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Original article

Confirmation of phenolic acids and flavonoids in honeys by UPLC-MS*

Sophie TRAUTVETTER, Isabelle KOELLING-SPEER, Karl SPEER

Technische Universität Dresden, Institute of Food Chemistry, Bergstrasse 66, 01069 Dresden, Germany

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Abstract – Certain phenolic acids and flavonoids are described in the literature as marker substances for several unifloral honeys. As not all authors utilised the same methods for extraction and determination, there are remarkable discrepancies in the published data concerning these substances. Ethyl acetate extracts which, aside from phenolic acids, also contain flavonoids were analysed by Ultra Performance Liquid Chromatography-Quadrupole/Time of flight-mass spectrometry (UPLC-Q/TOF-MS). First, the mass spectra of 37 phenolic acids and flavonoids described in the literature were recorded. Consequently, sunflower honeys, lime honeys, clover honeys, rape honeys, and honeydew honeys were analysed in regard to these substances. By employing the ChromaLynx^{TM} software, 34 of the 37 substances were identified quickly and clearly. By combining the retention time and the accurate molecular mass, it was even possible to identify several compounds which cannot be detected by diode array detection.

flavonoids / honey / phenolic acids / Time of flight (TOF) / Ultra Performance Liquid Chromatography (UPLC)

1. INTRODUCTION

Honey is one of man's last natural foods. Legal restrictions take place in the Honey Regulations ("Honigverordnung") of the German Food and Animal Feed Code of Law ("Lebensmittel- und Futtermittelgesetzbuch"). According to these regulations the exact labelling of the botanical origin of honey is only permitted "if the honey has its seeds completely or predominantly in the named blossom or living part of plants and if the honey possesses congruous organoleptic, physical-chemical, and microscopic characteristics" (Honigverordnung, 2006). Therefore, labelling honey as sunflower honey, lime honey, or honeydew honey is protected. To what extent such a declaration is justifiable until now has depended on the authentication of the various honeys by microscopic analysis of the pollen (melissopalynology). However, repeatable and definite results of melissopalynology can only be carried out by especially trained personnel (Louveaux et al., 1978).

Furthermore, ultrafiltered honeys do not allow for a conclusion of their origin by pollen analysis since all pollen are totally removed from the honey. Therefore, for a few years, every effort was made to accomplish a definite characterisation of individual kinds of honeys by chemical substances. Those indicators which can be directly associated with the nectar are of special interest. By using these substances, it may be possible to determine the individual kinds of honey and to classify them in regard to authenticity. Suitable substances in honey are phenolic acids and flavonoids as they are enriched minor components of nectar and honeydew.

Corresponding author: K. Speer,
karl.speer@chemie.tu-dresden.de
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Already in 1984, Speer and Montag determined higher contents of benzoic and phenylacetic acids by GC-MS in heather honeys compared to other kinds of honey. These results were confirmed by Steeg and Montag (1988a, b), who also identified mandelic and β-phenyllactic acid as markers for heather honey, hydrocinnamic acid for rape honey, protocatechuic acid for honeydew honey, and 4-hydroxybenzoic acid for buckwheet honey (Steeg and Montag, 1988b). Using coulometry, Jörg and Sontag (1992) confirmed protocatechuic acid as a marker for honeydew honey and identified p-coumaric acid and ferulic acid as indicators for chestnut honey. Apart from ferulic acid, Dimitrova et al. (2007) for chestnut honey also characterised 4-hydroxybenzoic, 4-hydroxyphenyllactic, and phenylacetic acid and reconfirmed contents of β-phenyllactic, phenylacetic, and benzoic acid in heather honey, using HPLC-UV-Vis detection. Furthermore, they described benzoic acid derivatives as specific compounds to eucalyptus honey whereas cinnamic acid derivatives are characteristic of acacia honey (Dimitrova et al., 2007); in sunflower honey the presence of p-coumaric, ferulic, and caffeic acid and the absence of phenyllactic and o-coumaric acid is the deciding factor in the HPLC-chromatograms (Dimitrova et al., 2007). This applies also to lime honey which does not contain o- and m-coumaric acid but instead 3-hydroxybenzoic acid and to lavender honey which contains gallic acid and caffeic acid (Dimitrova et al., 2007). As a marker for strawberry tree honey Cabras et al. (1999) stated homogentisic acid. In addition to benzoic, phenylacetic and β-phenyllactic acid (Speer and Montag, 1984; Steeg and Montag, 1988b; Dimitrova et al., 2007) ellagic acid and abscisic acid were also described as indicators for heather honey (Soler et al., 1995; Ferreres et al., 1996). Both of the phenolic acids, gallic and abscisic acid, were proposed as marker substances in eucalyptus honey (Yao et al., 2003, 2004b, 2005). In the group of flavonoids the flavanone hesperetin was identified by HPLC with diode array detector (HPLC-DAD) as a marker for citrus honey (Ferreres et al., 1993), and kaempferol was analysed for rosemary honey (Gil et al., 1995). Quercetin, a main flavonoid in sunflower honey, is being discussed as a characteristic substance (Soler et al., 1995; Ferreres et al., 1996). In eucalyptus honey the flavonoids myricetin, tricetin, luteolin, and quercetin were detected by Martos et al. (2000a, b) and Yao et al. (2004a, c).

Due to the minor content of phenolic acids and flavonoids in honey it is necessary to enrich the substances before proper analysis. Then it is possible to selectively analyse phenolic acids as trimethylsilylderivatives by GC-MS after liquid/liquid-extraction (Steeg and Montag, 1988b). A Headspace-GC method presented by Radovic et al. in 2001 proved to be unsuitable for the determination of the variety of the aromatic carboxylic acids. Another possibility for cleanup is solid phase extraction (SPE). However, the great amounts of starting material and solutions are disadvantages when enriching the flavonoids in methanol (Ferreres et al., 1991; Tomás-Barberán et al., 1992, 2001). Acetonitrile and tetrahydrofuran are used selectively for the isolation of phenolic acids (Dimitrova et al., 2007) but only to a lower degree for the flavonoids.

To simultaneously analyse the two groups of phenolic substances, HPLC-DAD is the method of choice (Tomás-Barberán et al., 2001; Yao et al., 2003) as flavonoids are difficult to detect by GC. Nevertheless, due to the quantity of compounds to be detected which possess rather similar structures within the same group, it is difficult to accomplish short elution times where the separation is sufficient (Tomás-Barberán et al., 1993, 2001; Gheldof et al., 2002; Yao et al., 2003). In addition the different UV-sensitivity of some compounds complicates the evaluation.

The aim of the present study was to identify the substances discussed in the literature due to the discrepancy in the detection of substances cleaned up with various methods. For this purpose, Ultra Performance Liquid Chromatography (UPLC) was applied for short run times combined with a quadrupole / time of flight mass spectrometer (Q/TOF-MS) which offers high mass accuracy. Extractions of ethyl acetate of five different kinds of honey were analysed and the substance definitely identified by this measuring method will be presented.

2. MATERIALS AND METHODS

2.1. Honey samples

The honey samples (4 honeydew honeys, 4 sunflower honeys, 3 lime honeys, 5 rape honeys, and 3 clover honeys) were stored at 6.5 °C. The botanical origin of each honey was verified by melissopalynology (Louveaux et al., 1978) at the Institut für Honig-Analytik, Quality Services International GmbH, Bremen (Germany).

2.2. Reagents

Benzoic, gentisic, and quinic acid, 4-methylpyrocatechol and phloroglucinol were purchased from Merck (Darmstadt, Germany). 4-Methoxycinnamic, caffeic, chlorogenic, tr-cinnamic, homogentisic, syringic, and vanillic acid, and kaempferol and myricetin were purchased from Fluka (Buchs, Switzerland). Gallic, mandelic, and p-anisic acid were obtained from VEB Laborchemie (Jena, Germany). Acetylsalicylic, ellagic, hydrocinnamic, 4-hydroxyphenylpropionic, protocatechuic, salicylic, tr-sinapic, and 3,4-dihydroxyphenylacetic acid were purchased from Acros (New Jersey, USA), 4-hydroxyphenylacetic acid from Serva (Heidelberg, Germany), 4-hydroxybenzoic acid from Pharmazeutisches Werk Oranienburg (Germany), and quercetin dihydrate from Riedl de Haën (Seelze, Germany). Tr-ferulic, phenylacetic, p-coumaric, and beta-phenyllactic acid came from Sigma (St. Louis, MO, USA). Cis, trans-abscisic acid and 4-hydroxyphenylpyruvic acid were obtained from Lancaster (Karlsruhe, Germany) and apigenin and chrysin from Roth (Karlsruhe, Germany). These standard substances were dissolved in methanol HPLC-Grade from Prolabo VWR (Darmstadt, Germany). Bi-distilled water was used for dilutions. Sodium chloride from Prolabo VWR was also dissolved in bi-distilled water. Ethyl acetate was obtained from Biesterfeld Chemiedistribution GmbH (Hamburg, Germany) and sodium sulphate from Grüssing GmbH, Filsum, Germany. HPLC-eluents were methanol from Riedel de Haën and 2% acetic acid (Fluka) in purified water (Millipore, Billerica, MA, USA). Leucine enkephalin from Sigma-Aldrich was permanently injected as an internal reference in the Q/TOF-MS to continuously control the accurate mass.

2.3. Sample preparation

10 g of homogenised honey was diluted with 10 mL of 2% sodium chloride solution. After being mixed for 1 min with an Ultra-Turrax, the diluted honey was extracted five times with 20 mL ethyl acetate, respectively. The combined organic phases were dried with sodium sulphate for at least 15 minutes. After concentration to about 1 mL by a rotary evaporator (40 °C, 240 bar) the solution was carefully dried under nitrogen. The residue was dissolved in 5 mL methanol/ water (3/2, v/v) and an aliquot was analysed by UPLC/MS after microfiltration.

2.4. Chromatographic equipment and conditions

UPLC-conditions

5 μL of prepared solution were separated on a Waters Acquity UPLC^{TM} System and detected by a Micromass Q-TOF Premier mass spectrometer, Waters (Almere, Netherlands), using an Acquity UPLC^{TM} BEH C_{18} column (2.1 × 100 mm, particle size 1.7 μm) at 40 °C. The elution was carried out at a flow rate of 0.3 mL/min with 2% acetic acid (eluent A) and methanol (eluent B). The gradient started with 8% B, reached 60% B in 12 minutes and 97% B after 15 minutes; 97% B was kept for 2.5 minutes.

MS-conditions

The injected samples were ionised with an Electro Spray Ionisation (ESI) LockSpray source in the negative mode (–40 V). The mass range was defined to 115–1000 m/z. The mass tolerance was 0.01 Dalton and the resolution was 5.000. Leucine enkephalin was used as the internal reference compound during ESI-MS accurate mass experiments and was permanently introduced via the LockSpray channel using a Hamilton pump. The Lock Mass Correction was +/– 1.000 for Mass Window.

All TOF-MS-chromatograms are displayed as Base Peak Intensity (BPI) chromatograms and scaled to 12400 counts per second (cps) (= 100%).

2.5. Experimental

First of all, the 37 phenolic acids, flavonoids, and additional compounds listed in Table I, were injected one by one at a concentration of 10 μg/mL.

Table I. List of all standards with retention time and exact mass after ionisation, and their occurrence in the five honeys.

ret.-time	substance	$[M-H]^-$	m/z	honey dew	sun-flower	lime	rape	clover
0.81	quinic acid	$C_7H_{11}O_6$	191.0556	+	-	-	-	-
0.85	shikimic acid	$C_7H_9O_5$	173.0450	-	-	-	-	-
1.12	phloroglucinol	$C_6H_5O_3$	125.0239	L	-	-	-	L
1.26	gallic acid	$C_7H_5O_5$	169.0137	+	L	+	L	L
1.44	5-(hydroxymethyl)furfural	$C_6H_5O_3$	125.0239	+	+	L	+	L
1.59	homogentisic acid	$C_8H_7O_4$	167.0344	+	+	+	+	-
2.02	protocatechuic acid	$C_7H_5O_4$	153.0188	++	+	+	+	+
2.42	3,4-dihydroxyphenylacetic acid	$C_8H_7O_4$	167.0344	+	L	L	L	L
3.00	gentisic acid	$C_7H_5O_4$	153.0188	+	L	L	L	+
3.06	4-hydroxybenzoic acid	$C_7H_5O_3$	137.0239	++	++	+	++	++
3.20	chlorogenic acid	$C_{16}H_{17}O_9$	353.0873	+	+	+	L	L
3.26	mandelic acid	$C_8H_7O_3$	151.0395	+	L	L	L	+
3.49	4-hydroxyphenylacetic acid	$C_8H_7O_3$	151.0395	+	+	L	+	L
3.77	caffeic acid	$C_9H_7O_4$	179.0344	+	+	+	+	+
3.84	vanillic acid	$C_8H_7O_4$	167.0344	+	+	+	+	+
4.29	syringic acid	$C_9H_9O_5$	197.0450	+	+	L	+	+
4.84	4-hydroxyphenylpropionic acid	$C_9H_9O_3$	165.0552	L	L	-	L	L
4.88	4-methylpyrocatechol	$C_7H_7O_2$	123.0446	L	L	L	L	L
5.25	p-coumaric acid	$C_9H_7O_3$	163.0395	+	+	+	+	+
5.64	beta-phenyllactic acid	$C_9H_9O_3$	165.0552	++	++	+	+	+
5.90	tr-ferulic acid	$C_{10}H_9O_4$	193.0501	+	+	+	+	+
6.01	tr-sinapic acid	$C_{11}H_{11}O_5$	223.0606	-	L	-	-	L
6.41	acetylsalicylic acid	$C_9H_7O_4$	179.0344	-	-	-	-	-
6.72	phenylacetic acid	$C_8H_7O_2$	135.0446	+	+	+	+	+
7.01	ellagic acid	$C_{14}H_5O_8$	300.9984	+	-	L	-	-
7.13	benzoic acid	$C_7H_5O_2$	121.0290	+	+	+	++	+
7.48	salicylic acid	$C_7H_5O_3$	137.0239	+	+	+	+	+
7.78	p-anisic acid	$C_8H_7O_3$	151.0395	L	-	-	L	-
7.85	myricetin	$C_{15}H_9O_8$	317.0297	-	-	-	-	-
9.12	hydrocinnamic acid	$C_9H_9O_2$	149.0603	L	L	L	+	L
9.18	abscisic acid	$C_{15}H_{19}O_4$	263.1283	++	+	++	++	+
9.52	quercetin	$C_{15}H_9O_7$	301.0348	L	L	L	L	+
9.73	tr-cinnamic acid	$C_9H_7O_2$	147.0446	L	L	L	L	-
9.91	4-methoxycinnamic acid	$C_{10}H_9O_3$	177.0522	L	-	L	L	-
10.93	kaempferol	$C_{15}H_9O_6$	285.0399	+	L	L	+	++
11.12	apigenin	$C_{15}H_9O_5$	269.0450	+	+	+	+	+
13.37	chrysin	$C_{15}H_9O_4$	253.0501	++	++	+	+	+

ret.-time = retention time [min]; - = not detected; L = <100 counts; + = 100–2000 counts; ++ = >2000 counts.

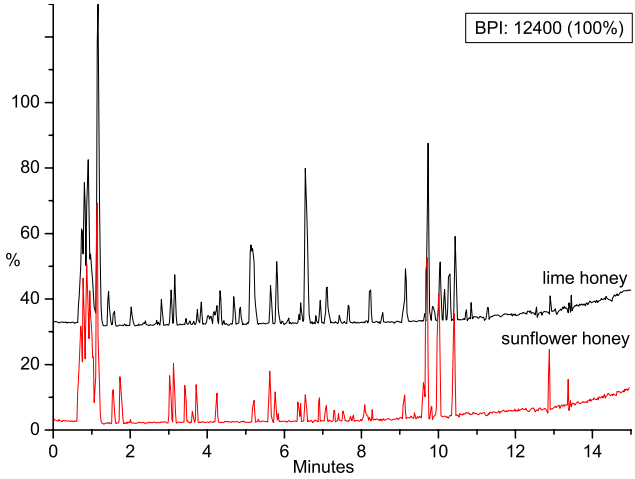

Figure 1. UPLC-chromatograms of sunflower honey and lime honey (both scaled to 12400 counts).

The characterisation of the single components was carried out via the retention time and the accurate molecular masses. Each compound was optimised to its estimated molecular mass $[M\text{-}H]^-$ in the negative mode preventing fragmentation. The data obtained from UPLC/MS were subsequently entered into the MassLynx 4.0 ChromaLynx^{TM} Application Manager software. Based on these data, the software is able to scan different honey samples (4 honeydew, 4 sunflower, 3 lime, 5 rape, and 3 clover) for the characterised substances.

3. RESULTS

After analysing all the honey samples by UPLC-Q/TOF-MS, the ChromaLynx^{TM} software scanned the extracts for the already integrated data of standard substances within three minutes. By adjusting the parameters for peak recognition, the automatic search was refined to such a degree that the manual search was no longer necessary. A quick identification of 34 of the 37 compounds in the honey extracts was possible even though they were overlapped by known or unknown substances in the complex honey matrix. Furthermore, due to the simultaneous cleanup of all honeys and the same volume of injection the results were accomplished semi-quantitative in Table I although a Q/TOF-MS was not the detector of choice for quantification. According to their intensity they were marked with "L" for less than 100 counts per second (cps), "+" for 100 to 2000 cps and "++" for more than 2000 cps.

Honeys of the same biological origin showed similar profiles in regard to their determined phenolic compounds (FEI-Schlussbericht, 2007). Even their relative proportions to each other were almost identical. Therefore, the five presented honeys in Table I are representatives for the other honeys of the same origin.

Honeydew honeys were the richest in phenolic compounds. In all four samples, 33 of the 37 standard compounds were determined, followed by rape honeys (30 compounds), sunflower honeys and lime honeys (both 29 compounds) as well as clover honeys (28). Moreover, honeydew honey was not only the one containing the most different phenolic standard substances, but it also contained five substances with an intensity of over 2000 counts, in particular protocatechuic acid and abscisic acid which are known as marker substances, 4-hydroxybenzoic acid, beta-phenyllactic acid, and chrysin. Comparing Figures 1–3 it is obvious that the profile of honeydew honey contains the most peaks. Nevertheless, the profiles of sunflower honey, lime honey, clover honey, rape honey, and honeydew honey vary greatly not only in regard to the different compounds but also in regard to their quantitative weighting. Thus,

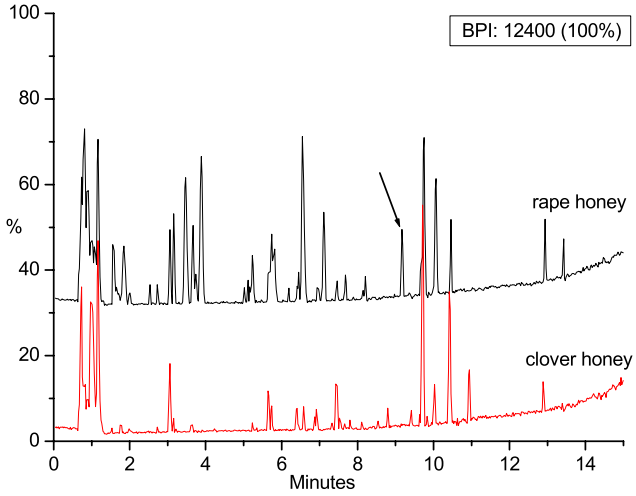

Figure 2. UPLC-chromatograms of clover honey and rape honey (both scaled to 12400 counts). Arrow marks peak of hydrocinnamic acid and abscisic acid at 9.16 minutes.

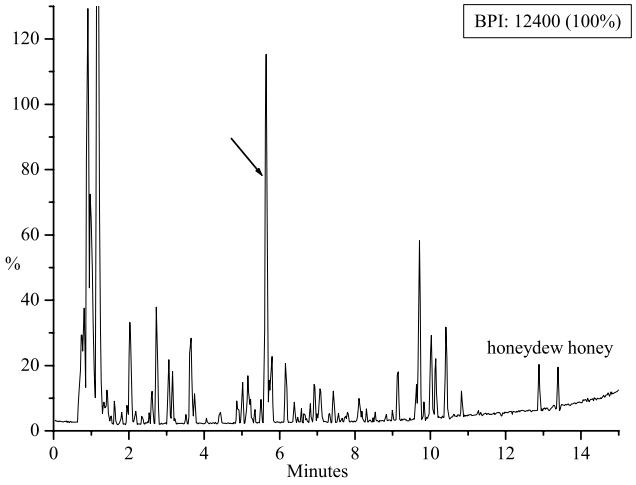

Figure 3. UPLC-chromatogram of the honeydew honey (scaled to 12400 counts). Arrow marks beta-phenyllactic acid at 5.64 minutes.

the UPLC-Q/TOF-MS-system showed that a differentiation of various kinds of honeys is possible. To prove this first impression, some results will especially be considered in the following.

Better separation and resolution results are obtained by utilising particles of only 1.7 μm and pressure-tolerant reversed-phase-columns ($\leq$ 800 bar). As a result, a noticeably shorter chromatography time with defined peaks will be achieved in comparison to traditional HPLC-systems. The previous analysis of honeys, which shows run-times of approximately one hour in the literature (Tomás-Barberán et al., 2001; Gheldof et al., 2002; Yao et al., 2003), takes 95 minutes by HPLC-analysis, especially when it is optimised for simultaneous determination of phenolic acids and flavonoids (Trautvetter et al., 2006). Respectively, the complete analysis by UPLC takes 20 minutes. The presented HPLC-chromatogram of the honeydew honey (Fig. 4)

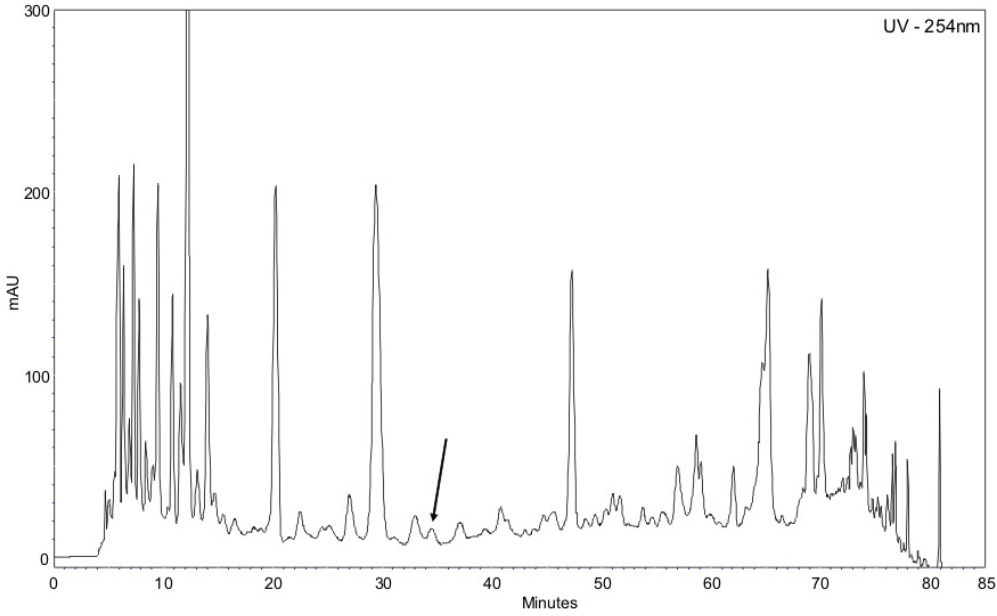

Figure 4. HPLC-chromatogram of the honeydew honey. Arrow marks beta-phenyllactic acid at 34.6 minutes.

and the UPLC-chromatogram of the same honeydew honey sample (Fig. 3) exemplify the time difference needed for analysis.

Aside from the time of analysis the detectors used play an important role. The selectivity and sensitivity of TOF-MS detectors are a good alternative to determine low UV-sensitive substances which are difficult to analyse in small amounts by DAD. β-Phenyllactic acid is one of these UV-sensitive compounds. Comparing the TOF-MS-chromatogram (Fig. 3) with the DAD-chromatogram (Fig. 4) of the honeydew honey, the great differences regarding the detection method can be ascertained. β-Phenyllactic acid elutes in the HPLC system at 34.6 minutes. At this point in the chromatogram β-phenyllactic acid is hardly detectable (Fig. 4 marked by an arrow), even though its UV-maximum is at the displayed wavelength λ = 254 nm. In the BPI-chromatogram, however, a very intensive signal is obtained from the phenolic acid at 5.64 minutes.

Another example of a less UV-sensitive compound is mandelic acid (3.26 min). The detection with DAD was not successful for determining the substance in any of the samples. Only by applying TOF-MS, traces of mandelic acid could be clearly detected in all of the honeys (compare Tab. I). Here, it becomes obvious that the TOF-MS-detector is more sensitive than a DAD, unless the substances can be easily ionised in the negative mode by ESI.

On the basis of the chromatograms (Figs. 3 and 4) it is obvious that the almost constant flow rate of 0.3 mL/min in combination with columns of a smaller internal diameter – as they cannot be used in traditional HPLC – has considerable influence on the retention time of some substances. Therefore, it appears that there are several peak overlaps in the UPLC-chromatograms due to the decrease in elution time, the similar structures within each group of phenolic acids and flavonoids, and the unspecific column for phenolic substances. As the hybrid Q/TOF-mass detector offers not only high selectivity and sensitivity but also great mass accuracy (ensured by permanent injection of leucine enkephalin), the assumed peaks can be assigned to individual substances. The sensitivity of the analysis

and to what extent the adjustments of the software allow for still identifying substances will be clearly exemplified by abscisic acid and hydrocinnamic acid in rape honey. Steeg and Montag (1988b) already detected the second acid as a characteristic marker for rape honey by GC/MS. Employing the optimised HPLC-DAD analysis (Trautvetter et al., 2006), the two standards are eluted completely separate from each other so that there is the opportunity to simultaneously determine both substances occurring in the honey, even if they obtain quite similar UV-maxima and the UV-sensitivity of hydrocinnamic acid is low. Therefore, hydrocinnamic acid was clearly detectable in rape honey analysed by HPLC-DAD (figure not shown). Using UPLC, the retention times of the phenolic acids differ by 0.06 minutes (Tab. I). Thus, it is not possible to be certain that both substances are eluting in the peak at 9.16 minutes in the chromatogram of rape honey (Fig. 2). But the displayed chromatograms of the extract masses of hydrocinnamic acid (m/z = 149.0603) and abscisic acid (m/z = 263.1283) (Fig. 5) reveal that hydrocinnamic acid is definitely identified in rape honey, even when the peak is overlapped by a considerably high content of abscisic acid. Thus, the performance of Q/TOF-MS affirms the results of the HPLC-DAD-analysis but within less analysis time.

Aside from the overlaps of known substances exemplified by abscisic and hydrocinnamic acid in rape honey, there are great overlaps in all analysed honeys (Figs. 1–3) especially in the first part of the chromatogram (until 1.50 min). These are mainly attributed to polar honey components such as the monosaccharides glucose and fructose. Despite several extraction steps, it is not possible to completely separate phenolic substances from the complex honey matrix without carrying over carbohydrates. The remaining high content of sugar molecules which elute from 0.75 to 0.87 minutes with an ionised mass of 179.0549 m/z interfere with the characteristic signal of shikimic acid, eluting at 0.85 minutes, and possessing the ionised mass of 173.0450 m/z in the negative mode. In spite of its selectivity and sensitivity the hybrid Q/TOF-mass detector reached its limit regarding the accurate mass. Thus, shikimic acid, definitely identified by LC-triple quadrupole in honeydew and sunflower honeys (results are not shown in this paper) could not be determined in either honey by the use of the UPLC-MS-system. Likewise, acetylsalicylic acid, which quickly decomposes to salicylic acid, as well as myricetin, which is difficult to detect in the ESI negative mode even in the standard solution, could not be detected in honeys due to the complex matrix. Therefore, in spite of the matrix cleanup, the analysis limit of the system is reached in regard to phenolic substances.

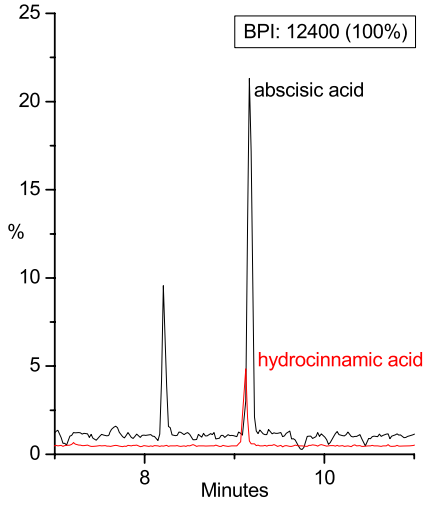

Figure 5. Chromatogram of extracted masses of hydrocinnamic acid and abscisic acid (scaled to 12400 counts).

4. DISCUSSION

Steeg identified 24 phenolic acids as trimethylsilyl derivatives in honey by GC-MS (Steeg and Montag, 1988a). Since flavonoids are not detectable under these conditions but several of them are described as marker substances in different honeys (Ferreres et al., 1993; Gil et al., 1995; Soler et al., 1995), HPLC-DAD must be used for the simultaneous determination of both groups of phenolic substances. However, applying this system leads to a long analysis time as flavonoids

elute relatively late compared to phenolic acids (Tomás-Barberán et al., 2001; Gheldof et al., 2002; Yao et al., 2003; Trautvetter et al., 2006). Furthermore, the evaluation of DAD-chromatograms is a complicated matter. On the one hand there are the numerous overlaps of some phenolic acids and, on the other hand, there is the limit of detection of about 0.04 mg/mL of substances with low UV-sensitivity such as phenylacetic, β-phenyllactic, and mandelic acid. The determination and the evaluation of honeys is much simpler using the UPLC-Q/TOF-MS presented by Waters Corporation. The system offered the possibility of a fast screening of honey samples in regard to phenolic substances already described in the literature.

All in all, by using the presented UPLC-MS-system the occurrence of most of the phenolic acids and flavonoids was affirmed in the analysed honeys: sunflower honey, lime honey, clover honey, rape honey, and honeydew honey. All honeys of the same kind showed comparable profiles regarding the detected phenolic compounds and also their relative proportions to each other. Thus, 34 of the 37 standard substances were identified, although they were overlapped by known and unknown substances due to the short UPLC run time (20 minutes) needed for analysis. Even though they only occur in low concentrations, the combination of the retention time and the accurate molecular mass allows for a fast qualification of substances using the ChromaLynx^{TM} software. The knowledge that some substances are more easily detectable by mass spectrometry after ionisation than by DAD, HPLC combined with triple quadrupole (LC-MS/MS) is the method of choice for quantifying the reported substances.

However, the detection by TOF-MS of all of the substances in the extracted solution can offer a great advantage. Not only the known standards will be detected, but also all of the compounds extracted from honey. In that way, other peaks which may be characteristic for some kinds of honey can be recognised more easily and identified faster. Since a high resolution mass spectrometer always determines accurate molecular masses, the software can suggest an appropriate molecular formula. This significantly facilitates the search for a molecular structure.

In addition, the identification of unknown substances is simplified. In the case that the marked peaks occur characteristically in only one kind of honey they might be potential marker substances for that honey. At the moment we are already working on the identification of characteristic substances in lime, rape, and honeydew honey.

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Confirmation par UPLC-MS de la présence d'acides phénoliques et de flavonoïdes dans le miel.

acide phénolique / analyseur temps de vol / chromatographie liquide ultra performante / flavonoïde / miel / TOF / UPLC

Zusammenfassung – Nachweis von Phenolcarbonsäuren und Flavonoiden in Honig mittels UPLC-MS. Für die Authentifizierung der Sortenhonige sind insbesondere solche Indikatorverbindungen von Interesse, die direkt mit dem Nektar in Verbindung gebracht werden können. Mit unterschiedlichsten Extraktionsmethoden und Analysenverfahren in der Literatur beschriebene Phenolcarbonsäuren und Flavonoide wurden in Tabelle I zusammengefasst. Anschließend wurden Ethylacetatextrakte von fünf Honigsorten (4 Sonnenblumen-, 3 Linden-, 3 Klee-, 5 Raps- und 4 Waldhonige) auf das Vorkommen dieser Verbindungen mit einer Ultra Performance Liquid Chromatography (UPLC)-Anlage gekoppelt mit einem Time-of-flight-Massenspektrometer (TOF-MS) untersucht. Mit der automatisierten Auswertungssoftware ChromaLynx^{TM} konnten 34 der 37 gelisteten Verbindungen innerhalb weniger Minuten eindeutig identifiziert werden, obwohl es durch die gekürzte Analysenzeit auf 20 Minuten (s. Abb. 3 im

Gegensatz zu Abb. 4) zu zahlreichen Überlagerungen von bekannten als auch unbekannten Verbindungen kam. Aufgrund gleicher Aufarbeitung und gleicher Injektionsvolumina konnten die Honige vergleichend semi-quantitativ ausgewertet werden (s. Tab. I).

Honige einer Sorte zeigten vergleichbare Phenolprofile, in denen auch die Verhältnisse der einzelnen Verbindungen zueinander sehr ähnlich waren. Neben der eindeutig identifizierten Hydrozimtsäure (s. Abb. 5), die eine Markerverbindung für Rapshonig ist (Steeg und Montag, 1988b) und von Abscisinsäure überlagert wurde, konnten weitere wenig UV-sensitive Verbindungen wie β-Phenylmilchsäure (s. Abb. 3 und Abb. 4) und Mandelsäure mittels TOF-MS und negativer ElectroSprayIonisation (ESI) detektiert werden. Die Abbildungen 1–3 belegen zudem, dass eine Unterscheidung der Honigsorten über die phenolischen Verbindungen möglich ist, da sich die Verteilung als auch die Gewichtung der einzelnen Substanzen stark unterscheiden. Durch die Selektivität und Sensitivität des Quadrupol/Time-of-flight (Q/TOF)-Detektors war es nicht nur möglich, alle bekannten Substanzen zu erfassen, sondern auch alle aus dem Honig extrahierten Verbindungen. Durch die Angabe der genauen Masse, für die die Software eine mögliche Summenformel vorschlägt, ist damit die Suche und Identifizierung nach möglichen sortenspezifischen, noch unbekannten Markerverbindungen erheblich erleichtert. An der Identifizierung markanter Peaks aus Linden-, Raps- und Waldhonig wird zurzeit gearbeitet. Da einige Verbindungen besser durch Massenspektrometrie erfasst werden können als über DAD, wird eine quantitative Auswertung über HPLC-MS/MS empfohlen.

Flavonoide / Honig / Phenolcarbonsäuren / Time of flight (TOF) / Ultra Performance Liquid Chromatography (UPLC)

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