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Analysis of Phenolic and Other Aromatic Compounds in Honeys by Solid-Phase Microextraction Followed by Gas Chromatography−Mass Spectrometry

SAWSAN DAHER* AND FAZIL O. GÜLAÇAR

Laboratory of Mass Spectrometry, Physical Chemistry, University of Geneva, Sciences I, Boulevard d'Ivoy 16, CH-1211 Geneva 4, Switzerland

The solid-phase microextraction (SPME) followed by gas chromatography−mass spectrometry (GC−MS) was used for the analysis of phenolic and other aromatic compounds in honey samples from different floral origin. Different parameters affecting the efficiency of the extraction, such as the type of the stationary phase of the fiber, NaCl and acetic acid addition, and extraction time, were optimized for the detection of the maximum number of compounds in the shortest analysis time. A total of 31 compounds were detected, with most of them identified and quantified by GC−MS. The principal component analysis (PCA) was applied to the data matrix; the results allowed for the differentiation between honeydew and nectar honeys on the basis of the salicylic acid concentration. It was found that this acid has a high contribution in the honeydew group (71.2−705.9 μg/100 g of honey) compared to the nectar honey group (0−47.6 μg/100 g of honey). The comparison of data in each honey group enabled us to characterize the floral source of some honeys using some aromatic compounds as markers.

KEYWORDS: Phenolic compounds; aromatic compounds; solid-phase microextraction; gas chromatography−mass spectrometry; honeydew honey; nectar honey; salicylic acid

INTRODUCTION

Honey is an important natural product that provides beneficial effects on human health. It has been reported to have an antioxidant capacity (*1, 2*). It is used as a food preservative (*3*) and a dressing for chronic wounds, burns, or skin ulcers because of its antibacterial activity (*4, 5*).

Phenolic compounds are among the important functional constituents of honey having an antioxidant activity (*6, 7*). These include phenolic acids and flavonoids. Analysis of these compounds has been suggested to study their biological effects (*8–15*) or floral origins of honeys (*8, 10, 15–19*). For this purpose, different methods, consisting of several steps, were developed to extract these compounds from the honey matrix: liquid−liquid extraction, column chromatography (*16, 18*), or solid-phase extraction using Amberlite XAD-2 column (*20*) or SPE-C18 cartridge (*13, 21*). The solid-phase microextraction (SPME), introduced by Pawliszyn and his group (*22, 23*), is a fast, simple, and more or less selective extraction method. It eliminates the use of organic solvents and combines sampling, isolation, and enrichment into one step that allows for a convenient automation of the extraction and desorption processes using a conventional autosampler (*24, 25*).

The object of the present study was to identify the phenolic and other aromatic compounds in honey and determine if the differences in their patterns could be related to their floral origin. The procedure used was the SPME followed by gas chromatography−mass spectrometry (GC−MS). Different parameters that could influence the extraction were also studied and optimized.

MATERIALS AND METHODS

Honey Samples. Different commercial types of honey from different regions were investigated: chestnut (*Castanea sativga*, two samples: France), fir (honeydew from *Abies alba* Miller, two samples: France and Italy), acacia (*Robinia pseudoacacia*, two samples: France and Hungary), Pyrenees (honeydew from the Pyrenean forestry massive, two samples: France), orange (*Citrus sinensis*, two samples: Spain and Italy), lavender (*Lavandula* spp., two samples: France), eucalyptus (*Eucalyptus camaldulensis*, two samples: Italy and Spain), forest (honeydew from latifoliae and coniferous trees, one sample: Italy), and oak (*Quercus* spp., one sample: France). Honey samples were purchased from two local markets. The beekeeper associations of these respective areas provide honey samples to these markets, ensuring their floral and geographical origins.

Standards and Fibers. Standards were purchased from Sigma-Aldrich Chemie, Buchs, Switzerland: 2,3-dihydrobenzofuran, benzoic acid, 1-(4-aminophenyl)-1-butanone, methyl syringate, galangin, chrysin, *n*-paraffin mix (C10, C12, C14, and C16), *n*-paraffin mix (C18, C20, C22, and C24), and *n*-paraffin mix (C24, C28, C32, and C36).

* To whom correspondence should be addressed. E-mail: sawsan.daher@chiphy.unige.ch.

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Five different fibers, supplied by Sigma-Aldrich Chemie, Buchs, Switzerland, were used: 85 μm of polyacrylate (PA), 100 μm of polydimethylsiloxan (PDMS), 70 μm of carbowax−divinylbenzene (CW−DVB), 75 μm of carboxen−polydimethylsiloxane (CAR−PDMS), and 65 μm of polydimethylsiloxane−divinylbenzene (PDMS−DVB). The fibers were conditioned prior to use in the hot injector part of the GC according to the instructions provided by the manufacturer. The conditions for PA, PDMS, CW−DVB, CAR−PDMS, and PDMS−DVB are 300 °C for 2 h, 250 °C for 30 min, 220 °C for 30 min, 300 °C for 1−2 h, and 250 °C for 30 min, respectively.

SPME Extraction Procedure. Honey sample (1 g) was introduced into a 10 mL vial for SPME. After the addition of distilled water (4 mL), sodium chloride (1 g), and acetic acid (0.2 mL), the mixture was stirred at room temperature until homogeneity. The SPME fiber was immersed into the solution for 30 min to extract the compounds from honey. The fiber was then immediately inserted into the hot GC−MS injector to desorb and analyze the extract.

GC−MS Analysis. Analyses were carried out on a Hewlett-Packard 5890 gas chromatography (equipped with a split/splitless injector) coupled to a VG Masslab Trio-2 mass spectrometer. Experimental parameters were as follows: J&W DB-5HT column (30 m × 0.32 mm, 0.1 μm film thickness); temperature of injector at 300 °C for PA and CAR−PDMS fibers, 250 °C for PDMS, PDMS−DVB, and CW−DVB fibers; temperature program, 40 °C for 1 min, heated at 3 °C/min to 300 °C for PA and CAR−PDMS fibers, 250 °C for PDMS, PDMS−DVB, and CW−DVB fibers, and hold for 5 min; desorption time of 2 min (splitless); electron impact at 70 eV; *m/z* 45−600 full scan; cycle time of 1.2 s; carrier gas, helium, with a column flow of 1 mL/min.

Qualitative Determination and Quantification. The identification of compounds was made by a comparison of their mass spectra to those of the National Institute of Standards and Technology (NIST) mass spectral library. To confirm the identifications, Kovats retention indices (KI) reported in the literature (*26–28*) and injections of standards were also used, when possible.

The relative concentrations of compounds were calculated from the integrated peak areas on the total ion chromatogram (TIC) trace with respect that of 2,3-dihydrobenzofuran used as an external standard, assuming a response factor equal to 1. For this purpose, 40 μL of 2,3-dihydrobenzofuran in methanol (0.1 mg/mL) was extracted and analyzed using the same protocol as for running honey samples. The average peak area from triplicate injections of this standard was used to calculate the relative concentrations of the investigated compounds in honey samples.

Statistical Analysis. Average concentrations and relative standard deviations (RSDs) were calculated for each compound from triplicate consecutive analytical runs of the chestnut honey sample. Principal component analysis (PCA) was applied to reveal information on the main factors that control the composition and distribution of investigated compounds in honeys using the statistical program KyPlot version 2.0 (Kyence, Tokyo, Japan).

RESULTS AND DISCUSSION

Optimization for the Extraction Conditions. To determine the optimal conditions for the extraction, the effects of different parameters including the type of the stationary phase of the fiber, NaCl and acetic acid addition, and extraction time were investigated on the chestnut honey sample, which has been chosen arbitrarily.

First, five types of fiber, differing in the polarity and thickness of the coating stationary phase (PA, PDMS, PDMS−DVB, CW−DVB, and CAR−PDMS), were compared for their affinity for the aromatic and phenolic compounds. The aqueous solutions were acidified at pH 3 by adding acetic acid and saturated with NaCl. Among the investigated fibers, PDMS and CAR−PDMS coated ones appeared not efficient. With CAR−PDMS, no flavonoid was extracted, and with PDMS, only tectochrysin and pinostrobin chalcone were detected. The extraction using CW−DVB and PDMS−DVB fibers gave several phenolic compounds and aromatic acids. However, the most appropriate fiber type to extract the phenolic compounds as well as aromatic acids (polar compounds) appeared to be the polar PA fiber, because more products have been adsorbed and better chromatograms have been obtained (data not shown). Obviously, among the SPME fibers tested in this work, the polarity of the PA fiber matches best the polarity of the studied compounds. Therefore, the PA fiber was selected for further experiments.

The effect of the addition of NaCl and acetic acid separately or together was then examined using PA fiber. It appeared clearly that these additives had a significant effect on the relative abundance of the early eluting (more volatile) compounds compared to later eluting compounds. The total amounts of early eluting compounds of interest (component numbers **1−20** in **Table 1** with KI < 2270) as compared to the total amounts of later eluting components are shown in **Figure 1**. The addition of either 0.2 mL of acetic acid or 1 g of NaCl induces an increase in the recovery of early eluting components by a factor of ∼1.5−2, respectively. However, this is accompanied by a decrease in the recovery of later eluting components. The addition of both NaCl and acetic acid turned out to be a good compromise for the extraction of the whole of the aromatic compounds with different volatilities and polarities. The addition of salt increases the ionic strength and the surface tension of the aqueous solution, therefore increasing the hydrophobicity of the organic compounds. As a result, the partition coefficients and the SPME recovery organic compounds are enhanced (*29*). Acidification of the aqueous solution is necessary for the extraction of carboxylic and phenolic acids, with the pK_a values higher than ∼4, because the extraction will be more effective if they are in their undissociated forms (*30, 31*).

Salicylic acid (SA) was extracted only when both NaCl and acetic acid were added to the chestnut honey sample, while it was completely absent in the other cases. The same result was observed for fir honey, which showed an intense peak corresponding to SA only after the addition of salt and acetic acid. The effect of salinity and pH on the availability of this acid to SPME fiber may be related to the intermolecular hydrogen bond O−H···O.

Taking into account the above results, the effect of the extraction time on the recovery was investigated using the PA fiber on the honey solutions with both NaCl and acetic acid. It should be noted that the addition of 50 μL of acetic acid (instead of 200 μL) does not change significantly the recoveries reported in **Figure 1**. However, to keep a relatively constant pH 3 in all samples, the addition of 200 μL of acetic acid was adopted for the subsequent work.

Figure 2 shows the variation of the extraction yields for some selected compounds with the extraction time. As expected, the yield increased with the extraction time for most of the analytes, and even after 60 min, the partitioning equilibrium between the sample solution and fiber is not reached. Therefore, to avoid long extraction times necessary to reach partitioning equilibrium, 30 min of extraction time was selected as a pre-equilibrium extraction for subsequent studies. However, to obtain reproducible data using a pre-equilibrium extraction approach, constant convection conditions and careful timing of the extraction are necessary (*24*).

SPME/GC−MS Analysis of Honey Samples. Results of the analyses of 16 commercially available honeys of 9 different floral origin (2 chestnut, 2 acacia, 2 fit, 2 Pyrenees, 2 lavender, 2 eucalyptus, 2 orange, 1 forest, and 1 oak) using the above selected conditions of SPME and GC−MS are reported in **Table 1**. Kovats indices were determined from the retention times of

Table 1. Phenolic and Other Aromatic Compounds Contents (μg/100 g of Honey) of Honey Samples

compound	Nb[a]	KI[b]	chestnut (n = 3) (RSD %)[c]		acacia		orange		lavender		eucalyptus		Pyrenees		fir		forest	oak
benzoic acid	**1**	1197	206.8 (25)	666.3	178.3	125.1	99.6	43.5	52.3	118.9	78.8	360.4	213.0	146.1	305.9	169.7	116.9	93.3
2-methyl-benzaldehyde	**2**	1219	94.4 (17)	144.9	90.9	8.3	13.6	11.5	20.7	34.3	22.4	17.1	88.3	174.6	123.6	37.6	32.2	47.3
phenyl-acetic acid	**3**	1266	50.5 (9)	383.9	97.3	9.0	52.0	40.7	81.0	87.7	23.0	1362.2	23.4		55.6	114.1	35.0	17.0
2-methoxy-4-vinylphenol	**4**	1296	21.3 (10)	42.8	38.6		6.7		9.6		65.0		27.4		32.7	28.7	23.0	9.2
2,3,5-trimethyl- phenol	**5**	1309			74.1		29.8				65.0							
salicylic acid	**6**	1323	34.0 (15)	47.6	24.9				28.2		39.4	25.3	203.1	705.9	349.2	433.4	183.3	71.2
1-(3-methoxyphenyl)- ethanone	**7**	1336										159.8						
3-phenyl-propionic acid	**8**	1345	13.3 (19)	60.7	7.7				5.6				8.6	21.3	13.1	22.9	22.7	27.7
4-isopropyl-benzoic acid	**9**	1426	7.9 (16)	115.9	3.3					10.0			41.2		28.5	47.6		14.4
cinnamic acid	**10**	1443	148.8 (26)	260.1	44.2										79.5	59.2	13.3	28.1
1-(2 or 3-aminophenyl)-1-butanone	**11**	1489	24.3 (10)	64.9											24.0			
unknown	**12**	1527	240.9 (11)	769.0						271.2			122.8		148.0	194.4	35.9	79.1
5-phenyl-pent-4-enoic acid	**13**	1561	97.1 (3)	267.5	54.6			35.5		168.4		57.3		154.8	109.6	37.2	64.5	69.2
unknown	**14**	1629							31.7									
methyl syringate	**15**	1772	1.4 (35)	76.9	2.0	20.1	67.2	0.8	67.7	37.2	67.7	82.7	1.3	2.0	2.7	8.4	2.7	
ferulic acid	**16**	1887	14.8 (25)		28.0						15.7		21.8		17.3			
3,4-dimethoxycinnamic acid	**17**	1928	19.3 (26)	56.8	9.7		20.1	27.4		25.6	17.7		10.2		13.9	12.3	10.2	29.8
3,6-dimethyl-naphtho[1,2-*b*]furan-4,5-dione	**18**	1959							31.7									
unknown	**19**	2251										49.5						
unknown	**20**	2263										22.3						
2,4,6-trimethyl-*N*-(5-methyl-pyridin-2-yl) benzamide	**21**	2278	17.1 (12)	14.5	25.3								21.5	23.8	17.6	6.6		
pinostrobin chalcone	**22**	2348	56.3 (19)	113.6	42.5	25.1	71.5	118.9	45.4	105.0	122.7	49.5	24.0	240.2	46.2	72.1	117.9	46.8
oxalic acid dibenzyl ester	**23**	2368	6.8 (10)	19.1	8.6	6.6	13.1	31.6	9.1	168.4	11.8	22.7	6.1		12.8	20.6	31.1	12.9
5-methoxy-3,7-dihydroxyflavanone	**24**	2425	10.7 (13)	35.9	10.7	11.7	17.7	15.2	8.8	35.3	18.9	24.6	5.9	25.5	8.8	12.8	14.2	13.0
pinocembrin	**25**	2450	277.0 (9)	996.9	667.5	585.8	392.6	1105.9	667.5	626.6	565.9	684.8	355.4	674.9	454.5	678.6	764.1	476.8
tectochrysin	**26**	2528	31.0 (23)	131.3	49.4	56.1	54.2	157.3	52.6	137.5	88.4	85.2	27.4	139.9	39.6	76.5	83.1	54.2
pinobanksin chalcone	**27**	2541	53.6 (11)	156.0	159.8	68.4	0.6	221.0	95.7	176.3	104.0	58.2	67.9	61.9	81.2	59.4	52.4	55.5
2′-methoxychrysin	**28**	2561	11.2 (11)	45.2	9.2	17.0	4.5	4.4	11.2	7.9	16.7	5.7	7.4	66.3	13.1	17.2	23.7	5.8
chrysin	**29**	2641	152.6 (24)	634.1	286.1	174.6	255.1	613.0	267.5	318.3	341.8	199.4	142.4	315.8	220.4	319.5	470.6	289.8
acacetin and galangin	**30**	2679	36.2 (11)	322.0	164.7	93.6	86.8	317.0	169.7	156.0	157.3	67.2	58.1	105.9	87.8	136.2	253.9	227.9
4′,5-dihydroxy-7-methoxyflavanone	**31**	2715	24.1 (17)	102.8	50.5	21.3	75.4	151.1	65.8	56.1	118.3	4.6	44.2	81.7	25.3	61.4	85.2	74.4

[a] Compound number. [b] Kovats retention indices. [c] Relative standard deviation.

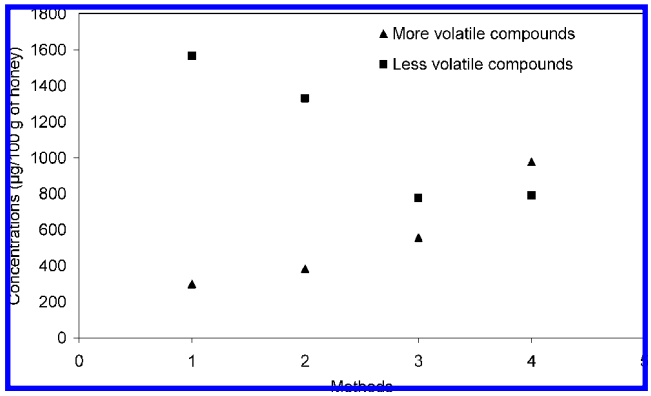

Figure 1. Graphic showing the effect of the addition of NaCl and acetic acid. Methods: (1) without NaCl and acetic acid, (2) without NaCl and with acetic acid, (3) with NaCl and without acetic acid, and (4) with NaCl and acetic acid.

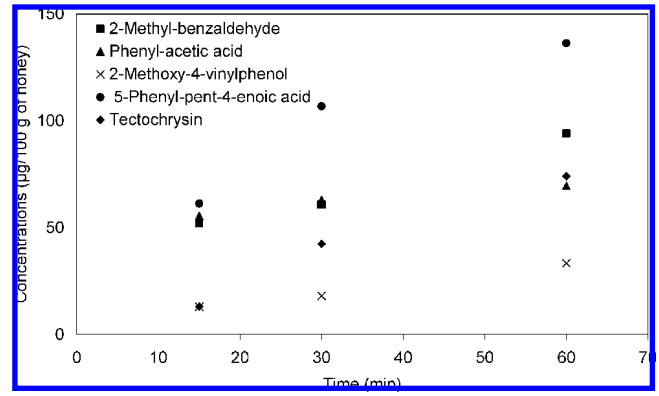

Figure 2. Extraction time profiles of selected compounds with PA SPME fibers.

the *n*-alkanes analyzed under identical chromatographic conditions. Two typical GC−MS chromatograms of phenolic and other aromatic compounds extracted from the honey samples using the PA fiber under the optimized conditions are shown in **Figure 3**.

For compounds that could not be formally identified, major fragments in their mass spectra are reported in **Table 2**. Compound **11** has a mass spectrum similar to that of 1-(4-aminophenyl)-1-butanone but a shorter retention time. It is probably an isomer with an amino group in *ortho* or *meta* position [1-(2 or 3-aminophenyl)-1-butanone].

The concentration range of the compounds reported in **Table 1** (0.6−1362.2 μg/100 g of honey) is in the linearity range of the MS detector. The chestnut honey sample was run in triplicate to assess analytical precision (RSD % in **Table 1**). The minimum value of RSD was 3% for 5-phenyl-pent-4-enoic acid (**13**), and the maximum was 35% for methyl syringate (**15**, low concentration level 1.4 μg/100 g of honey).

The PCA was applied to the analytical data to reveal the main factors controlling the compound distribution (*32*). The PCA was first applied to the complete set of compounds listed in **Table 1**; however, the scores for the first principal components were un-informative, and no clusters were observed (plots not shown here). As can be seen in **Table 1**, all honey samples contained the late eluting compounds **22**−**31**, but there were differences for the early eluting compounds from **1** to **21**. We therefore chose to perform PCA with the data matrix including only compounds **1**−**21**. In that case, the first four principal components accounted for 96% of the total variance, and the corresponding eigenvalues were larger than 2 for the first three components as shown in **Table 3**.

Figures 4 and **5** show the PCA loading and score plots respectively of component 1 versus component 2. The loading plot represents the direction of the original variables in the same

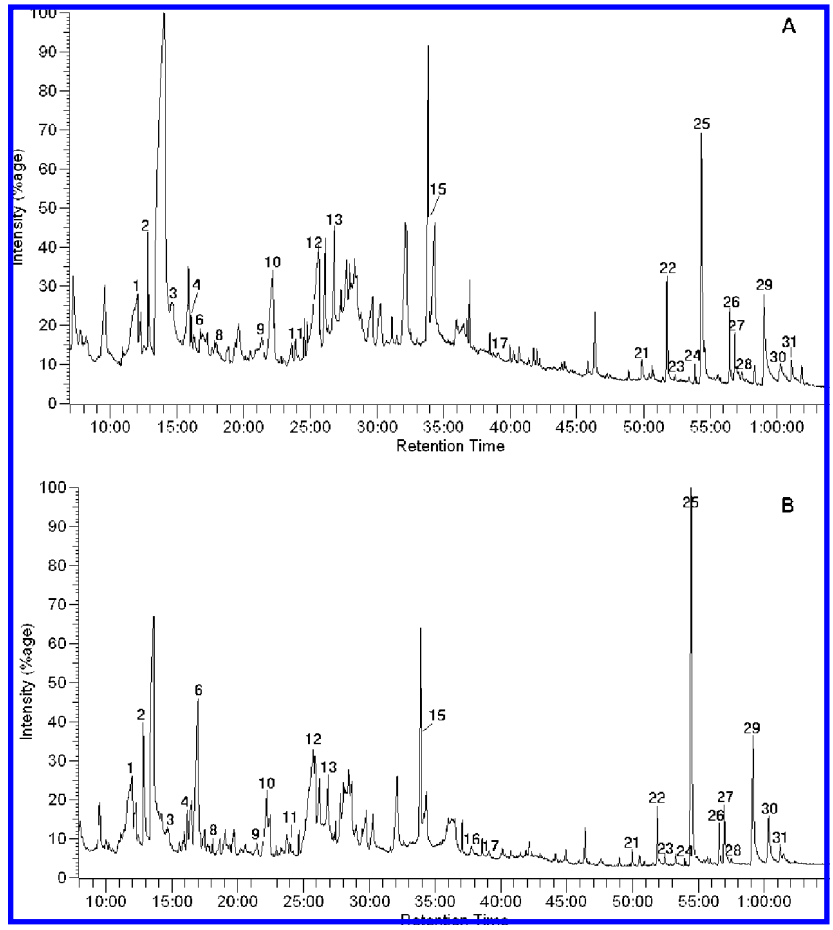

Figure 3. Two typical GC−MS chromatograms of phenolic and other aromatic compounds extracted from the honey samples using the PA fiber under the optimized conditions: (**A**) chestnut honey and (**B**) fir honey. The numbers refer to compounds listed in **Table 1**.

Table 2. Mass Spectral Data of Unknown Compounds Listed in **Table 1**

number of compound	mass (*m*/*z*) and intensity (%, in parentheses) of prominent MS peaks
12	77 (50), 79 (55), 91 (100), 105 (37), 119 (75), 123 (65), 164 (85)
14	57 (32), 91 (7), 233 (100), 247 (17), 262 (9)
19	65 (12), 91 (100), 119 (9), 220 (13), 266 (12)
20	140 (20), 168 (100), 207 (13), 222 (42)

Table 3. Eigenvalues of Correlation Matrix

	component 1	component 2	component 3	component 4
eigenvalue	7.939 133	2.985 430	2.134 403	1.162 338
percentage of total variance	49.62	18.66	13.34	7.26

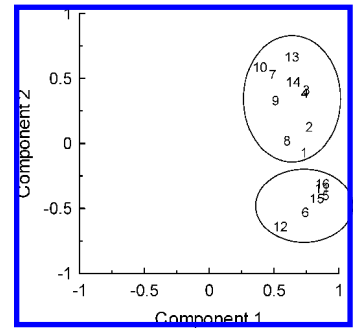

Figure 4. Loading plot for data listed in **Table 1**. Separation of different honey samples in nectar honey (at the top) and honeydew (lower part): (1 and 2) chestnut, (3 and 4) acacia, (5 and 6) fir, (7 and 8) lavender, (9 and 10) eucalyptus, (11 and 12) Pyrenees, (13 and 14) orange, (15) forest, and (16) oak.

plane. In **Figure 4**, it can be seen that two groups of honey samples are well-distinguished by the values of their second component scores. These groups are honeydew honeys (fir, Pyrenees, forest, and oak) with positive loadings on PC2 and nectar honeys (chestnut, acacia, orange, eucalyptus, and lavender) with negative loadings on PC2. Floral honey is made by honeybees from the nectar of blossoms, while honeydew honey is prepared from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants (*33*). The score plot (**Figure 5**) indicates the position of the objects (compounds **1**−**21**). The SA is positioned at the extreme negative side of the PC2, characteristic for the honeydew group in the loading plot. In **Table 1**, it can be seen that this acid has a high contribution in the honeydew group (71.2−705.9 μg/100 g of honey) compared to the nectar honey group (0−47.6 μg/100 g of honey). These results show that the SA can be used as a marker (weight or proportion) to distinguish honeydew from nectar honey. In the same field, Soria et al. (*34*) proved recently the usefulness of the application of the linear regression to the data obtained for volatile components by SPME/GC−MS for the differentiation of both honeydew and nectar honeys.

Differences in aromatic acid contents are also observed in each group. Regarding the nectar honey, cinnamic acid (**10**) has the highest concentrations (148.8−260.1 μg/100 g of honey) in the two chestnut honey samples and 1-(2 or 3-aminophenyl)-1-butanone (**11**) was only present in this type of honey (24.3−64.9 μg/100 g of honey). Therefore, these two com-

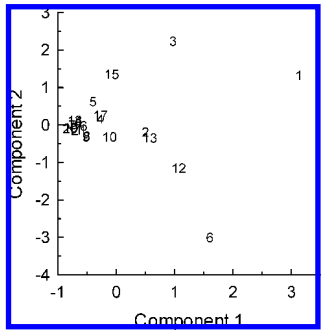

Figure 5. Scores plot for data listing in **Table 1**. The numbers refer to compounds listed in **Table 1**.

pounds may be used as helpful markers for identifying the chestnut honey in the nectar honey group. Some of the compounds in **Table 1** are present only in one of the two samples of the same honey type, and although absent in other honeys, they can not be considered as a marker of honey floral origin.

In the honeydew group, Pyrenees honey can be distinguished from the other honey types by the absence of cinnamic acid. Fir honey differs from other honeydew honeys by the content of SA and component **12**, which are present in concentrations 2−6 times higher than in forest and oak honeys. Further studies on more honey samples are necessary to confirm these findings with respect to floral origin and chemical composition in each group.

The flavonoids, pinocembrin, pinobanksin and chrysin, have been identified in all analyzed samples as already reported for most European honey samples (*10*) and show no correlation with honey origins.

The results obtained show that SPME/GC−MS can be used as a rapid and solvent-free method for the determination of phenolic and other aromatic compounds in honey samples. The PCA application to the measured data showed that the concentration of SA may be a useful tool for differentiation between two groups, honeydew and nectar honeys. Differences in some aromatic compound contents allowed for the characterization of some honeys in each group.

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