/kg) of naringenin, a compound which has been observed commonly in citrus fruits and leaves. It is of interest that other authors [3, 10, 12, 16, 20, 26] could not identify naringenin in citrus and orange honeys. In this investigation, naringenin was also found in rhododendron, rosemary, and in the highest concentration (0.27 mg/kg) in cherry blossom honey. The presence of naringenin together with kaempferol and galangin in honeys, which were harvested in Cervia (Emilia Romagna, Italy) and Zafferana Etnea (Sicily), could be explained by certain propolis amounts collected by bees from leaf buds and cracks in the barks of lemon and orange trees. Volpi [29] has analyzed phenolic compounds in propolis from the region Emilia Romagna and found—beside other phenolic compounds—naringenin, galangin, kaempferol, and quercetin, while luteolin and myricetin could not be detected. The highest concentration was observed for galangin and the relations between galangin/quercetin, galangin/kaempferol, and galangin/naringenin were about 2, 7, and 18, respectively. In the honeys harvested in this region, we found relations of 1.2, 3.5, and 15, respectively. Due to these data, it could not be confirmed that naringenin is a marker for lavender honey [1, 26]. We could not identify naringenin in lavender honey, either (Fig. 3).

Fig. 2 CEAD chromatogram of an extract of orange (organic) honey. *Qu* quercetin, *Nar* naringenin, *Lut* luteolin, *Hes* hesperetin, *Kae* kaempferol, *Irh* isorhamnetin, *Gal* galangin
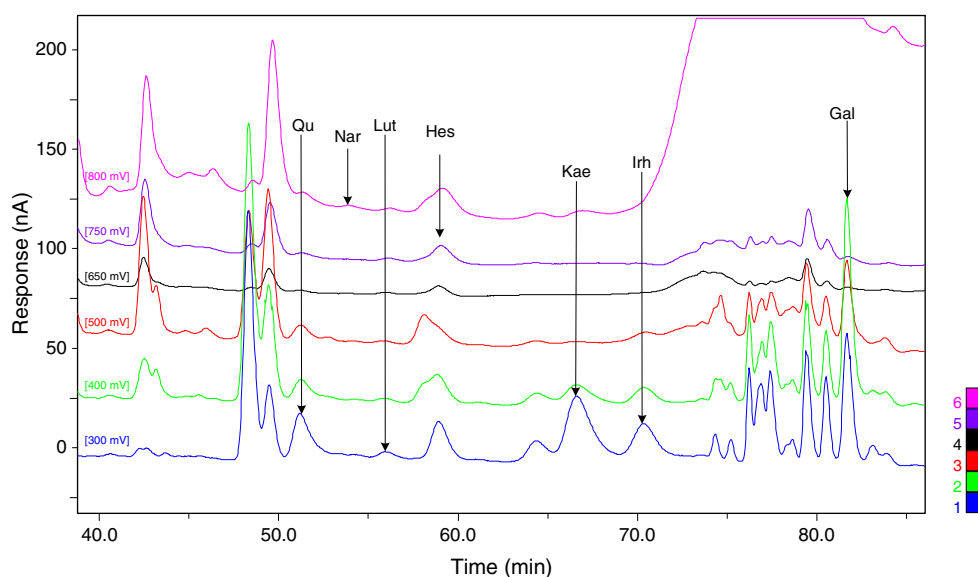
However, the absence of naringenin in dandelion and melon honeys harvested in the same region (Cervia, Italy) by the same apiculturist as rhododendron, rosemary, and cherry blossom honey (Table 2) shows that the interpretation of the data is difficult. Explanations might be that the concentration of naringenin was below the detection limit or that another kind of propolis had been transported into the hives by the bees. The propolis composition, as shown by Volpi et al. [42], is quite different and can influence the phenolic composition of honeys. On the other hand, melon honey from Cervia (Italy) and pumpkin honey harvested in Lower Austria show very similar flavonoid profiles (Fig. 3) combined with the highest kaempferol contents of 3.4 and 2.8 mg/kg, respectively. This could be a hint that these flavonoid profiles are characteristic for these kinds of honey and are not so much influenced by the propolis content.

Quercetin and kaempferol were proposed as markers for sunflower and rosemary honey, respectively, by Tomás-Barberán et al. [12] and Gil et al. [9]. Both compounds were found in all kinds of honeys under investigation, and their contents varied between 0.03 and 0.95 mg/kg for quercetin and 0.1 to 3.4 mg/kg for kaempferol. Therefore, these substances could not be indicated as marker substances, even though some honeys are characterized by a relatively high content of kaempferol (melon, pumpkin, rape seed, and cherry blossom honeys) in comparison to, e.g., herb honeys (Fig. 3).

Conclusion

The floral source appears to be the primary reason for large variations in the flavonoid content in honeys, but the flavonoid content is also largely influenced by the kind and content of propolis. The propolis composition depends—among other factors—on the geographical provenience, the plants growing there, and the climate predominating in these regions [29, 42]. For an objective authentication of the floral origins, the flavonoid profiles alone are not sufficient, but a number of other chemical parameters like metal ions [45] or volatile organic compounds [46] could

Table 3 Qualitative analysis of honeys by HPLC-ESI/MS

Positive mode (m/z)	Myricetin 319.2	Quercetin 303.2	Naringenin 273.3	Luteolin 287.2	Hesperetin 303.2	Kaempferol 287.2	Isorhamnetin 317.3	Galangin 271.2
Melon honey	−	+	−	+	−	+	+	+
Pumpkin honey	−	+	−	+	−	+	+	+
Cherry blossom honey	−	+	+	+	−	+	+	+
Dandelion honey	−	+	−	+	−	+	+	+
Maple honey	−	+	−	+	−	+	+	+
Pine tree honey	−	+	−	+	−	+	+	+

+ identified, − below the detection limit (0.8 to 4.6 μg/kg)

Fig. 3 Flavonoid profiles of honeys
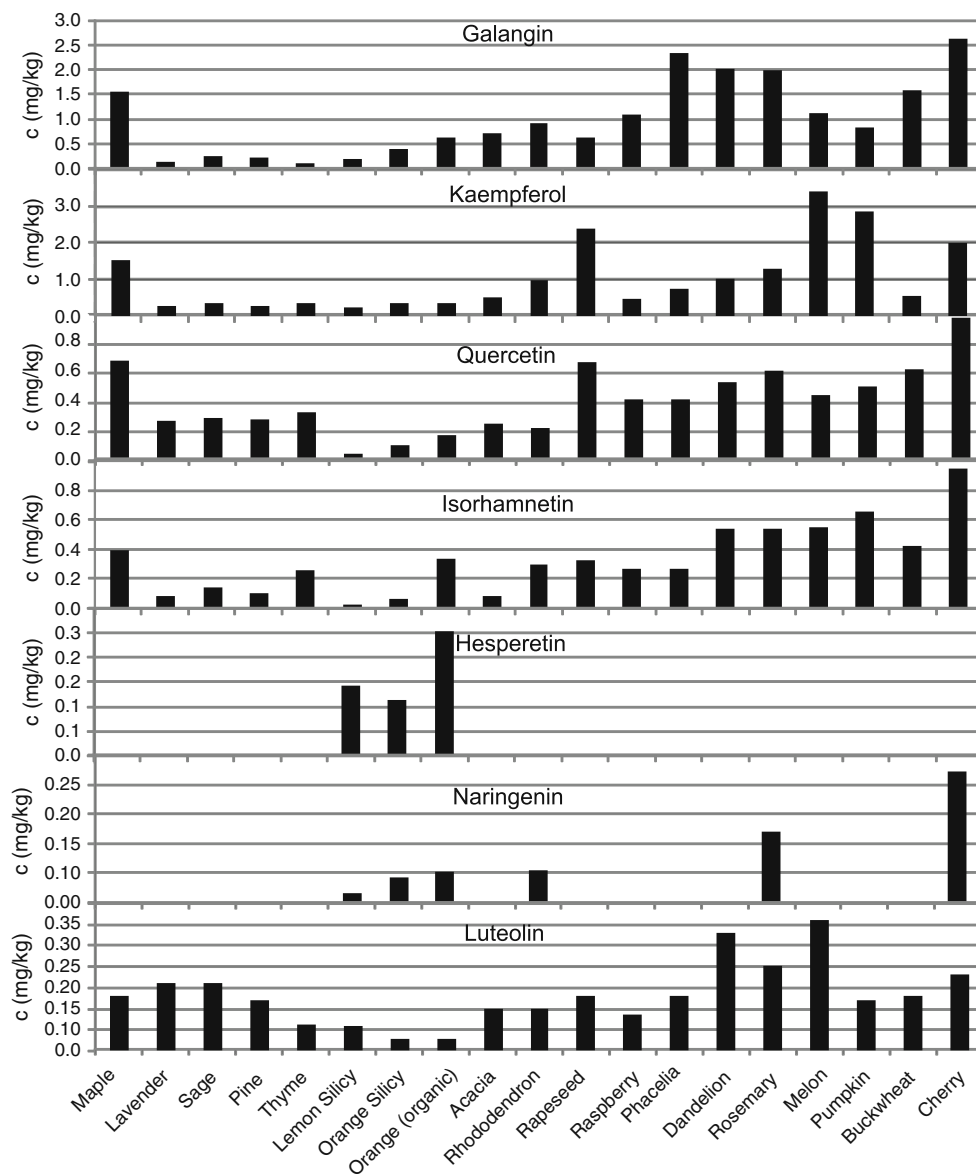
help to identify unifloral honeys. For instance, honeys with low flavonoid contents like lavender, sage, and thyme honeys can be easily distinguished from honeys containing relatively high flavonoid concentrations as, e.g., rosemary honey.

HPLC with coulometric electrode array detection proved to be a reliable method for determination of traces of flavonoids in honey with detection limits in the low ppb range. This detection mode offers more selectivity and detection of the compounds at lower potentials in comparison to amperometric detection at one working electrode. The optimal detection potential of a compound located in a peak maximum of the current voltage curve corresponds approximately to the half wave potential of a sigmoid hydrodynamic voltammogram established with a single cell electrochemical detector. In contrast, for single electrode

detection, a potential in the limiting current range has to be chosen. This means that a potential of about +1,100 mV against silver/silverchloride/sat. potassium chloride would be necessary to detect the investigated flavonoids. At such a high detector potential, the recognition of overlapping substances in the chromatogram would be difficult. Coulometric electrode array detection allows the quantification of substances simultaneously at lower potentials, and overlapping of substances can be recognized if their electrochemical behavior is different. The drawback of a long analysis time for one sample was compensated by the stability of the total system. The only maintenance steps required over a period of 6 months (including analysis of 200 samples) were electrochemical cleaning steps by polarizing the electrodes some minutes at −800 and then by +900 mV in mobile phase A.

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