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Analysis of Volatiles from Spanish Honeys by Solid-Phase Microextraction and Gas Chromatography−Mass Spectrometry

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Headspace solid-phase microextraction (SPME), followed by gas chromatography (GC)−mass spectrometry (MS) determination, has been used for the analysis of honey volatiles. Two SPME fibers were employed to study the composition of volatiles from various types of Spanish honeys. The best results were obtained with the Carboxen/PDMS fiber, using a homogenization time of 1 h at 70 °C and a sampling period of 30 min. A total of 35 compounds were detected, most of them identified by GC−MS and quantified using external standards. Differences in the composition of honey volatiles were obtained, and these results allowed the differentiation of honeys. However, further studies are necessary to confirm the utility of this technique as an alternative tool for the characterization of the floral origin of honeys.

KEYWORDS: Honey; volatiles; solid-phase microextraction; gas chromatography; mass spectrometry

INTRODUCTION

The floral origin of honeys is a very important characteristic of the quality of these food products. Unifloral honeys possess distinctive flavors, mainly derived from their nectar sources, indicating the presence of volatile components. Nevertheless, the floral origin of honeys is usually determined by pollen analysis, physicochemical parameters, and organoleptic properties. In fact, the characterization of various Spanish honeys has been reported based on palynological properties, physicochemical parameters, and composition of sugars (*1*, *2*). However, the presence of the specific pollen is low in some types of honey (citrus, lavender, and rosemary) making a wider range of markers necessary to ascertain the origin of honeys.

A large number of organic compounds have been described as components of different types of honeys (*3*−*10*). The main components or source specific honey volatiles belong, in general, to three principal categories such as terpenes, norisoprenoids, and benzene derivatives (*9*). Some of these substances have been described as characteristics of the floral source, and other compounds, like some alcohols, branched aldehydes, and fural derivatives, may be related to the microbial purity of processing and storage conditions of honey (*11*).

The identification and quantification of volatile compounds from a complex mixture such as honey are difficult. Analysis of volatile and semivolatile components of honey has been carried out by simultaneous steam distillation−solvent extraction (*6*), although other techniques such as extraction with organic solvents (*9*, *12*), column extraction (*8*), and dynamic headspace analysis (*5*, *7*, *11*) have also been used.

Solid-phase microextraction (SPME) is a solventless extraction technique based on the exposure of an immobilized stationary phase into the matrix containing the analytes, which could be liquid, solid, or gaseous, followed by thermal desorption of the analytes in the injector of a gas chromatograph (*13*). This technique has been successfully used for the analysis of volatile flavor compounds (*14*−*17*). However, data on the application of SPME to the analysis of volatile compounds present in honey are scarce in the available literature (*18*).

The aim of the present work was to study the determination of volatiles from honey using headspace SPME and gas chromatography with mass spectrometric detection (GC−MS). This method was applied to determine the main volatile components of various Spanish unifloral honeys (orange, eucalyptus, rosemary, lavender, and thyme) and to evaluate the possibility of using this technique for the characterization of honeys.

MATERIALS AND METHODS

Materials. Dimethyl sulfide, 1-hydroxy-2-propanone, 3-methyl-3-buten-1-ol, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3-butanediol, 2-furaldehyde (furfural), 2-furanmethanol, 1-hexanol, benzaldehyde, benzyl alcohol, benzene acetaldehyde, 2-phenyl ethanol, and 3,5,5-trimethyl-2-cyclohexen-1-one were purchased from Sigma-Aldrich Chimica. Ethanol, acetone, and acetic acid were obtained from Panreac (Spain).

Four replicate samples of honeys from various producers and regions were collected for this study from commercial sources. The honeys studied were orange (*Citrus* spp.), eucalyptus (*Eucalyptus* spp.),

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rosemary (*Rosmarinus officinalis* L.), lavender (*Lavandula latifolia* Med.), and thyme (*Thymus* spp.). Another eight replicates of orange honeys were obtained directly from beekeepers of Valencia (Spain); these honeys were collected in a zone where the main varieties were Navel and Naveline oranges (*Citrus sinensis*, Osbeck). All honey samples originated from Spain.

Carboxen/PDMS and PDMS/DVB fibers (Supelco, Spain) were used to extract headspace volatiles of honey. Before analysis, the fibers were preconditioned in the injection port of a gas chromatograph according to the instructions provided by the supplier.

GC−MS. Analyses were performed using a Hewlett-Packard 6890 (Waldbronn, Germany) gas chromatograph equipped with a mass spectrometric detector (MSD) model HP 5973. Samples were injected splitless, and volatiles were separated using a fused silica capillary column (HP-5MS), diphenyl dimethylpolysiloxane as nonpolar stationary phase (30 m × 0.25 mm i.d.), and 0.25 μm film thickness, supplied by Agilent (Madrid, Spain) with helium as carrier gas at a flow rate of 1 mL/min. The injector port temperatures were 270 °C for Carboxen/PDMS and 250 °C for PDMS/DVB. The interface temperature was 250 °C. The oven temperature was maintained at 50 °C for 4 min, programmed at 10 °C/min to 200 °C, held for 0.5 min, programmed at 20 °C/min to 230 °C, and held 1 min.

The mass spectrometer was operated in the electron impact mode with an electron energy of 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; mass range *m*/*z* 25−500; scan rate, 3.62 s per scan; and EM voltage, 1150.

Compounds were identified based on NIST mass spectra library search. Most of these compounds were further confirmed by comparing their mass spectra and retention times with those obtained for standards.

Headspace SPME Analysis. Analyses of volatile compounds of honeys were carried out by weighing 1 g of honey in 4 mL vials (4.4 cm height × 1.1 cm i.d.) with PTFE/silicone septa and a stirring bar. The vials and septa were previously heated, at 150 °C during 24 h, to remove undesirable chromatographic signals able to interfere with honey volatiles. Samples were maintained and magnetically stirred for 1 h at 70 °C to allow equilibration. Sampling of the volatile honey compounds was done by inserting the sheathed fiber through the septum and exposing it to the headspace for 30 min. The fiber was then retracted and transferred to the injector port of the gas chromatograph where the compounds were desorbed for 5 min.

Quantification. The concentrations of honey volatiles were determined by comparing the ratios of the peak areas in the sample with those found for a known concentration of these compounds in the external standard mixture, prepared in ethanol with the compounds indicated above. The quantification of compounds not present in the standard mixture was performed with respect to benzyl alcohol, assuming a response factor equal to 1. The detector response was linear in the range of concentrations found. The limit of detection was 0.1 ng of benzyl alcohol, considering a signal higher than three times the background noise.

Statistical Analysis. The concentrations of the components detected in honeys were statistically tested. Univariate analysis (ANOVA, Kolmogorov-Smirnov test of good fitness) and multivariate analysis (stepwise discriminant analysis, canonical discriminant analysis, M. De Box test) were used in the analyses. These were conducted using the BMDP7M and CANDIS programs from BMDP Statistical Software release 7 (*19*) and SAS version 8 (*20*), respectively.

RESULTS AND DISCUSSION

SPME Analysis of Honey Volatiles. *Equilibration and Sampling.* Preliminary assays with Carboxen/PDMS were carried out in order to establish the experimental conditions for headspace SPME analysis of honey volatiles, particularly the temperature and the equilibration and sampling times.

Three temperatures were evaluated for headspace analysis: 50, 70, and 85 °C. The best results were obtained at 70 °C (data not shown). At this temperature, the number of chromatographic signals was higher than those obtained at 50 °C. When the analysis was carried out at 85 °C, or at a higher temperature, a high signal of 5-hydroxymethyl-2-furancarboxaldehyde was detected, as a result of the fructose decomposition. This signal has been described as an indicator of loss of honey quality by overheating during processing or storage or by honey adulteration with acid-hydrolyzed inverted sugar (*12*).

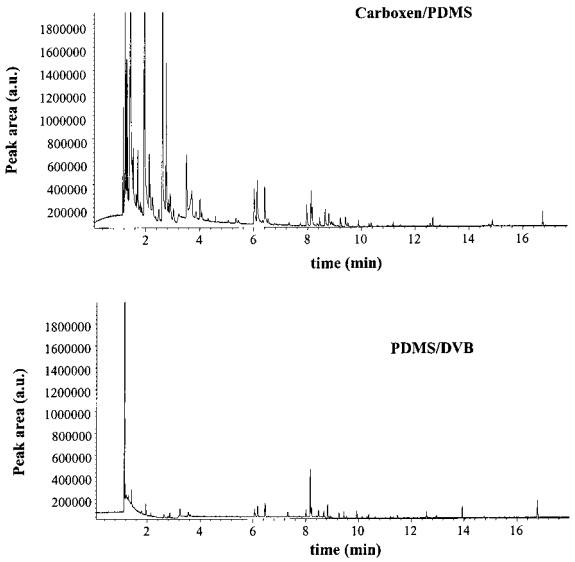

Figure 1. Chromatograms obtained by headspace SPME analysis of eucalyptus honey with Carboxen/PDMS and PDMS/DVB fibers.

The effect of the other experimental conditions, the equilibration and sampling times, was studied at 70 °C. To evaluate the equilibration time, SPME analyses were carried out after 30 or 60 min of equilibration. The obtained results showed a higher number of chromatographic signals after 60 min of equilibration (data not shown). This equilibration time is in agreement with the results previously reported by Bartelt for headspace SPME analysis of various organic compounds (*21*). The effect of the sampling time on the extraction efficiency was evaluated by sampling the headspace during 30 or 60 min, after 1 h of equilibration, and similar peak areas were obtained in both conditions.

These results indicated that a stirring time of 1 h at 70 °C, followed by a sampling period of 30 min, were adequate conditions for SPME analysis of honey volatiles.

Type of Fiber. Two different SPME fibers, Carboxen/PDMS and PDMS/DVB, were evaluated in the conditions described above. These fibers were selected based on the results previously obtained in our laboratory in the SPME analysis of organic volatiles (*22*). Both fibers were used in the headspace SPME analysis of honey samples with different floral origins. The results obtained with the Carboxen/PDMS fiber were better in all cases, due to the higher number and concentration of honey volatiles, mainly those with retention times between 0 and 4 min. **Figure 1** shows representative chromatograms obtained with these fibers for eucalyptus honey. As a result of these assays, the Carboxen/PDMS fiber was selected for the analysis of volatile compounds from honey.

Analysis of Honey Volatiles. Five commercially available Spanish honeys of different floral origin (orange, eucalyptus, rosemary, lavender, and thyme) were analyzed by headspace SPME and GC−MS. **Table 1** shows the volatile compounds detected in the studied honeys. These compounds were quantified, and the results obtained are summarized in that table. The quantification of honey volatiles has been carried out by comparing their peak areas with those of an external standard mixture. **Figure 2** shows representative total ion chromatograms of the analyzed samples.

Table 1. SPME Analysis of Volatiles from Spanish Honeys Using Carboxen/PDMS Fiber

				mean amount extracted by SPME, ng (SD)				
				honey samples				
peak no.	Id[a]	compd	retention time (min)	orange	eucalyptus	rosemary	lavender	thyme
1	A	ethanol	1.24	14.4 (1.0)	30.0 (7.1)	105.8 (10.1)	21.3 (6.0)	98.3 (7.7)
2	A	acetone	1.27	32.4 (2.3)	6.0 (2.2)	10.1 (2.5)	11.5 (1.2)	11.4 (5.0)
3	A	dimethyl sulfide	1.30	4.7 (0.5)	5.3 (1.9)	8.0 (2.2)	81.6 (5.6)	42.0 (5.0)
4	A	acetic acid	1.40	36.0 (7.3)	61.2 (18.0)	44.0 (3.4)	57.5 (4.0)	77.7 (5.9)
5	B	2-butanol	1.46					11.9 (1.4)
6	B	2-methyl-3-buten-2-ol	1.49				20.1 (2.3)	3.0 (0.8)
7	B	2-methyl-1-propanol	1.54		2.4 (0.8)	2.8 (0.2)		3.1 (0.4)
8	B	3-methyl butanal	1.65					2.8 (1.1)
9	A	1-hydroxy-2-propanone	1.69	28.4 (5.0)	17.7 (3.4)	27.6 (4.7)	41.1 (5.7)	43.8 (3.5)
10	A	3-hydroxy-2-butanone	1.95	2.2 (0.9)	117.6 (11.1)	2.0 (0.2)	3.4 (0.1)	14.5 (3.9)
11	A	3-methyl-3-buten-1-ol	2.13	1.8 (0.9)		2.7 (0.9)	33.2 (2.5)	2.8 (0.8)
12	A	3-methyl-1-butanol	2.15		4.6 (1.8)	2.1 (0.6)		5.5 (0.6)
13	A	2-methyl-1-butanol	2.18		1.7 (0.4)	2.7 (1.2)	1.6 (0.5)	2.6 (0.6)
14	B	dimethyl disulfide	2.26		1.6 (0.7)	1.8 (0.3)		
15	B	2-methyl-2-buten-1-ol	2.53				4.2 (0.6)	
16	B	3-methyl-2-buten-1-ol	2.55				5.2 (0.1)	
17	A	2,3-butanediol	2.64; 2.78	10.2 (2.1)	132.7 (37.4)	29.0 (3.4)	853.0 (57.3)	115.9 (14.7)
18	A	furfural	3.51	55.0 (5.7)	15.6 (1.9)	39.4 (4.4)	24.6 (5.9)	35.6 (2.6)
19	B	3-methyl butanoic acid	3.74		12.4 (2.6)			
20	A	2-furanmethanol	4.03			3.0 (0.9)	23.4 (0.7)	6.0 (0.2)
21	A	1-hexanol	4.34				20.4 (2.4)	
22		unknown 1	6.06		8.8 (1.7)			
23		unknown 2	6.17		9.6 (1.3)			
24	A	benzaldehyde	6.46	4.2 (0.8)	6.4 (1.7)	4.2 (1.0)	16.8 (4.4)	3.8 (0.8)
25	A	benzene acetaldehyde	8.17	2.2 (0.7)	4.3 (1.2)	10.3 (4.2)	8.2 (1.1)	13.7 (5.8)
26		unknown 3	8.82	25.3 (2.2)	2.3 (0.6)	5.2 (1.0)	2.2 (0.7)	
27	B	3,7-dimethyl-1,5,7-octatrien-3-ol	9.27				12.7 (1.1)	
28	A	2-phenyl ethanol	9.45	3.7 (1.6)	1.8 (0.4)	4.5 (1.0)	9.6 (2.5)	1.6 (0.1)
29	A	3,5,5-trimethyl-2-cyclohexen-1-one	9.53				11.5 (2.0)	
30	B	lilac aldehyde 1	9.85	5.2 (1.5)				
31	B	2,6,6-trimethyl-2-cyclohexene-1,4-dione	9.93		0.9 (0.2)	1.1 (0.4)	1.1 (0.2)	
32	B	lilac aldehyde 2	10.07	14.8 (5.0)				
33	B	lilac aldehyde 3	10.31	7.8 (2.4)				
34	B	2-amino methyl benzoate	12.95	4.3 (1.2)				
35	B	4,5,6,7-tetrahydro-3,6-dimethyl benzofuran	16.76		1.5 (0.2)			

[a] Identification: (A) comparison of retention time and mass spectrum with that of an authentic sample recorded under the same conditions. (B) Tentative identification by comparison of mass spectrum with NIST library (computer) spectrum.

A total of 35 signals were identified in the studied honeys and 11 of these compounds were detected in all of the honeys: ethanol, acetone, dimethyl sulfide, acetic acid, 1-hydroxy-2-propanone, 3-hydroxy-2-butanone, 2,3-butanediol, furfural, benzaldehyde, benzene acetaldehyde, and 2-phenyl ethanol. Some of these 11 compounds, ethanol, furfural, benzene acetaldehyde, acetone, and dimethyl sulfide, have been reported as common components of various unifloral honeys at variable concentrations (*5*, *11*). The amount of these compounds found in the studied Spanish honeys was quite different depending on the floral origin.

Some of the compounds shown in **Table 1** can be used to distinguish the different types of honey. Thus, the presence of three signals of lilac aldehydes (α,5-dimethyl-5-ethylenyl-2-tetrahydrofuran acetaldehydes), floral odor, seems to be a particular characteristic of the orange honey. Lavender honey could be distinguished by the detection of 3,7-dimethyl-1,5,7-octatrien-3-ol and 3,5,5-trimethyl-2-cyclohexen-1-one, together with a high amount of 2,3-butanediol and a certain presence of 2-methyl-3-buten-2-ol, 3-methyl-3-buten-1-ol, 1-hexanol, 2-methyl-2-buten-1-ol, and 3-methyl-2-buten-1-ol. A high content of 3-hydroxy-2-butanone and the presence of dimethyl disulfide, 3-methylbutanoic acid, and 4,5,6,7-tetrahydro-3,6-dimethyl benzofuran seem to be characteristic of the eucalyptus honey. The chromatograms obtained for rosemary honey, **Figure 2**, showed few representative signals; the high content of ethanol and the

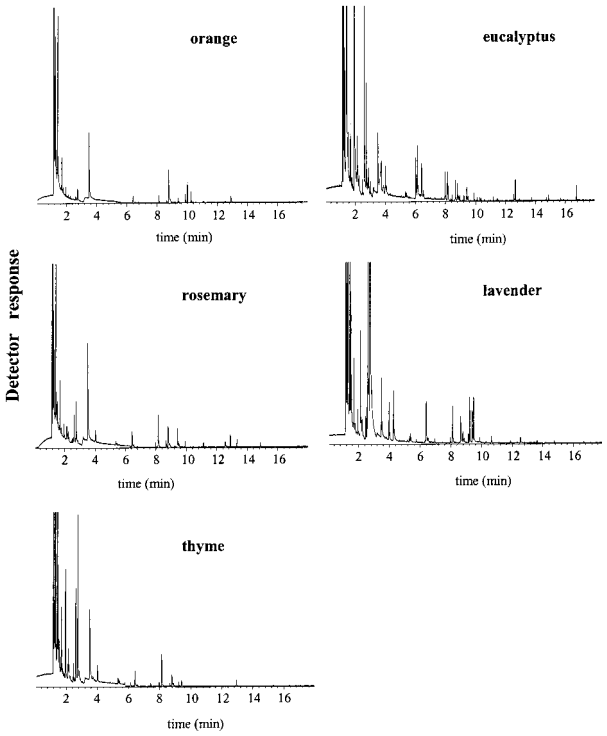

Figure 2. Representative chromatograms obtained by headspace SPME analysis with Carboxen/PDMS for orange, eucalyptus, rosemary, lavender, and thyme honeys.

presence of dimethyl disulfide were the most representative. Thyme honey also showed a high content of ethanol and few chromatographic signals at retention times higher than 3 min, although the presence of 2-butanol and 3-methyl butanal has been detected only in this honey type.

Various factors, such as plant source, seasonal and climatic conditions, and processing or storage circumstances, may affect the composition of honey volatiles. Therefore, additional SPME analyses were carried out with samples of orange honeys obtained directly from beekeepers, to compare the volatile compounds detected for these honeys with those of the previously analyzed commercial orange honeys. In these analyses, three signals identified as lilac aldehydes were observed in the samples, as found previously in the commercial orange honey. Therefore, these components seem to be a characteristic of Spanish orange honeys.

Differentiation of Spanish Honey Types. The results of SPME−GC analysis, summarized in **Table 1**, were used as variables in the statistical analysis. Previously to the discriminant analysis, the Kolmogorov−Smirnov test was done to evaluate the normality. As a result of this previous test and the M. De Box test, no transformation was done. Canonical discriminant and stepwise discriminant analyses were made in order to evaluate the studied honeys and to establish the characteristic variables of each honey type (*19*, *20*, *23*).

The univariate and multivariate analyses showed very significant differences ($Pr < 0.0001$) among the average values for all of the variables in relation to honey type. The canonical discriminant analysis showed that the first four discriminant functions provided a good summary of the original data of the considered variables. Thus, the proportion of the total accumulated dispersion with the two and three first functions were 0.90 and 0.96, respectively, and the squared canonical correlations were 0.998, 0.990, 0.980, and 0.968 for these four functions. The correlations between the canonical variables and

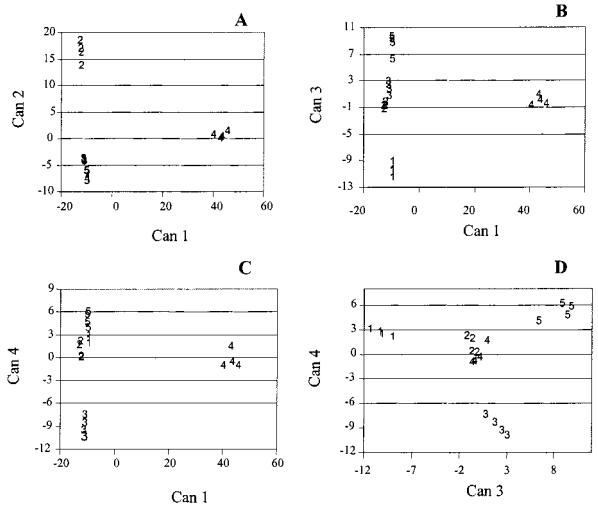

Figure 3. Plots of discriminant canonical functions. Honey codes: (1) orange, (2) eucalyptus, (3) rosemary, (4) lavender, and (5) thyme.

Table 2. Canonical Structure Values

variable	Can 1	Can 2	Can 3	Can 4
ethanol	–0.3935	–0.3552	0.7577	–0.3759
2-butanol	–0.1767	–0.3655	0.7223	0.5579
3-hydroxy-2-butanone	–0.3094	0.9316	0.0259	0.1875
3-methyl-2-buten-1-ol	0.9988	0.0437	0.0076	–0.0164
2,3-butanediol	0.9676	0.1452	0.1064	0.0583
lilac aldehyde	–0.2140	–0.3983	–0.8201	0.2670

the original variables, called canonical structures (**Table 2**), were used in conjunction with plots of discriminant canonical functions to aid interpretation of group differences. The plots obtained are shown in **Figure 3**.

Lavender (number 4) and eucalyptus (number 2) honeys were clearly separated from the other honeys in plot A. Lavender honey was positioned at the extreme positive side of the first canonical function (Can 1). The high positive canonical structure values (**Table 2**) were observed for 3-methyl-2-buten-1-ol and 2,3-butanediol, and according to this, both compounds are important to distinguish lavender honey from the others. Other compounds, like 3,7-dimethyl-1,5,7-octatrien-3-ol and 3,5,5-trimethyl-2-cyclohexen-1-one, may also be used in the characterization of lavender honey. Eucalyptus honey was positioned at the extreme positive side of the second canonical function (Can 2); 3-hydroxy-2-butanone was the characteristic variable of this honey. The high content of 3-hydroxy-2-butanone is in agreement with the results obtained by D'Arcy (*9*) in unmethylated eucalyptus Australian honeys.

Plots B and D allow the differentiation of thyme (number 5) and orange (number 1) honeys from the others, considering the third discriminant canonical function (Can 3). As a result from the canonical structure values of function 3, orange honey was defined by the variable corresponding to lilac aldehyde and thyme honey by the ethanol and 2-butanol variables.

Finally, rosemary honey (number 3) could be distinguished from the other honeys, in plots C and D, by the discriminant canonical function 4 (Can 4), which shows high negative values for ethanol in this honey type. Although, thyme also has a high ethanol content, these honey types can be differentiated by the 2-butanol content. Nevertheless, other markers are necessary to better characterize these honeys.

The five honeys studied were correctly classified by using these canonical functions. In addition, a Jack-knifed classifica-

tion matrix (*19*) was used as a special case of the general cross-validation, and again, all of the honeys were correctly classified. Finally, eight samples of orange honey from apiaries placed in a citrus zone were considered. These honeys were classified using the canonical functions obtained above, and all of the samples were correctly classified as orange honeys.

The headspace SPME−GC analysis of different types of Spanish honeys described above allows the identification and quantification of a wide range of volatile components of honey. The best results were obtained with the Carboxen/PDMS fiber. A temperature of 70 °C, 1 h of sample equilibration, and 30 min of SPME sampling were established as adequate conditions for SPME analysis of honey volatiles. A total of 35 compounds were detected, most of them identified by GC−MS and quantified using external standards.

The results obtained show that SPME followed by GC−MS can be successfully used in the analysis of volatile components of honey samples. Differences in the composition of volatiles from various unifloral Spanish honeys were observed, and the comparative analysis of the volatile composition shows that some compounds can be used for the characterization of the floral source. The results obtained so far indicate that this technique may be a useful tool for the authentication of the floral origin of honeys. Further studies including other honey types are however necessary in order to confirm the utility of this technique as an alternative tool for the characterization of honeys.

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