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HS-SPME-GC-MS Analysis of the Volatile Composition of Italian Honey for Its Characterization and Authentication Using the Genetic Algorithm

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Abstract: Honey's chemical and sensory characteristics depend on several factors, including its botanical and geographic origins. The consumers' increasing interest in monofloral honey and honey with a clear indication of geographic origin make these types of honey susceptible to fraud. The aim was to propose an original chemometric approach for honey's botanical and geographic authentication purposes. The volatile fraction of almost 100 Italian honey samples (4 out of which are from Greece) from different regions and botanical origins was characterized using HS-SPME-GC-MS; the obtained data were combined for the first time with a genetic algorithm to provide a model for the simultaneous authentication of the botanical and geographic origins of the honey samples. A total of 212 volatile compounds were tentatively identified; strawberry tree honeys were those with the greatest total content (i.e., 4829.2 ng/g). A greater variability in the VOCs' content was pointed out for botanical than for geographic origin. The genetic algorithm obtained a 100% correct classification for acacia and eucalyptus honeys, while worst results were achieved for honeydew (75%) and wildflower (60%) honeys; concerning geographic authentication, the best results were for Tuscany (92.7%). The original combination of HS-SPME-GC-MS analysis and a genetic algorithm is therefore proposed as a promising tool for honey authentication purposes.

Keywords: geographic origin; botanical origin; metabolomic; volatile compounds; acacia honey; chestnut honey; eucalyptus honey; honeydew honey; wildflower honey

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1. Introduction

Honey and honeydew honey are the products of bees' metabolism from flower nectar and the sweet secretion of living parts of plants, respectively [1]. The botanical and geographical origins are the main factors that influence the properties and characteristics of honey. Concerning botanical origin, the difference between honey and honeydew honey is primary; the classification into monofloral honey (which contains more than 45% pollen from a specific botanical species, with some exceptions) and multifloral honey (which is derived from various botanical species) is increasingly important, especially due to the peculiar sensory characteristics and nutritional properties (one emblematic example is of Manuka honey, whose great popularity is due to its significant health benefits) [2–9]. Furthermore, the geographical origin is an important parameter for the differentiation and valorization of honeys and has become even more important in recent years due to consumers' increasing interest in geographical indications such as Protected Designation of

Origin (PDO) and Protected Geographical Indication (PGI). Declaration of honey origin on the label and quality schemes for PDO/PGI are present in the EU legislation [10,11]. Due to their distinct and appreciated characteristics, and the absence of reliable authentication markers, monofloral and PDO/PGI honey are easily subject to adulteration and fraud [12]; for this reason, it is really important to identify increasingly high-performance authentication methods [2,7,9,13–22].

The composition of honey has been extensively studied to find chemical markers to be used for authentication. Concerning authentication of the botanical origin, an official method does exist (i.e., the melissopalynological analysis), but it has some limitations such as the necessity of expert analysts and of sufficient amounts of pollen grain in the honey sample (e.g., the filtered honey cannot be analyzed) [9,23,24]. For these reasons, instrumental analysis is recently the focus in research focused on honey authenticity [9]. Many studies have focused on VOCs composition due to their strong relationship with aroma, which is a very important property in honeys. In food science and technology, the importance of volatile organic compounds (VOCs) in the characterization and determination of quality is undisputed [25–27]. In some foodstuffs, VOCs are very important due to their relationship with sensory characteristics (and consequently with consumer acceptability) and, in some cases, with legal parameters (e.g., extra virgin olive oil and wines with geographical indications) [28–30].

In the last decade, the techniques used for the determination of VOCs in foodstuffs, and so in honeys, have evolved. Currently, headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS) is commonly used for the determination of VOCs in honeys. HS-SPME is selected due to its solvent-free extraction, time-saving isolation capacity in complex matrices, sensitivity and versatility (especially when fibers with multi-coating are used), GC for its high separation ability of molecules in complex biological systems and MS for its great sensitivity, resolution and selectivity [7,9,29,31–33].

More than 600 VOCs, including alcohols, aldehydes, ketones, carboxylic acids, esters and lactones, terpenes, hydrocarbons and benzene derivates, have been identified in honey samples [9,13,15,34–38]. Correlations between some specific VOCs or groups of VOCs, and the botanical and/or geographical origins of honeys, have been proposed [7,9,13,15]. Several chemometric assays have been used for honey authentication based on VOCs' content including, for example, a combination of multivariate analysis of variance (MANOVA), stepwise linear discriminant analysis (SLDA) and *k*-nearest neighbor (*k*-NN), a combination of orthogonal partial least square discriminant analysis (PLS-DA) and principal component analysis (PCA), hierarchical cluster analysis (HCA), analysis of variance (ANOVA), ascending hierarchical classification (AHC), principal component regression (PCR), linear discriminant analysis (LDA) and cluster analysis (CA) [7,9,39–47]. Good results have been obtained in the botanical and geographic authentication of honey, but contradictory results are sometimes published in different articles due to a non-correct use of statistical approaches and multivariate analysis (for example, a good LDA-based model must be combined with a suitable stepwise algorithm for the selection of variables), but sometimes also poor analytical data (in this case, not even a very powerful statistical tool can compensate for them) [7], and therefore, further explorations are needed combining reliable analytical data with suitable statistical tools. The application of increasingly high-performance and powerful chemometric assays, such as the genetic algorithm (GA), is a goal. The GA is an algorithm inspired by biological evolution theory and natural selection [48]; it seeks solutions represented by those combinations of variables that best suit sample classification. It was sometimes applied on different analysis outputs for honey authentication purposes [49–52]. However, to the authors' knowledge, GA was never applied to honey's VOC profile to select suitable variables to be used for developing a chemometric approach for authentication of the botanical and geographic origins of honey.

The aim of this study was to propose a combination of HS-SPME-GC-MS analysis of VOCs and a genetic algorithm to simultaneously authenticate the botanical and geographical origins of Italian honey and honeydew honey. Ninety-eight honey samples from different Italian regions (4 were from Greece) and floral origins were collected and a multivariate technique such as LDA was used as the fitness function of the genetic algorithm.

2. Materials and Methods

2.1. Chemicals

4-Methyl-2-pentanol, trimethylacetaldehyde, ethyl acetate d_8, toluene d_8, 1-butanol d_{10}, 3-octanone, 6-chloro-2-hexanone, hexanoic acid d_{11} and 3,4-dimethylphenol, and the linear alkanes (C7–C30) mixture in hexane were purchased from Merck (Saint Louis, MO, USA). The latter mixture was used to calculate the linear retention indices of the identified molecules. The inert gasses, such as helium and nitrogen (purity: 99.999%), were purchased by the SOL company (Monza, Italy).

2.2. Samples

A total of 98 honey samples from the 2017 production year were collected. They were supplied by Italian honey producers, with the exception of 4 Greek samples, and were stored at 20 °C under dark conditions until the analyses were carried out. The 4 Greek samples were of course not considered in the study of the geographic origins in terms of Italian regions, but they were kept for improving the dataset with more samples of some botanical origins. Apart from some preliminary trials, carried out using a couple of samples from 2 weeks before, all samples were analyzed at the same time. Their distribution in terms of botanical and geographic origins is summarized in Table 1. They included varieties and origins with at least 5 samples (i.e., the varieties honeydew, wildflower (also known as a multiflower), chestnut, acacia and eucalyptus, and the origins Tuscany, Trentino-Alto Adige and Veneto) and varieties and origins with no more than 4 samples (see Table 1). Concerning varieties, this sample set was representative of the distribution of the main varieties present in the Italian market. All collected samples were analyzed, and the varieties and origins with at least five samples were used for statistical evaluations.

Table 1. Distribution of the honey samples collected in terms of botanical and geographic origins.

Origin	Total	Tuscany	Trentino-Alto Adige	Veneto	Greece	Emilia Romagna	Sicily	Sardinia	Calabria	Lombardia	Piemonte
Honeydew	**19**	**13**				**1**	**2**		**1**	**1**	**1**
Wildflower	**15**	**9**	**2**	**1**	**2**	**1**					
Chestnut	**14**	**12**	**1**	**1**							
Acacia	**12**	**10**	**1**			**1**					
Eucalyptus	**5**		**2**	**2**				**1**			
French honeysuckle	3	1	2								
Ivy	3	2	1								
Linden	3	2	1								
Clover	2	1	1								
Coriander	2	1	1								
Heather	2	1	1								
Orange tree	2		1	1							
Strawberry tree	2		1		1						
Sunflowers	2	1	1								
Alianthus	1	1									
Alfalfa	1		1								
Alps flower	1		1								
Apple	1		1								
Bitter	1				1						
Fir	1		1								
Forest honey	1		1								
Lavander	1		1								
Marruca	1		1								
Paradise tree	1		1								
Sweet clover	1	1									
Thyme	1		1								
	98	55	25	5	4	3	2	1	1	1	1

2.3. HS-SPME-GC-MS Analysis of Volatile Organic Compounds

Volatile organic compounds (VOCs) were analyzed using HS-SPME-GC-MS. After preliminary trials aimed at optimizing sample and internal standard amounts, time and temperature of exposure of the fiber in the vial headspace, the conditions of the HS-SPME pre-concentration step were set as follows: Into a 20 mL screw cap vial fitted with a PTFE/silicone septum, 1 g of honey sample was dissolved in 5 g of water, and then 2 g of NaCl and 25 µL of internal standard solution (i.e., 4-methyl-2-pentanol 5 mg/L) were added. The vial was let to equilibrate for 5 min at 60 °C, then a 2 cm DVB/CAR/PDMS SPME fiber (Supelco, Bellefonte, PA, USA) was exposed to the vial headspace for 5 min at 60 °C under orbital shaking (500 rpm). The adsorbed VOCs were then immediately desorbed in a GC injection port at 260 °C for 1.7 min in splitless mode. The GC system was a 7890a GC system (Agilent Technologies, Santa Clara, CA, USA). The desorbed VOCs were separated using a DB InnoWAX column (0.4 µm × 0.2 mm × 50 m) while a quadrupole Mass Spectrometer Detector 5975c MSD (Agilent Technologies, Palo Alto, CA, USA) was used for their detection in EI mode at 70 eV. The oven temperature varied as follows: Starting temperature was 40 °C, stayed so for 1 min, and then raised at 5 °C/min up to 220 °C, and then at 10 °C/min up to 260 °C with a final stay at 260 °C for 4 min. Helium at 1.2 mL/min was the carrier gas. The working range of the mass spectrometer was *m/z* 29–350.

Tentative identification of VOCs was carried out for each peak, comparing the mass spectrum with that present in the mass spectra database NIST08 (https://webbook.nist.gov/chemistry/name-ser/, latest access 27 August 2024; minimum matching factor of 80%) and confirming the identification by comparison of the linear retention index calculated after the injection of the mixture of linear alkanes (C7–C30) in the same analytical conditions of samples according to the generalized equation [53], with the one present in the literature [54].

For quantitative purposes, quantifier and qualifier ions were selected for each peak, which allowed us to achieve suitable peak separation even when partial coelution occurred. Quantitative data were obtained in terms of the relative concentration of each identified VOC according to the following formula:

$$\left[VOC\left(\frac{\mathrm{ng}}{\mathrm{g}}\right)\right] = \left.\frac{A_{VOC}}{A_{ISTD}} \times \frac{m_{ISTD}}{m_{sample}}\right)$$

with A_{VOC} as quantifier peak area of the VOC, A_{ISTD} as quantifier peak area of the internal standard (4-methyl-2-pentanol), m_{ISTD} and m_{sample} as amounts of internal standard and of sample into the vial, respectively. A response factor equal to 1 was assumed for all VOCs to be quantitated, according to the previous literature [55].

2.4. Statistical Analysis

A matrix was created with relative quantitative data of 212 VOCs obtained by HS-SPME-GC-MS analysis. In the matrix rows there were the samples, while in the matrix columns there were the data in ng/g of each volatile molecule.

For the first characterization of the samples, the sum of VOCs belonging to different chemical classes was calculated for each sample, and average and medium contents of each class were calculated for each botanical and geographic origin of the honey samples. After application of the Shapiro–Wilk test and verification that most of the distribution were not normal, the median values were calculated and the nonparametric Kruskal–Wallis test was applied to the origins (botanic and geographic, separately) with at least 5 samples to assess which chemical classes were capable to differentiate them at $p < 0.05$. The Dunn's test was also applied, so as to assess, for each chemical class, the significance of the differences between each origin.

Then, a chemometric approach for the botanic and geographic authentication of the honey samples was developed based on a genetic algorithm (GA) to find the best combination of VOCs that, when used to run a linear discriminant analysis as the fitness function, allowed us to achieve such an aim. The adopted GA (that was run independently for botanical and geographic authentication) is represented by the flowchart in the scheme in Figure 1.

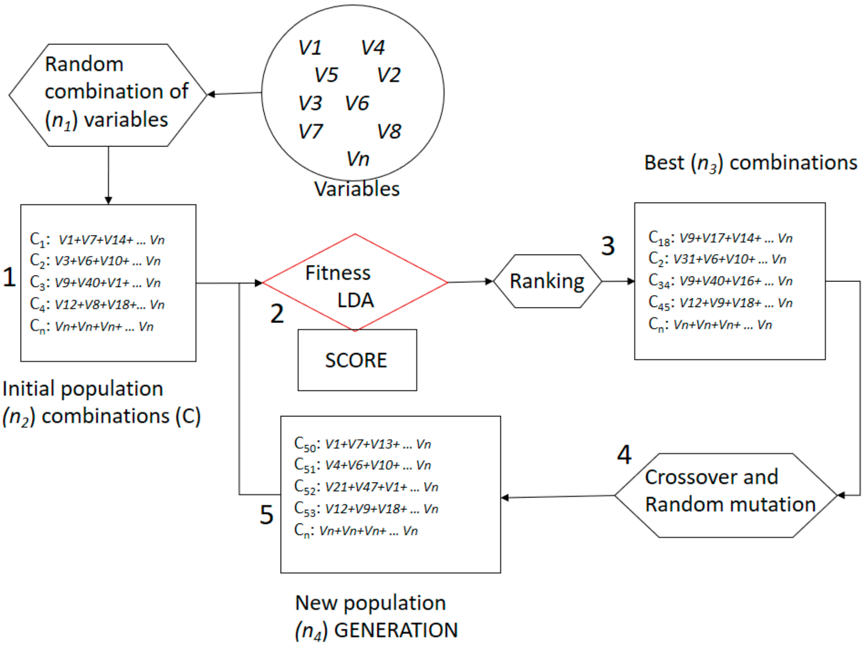

Figure 1. Scheme of the genetic algorithm employed for authentication of the geographic and botanical origins of the honey samples.

In this study, the variables were the VOCs' relative concentrations, while the fitness function was a linear discriminant analysis (LDA), which is the calculation of the best discriminant function capable to classify honey samples in terms of botanic or geographic origin based on the best combination of VOCs. The "score" used in the GA was given by the error rate (ER), which is the percentage error obtained during samples classification via leave-one-out cross-validation (LOO-CV). The score is calculated as

$$\text{score} = 100 - \text{ER}$$

representing the percentage of correct classification.

The parameters n1, n2, n3 and n4 are the number of variables (i.e., the VOCs), the number of initial combinations, the number of the best combinations selected for the "crossover" and the number of generations, respectively.

Since in this research we excluded from the models the origins with less than 5 samples, and taking into account that, when linear discriminant analysis is used as the pattern recognition technique as in this study, the number of variables must not be greater than the lowest number of samples belonging to a single class (i.e., 5 for the botanical origin "eucalyptus", 5 for the geographic origin "Veneto") [56], the number of variables selected was 5.

In order to evaluate the reproducibility of the approach, the genetic algorithm was run two times both for the botanic and geographic authentications.

3. Results and Discussion

From a preliminary qualitative HS-SPME-GC-MS analysis of a few honey samples from the 2016 production year, a high number of volatile compounds was pointed out, resulting in agreement with the previous literature [15,55]. Two further findings emerged from this analysis: (i) the intensity of the peaks present in the chromatograms was quite low, suggesting that the identified VOCs were present in low amounts; (ii) clear differences in the volatile profile of honey from different botanic origins were pointed out. Both findings were supported by the literature [9,55,57]. Starting from these results, we analyzed the collected honey samples (Table 1) with the aims of (i) a qualitative and semi-quantitative characterization of the honey samples of different geographic and botanical origins, and (ii) proposing a new combination of chemical and statistical approaches (based on HS-SPME-GC-MS analysis of VOCs and the genetic algorithm (GA)) for the authentication of the geographic and botanical origins of Italian honey samples.

The first step towards this goal was defining a suitable internal standard to be added in a suitable amount in the headspace vial for peaks area normalization. The internal standard had to be a volatile molecule absent in the honey samples, falling in a zone of the chromatogram so as to not interfere with the analytes and to be added at a concentration suitable to give signals at similar intensities of that of the analytes. To this aim, after having selected a group of molecules absent in the honey samples' chromatographic profiles from the preliminary analysis, an EtOH solution of these molecules was prepared and added to a honey sample in the vial at a concentration of 10 mg/L. The honey sample used was constituted by a mixture of equal amounts of three honey samples from 2016: a wildflower, a chestnut and an acacia honey sample. The analysis of such a sample highlighted that (i) the concentration of 10 mg/L was (as expected) too high; (ii) the internal standard 4-methyl-2-pentanol was the one that fell in a freer area of the chromatogram than the other internal standards; and (iii) some ethyl esters such as ethyl octanoate and ethyl nonanoate were present in the chromatogram. These molecules were absent when the samples were analyzed in the absence of the ethanolic solution of the internal standard during the preliminary analysis; therefore, their presence was due to the reaction of carboxylic acids present in the honey sample with the excess of ethanol. Therefore, in the next steps, we focused the attention on 4-methyl-2-pentanol, strongly diluting the solution with water to avoiding the formation of ethyl esters during analysis due to the presence of ethanol. After several attempts, the final solution had a concentration of 4-methyl-2-pentanol after adding 25 µL to the sample vial, and the internal standard concentration was 0.025 mg/L (i.e., 25 µg/L). In this way, the peak of the internal standard 4-methyl-2-pentanol was of the same order of magnitude of many of the peaks present in the honey samples (Figure 2), and peaks relating to the presence of ethyl esters were no longer detected.

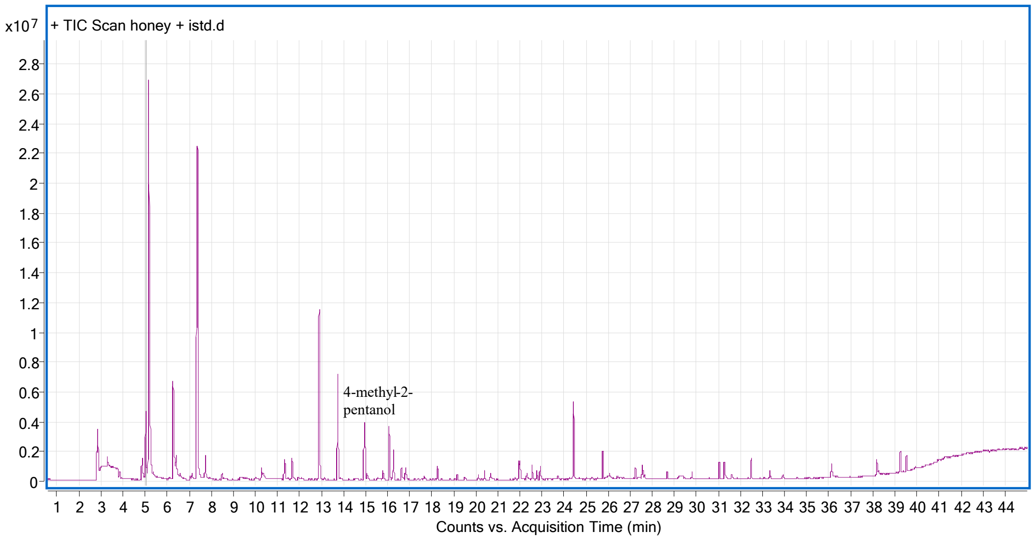

Figure 2. Total ion current chromatogram of a mixed honey sample (consisting of a mixture of a wildflower, a chestnut and an acacia honey sample) in the presence of the internal standard 4-methyl-2-pentanol at a concentration of 25 µg/L.

Therefore, we proceeded to analyze all the collected honey samples in the presence of that amount of 4-methyl-2-pentanol and calculate the relative concentration of the identified compounds using such an internal standard.

3.1. Characterization of the Volatile Fraction of Honey by HS-SPME-GC-MS

Honey's VOCs mainly originate from plant nectar, and therefore, the volatile profile of honey surely depends on the botanical origin [9], but geographic origin also affects honey's volatile profile [15]. When describing the volatile profile of honey or using such a profile for purposes such as authentication, it must be kept into account that it is very complex since bees visit more than one plant species; nevertheless, the literature data have showed specific features for honey of different botanic (and geographic) origins [15].

In the samples of this research, collected in Italy in the 2017, it was possible to identify up to 212 chromatographic peaks, which were tentatively identified as described above. Among them there were 25 alcohols, 5 aromatic alcohols, 14 aldehydes, 15 aromatic aldehydes, 22 ketones, 6 aromatic ketones, 12 benzene derivatives, 14 carboxylic acids, 3 chloro derivatives, 13 esters, 10 furanes, 8 hydrocarbon compounds, 41 monoterpenes, 5 nitriles, 5 volatile phenols and 14 other compounds (Table 2). The presence of chloro derivatives (i.e., dichloromethane (6.34–34.29 ng/g), chlorobenzene (0.36–1.34 ng/g), 1-chloro octane (0.02–2.11 ng/g)), even if in quite low amounts, might suggest some contamination from pesticides or solvents, which could be a topic for future research.

Table 2. List of the volatile organic compounds detected in honey samples via HS-SPME-GC-MS. The molecules whose identification was not confirmed by linear retention index are in italic.

Compound Name	ri calc	ri rif	Quantifier Ion	Qualifier Ion	Chemical Class
tert-butanol	899	900	59	31	alcohol
2-methyl-2-butanol	1008	1000	73	59	alcohol
2-methyl-3-buten-2-ol	1032	1031	71	59	alcohol
2-methyl-1-propanol	1082	1085	74	73	alcohol
2-methyl-2-pentanol	*1087*	-	*87*	*59*	*alcohol*
pentan-3-ol	1128	1108	59	41	alcohol
butanol	1135	1132	56	55	alcohol
2-methyl-3-pentanol	1140	1121	73	59	alcohol
2-methyl-1-butanol + 3-methyl-1-butanol	1196	1206	70	41	alcohol
3-methyl-3-buten-1-ol	1242	1244	86	68	alcohol
2-heptanol	1310	1301	45	55	alcohol
2- + 3-methyl-2-buten-1-ol	1313	1311/1313	71	86	alcohol
3-methyl-1-pentanol	1320	1328	69	56	alcohol
1-hexanol	1345	1344	56	69	alcohol
2,4-dimethylpentan-3-ol	*1372*	-	*73*	*55*	*alcohol*
2,4,4-trimethyl-cyclopentanol	*1377*	-	*95*	*85*	*alcohol*
(*Z*)-3-hexenol	1379	1384	67	82	alcohol
octan-3-ol	1385	1383	101	83	alcohol
1-octen-3-ol	1442	1442	57	72	alcohol
6-methyl-5-hepten-2-ol	1454	1466	128	95	alcohol
2-ethyl-1-hexanol	1482	1481	57	98	alcohol
1-octanol	1552	1552	84	70	alcohol
1-nonanol	1652	1654	56	57	alcohol
4-isopropyl cyclohexanol	1681	1683	124	81	alcohol
decanol	1754	1766	112	97	alcohol
butanal	883	875	72	57	aldehyde
2-methyl-butanal	917	916	57	41	aldehyde
3-methyl-butanal	921	918	44	71	aldehyde
pentanal	984	984	57	44	aldehyde
3-methyl-pentanal	*1035*	-	*56*	*57*	*aldehyde*
hexanal	1076	1076	56	82	aldehyde
2-methyl-(*E*)-2-butenal	1090	1088	84	55	aldehyde
heptanal	1181	1181	70	96	aldehyde
3-methyl-2-butenal	1200	1200	84	55	aldehyde
(*E*)-2-hexenal	1227	1220	98	83	aldehyde
octanal	1292	1293	84	100	aldehyde
nonanal	1399	1398	98	82	aldehyde

Table 2. *Cont.*

Compound Name	ri calc	ri rif	Quantifier Ion	Qualifier Ion	Chemical Class
(*E*)-2-octenal	1439	1437	70	83	aldehyde
decanal	1505	1505	112	82	aldehyde
α-methyl-benzenemethanol	1812	1818	122	107	aromatic alcohol
p-cymen-8-ol	1848	1850	135	132	aromatic alcohol
benzyl alcohol	1876	1876	108	107	aromatic alcohol
phenylethyl alcohol	1915	1920	122	91	aromatic alcohol
p-cymen-7-ol	*2143*	*2090*	*135*	*119*	*aromatic alcohol*
(E)-(3,3-dimethylcyclohexylidene)-acetaldehyde	*1226*	-	*109*	*152*	*aromatic aldehyde*
cyclohexanecarboxaldehyde	*1281*	-	*83*	*112*	*aromatic aldehyde*
furfural	1467	1465	96	95	aromatic aldehyde
benzaldehyde	1539	1537	106	105	aromatic aldehyde
5-methyl-2-furancarboxaldehyde	1581	1580	110	109	aromatic aldehyde
4-methyl benzaldehyde	1642	1654	119	120	aromatic aldehyde
benzeneacetaldehyde	1657	1652	120	91	aromatic aldehyde
3-methyl-benzaldehyde	*1668*	*1624*	*119*	*91*	*aromatic aldehyde*
2-hydroxybenzaldehyde (salicilaldehyde)	1693	1674	122	121	aromatic aldehyde
p-isopropylbenzaldehyde	1803	1789	148	133	aromatic aldehyde
2,5-furandicarboxyaldehyde	1985	1967	124	123	aromatic aldehyde
methoxy benzaldehyde	*2066*	-	*135*	*136*	*aromatic aldehyde*
(E)-cinnamaldehyde	*2084*	*2025*	*131*	*103*	*aromatic aldehyde*
dimethoxy-benzaldehyde	*2493*	-	*166*	*151*	*aromatic aldehyde*
trimethoxy-benzaldehyde	*2623*	-	*196*	*181*	*aromatic aldehyde*
1-(2-furanyl)-ethanone	1512	1511	95	110	aromatic ketone
acetophenone	1667	1669	105	77	aromatic ketone
1-(4-methylphenyl)-ethanone	*1794*	*1752*	*134*	*119*	*aromatic ketone*
1-(1a,2,3,5,6a,6b-hexahydro-3,3,6a-trimethyloxireno[g]benzofuran-5-yl)-ethanone isomer 1	*1812*	-	*179*	*95*	*aromatic ketone*
1-(1a,2,3,5,6a,6b-hexahydro-3,3,6a-trimethyloxireno[g]benzofuran-5-yl)-ethanone isomer 2	*1858*	-	*179*	*95*	*aromatic ketone*
1-(2-aminophenyl)-ethanone	2286	2270	120	92	aromatic ketone
toluene	1041	1041	91	92	benzene derivative
ethylbenzene	1121	1120	91	106	benzene derivative
(1-methylethyl)-benzene	1172	1177	105	120	benzene derivative
styrene	1262	1262	104	78	benzene derivative
p-cymene	1276	1276	119	134	benzene derivative
α-methylstyrene	1338	1326	118	103	benzene derivative
anisole	1349	1354	108	78	benzene derivative
p-cymenene	1446	1438	117	132	benzene derivative
α-ionene A	*1463*	-	*159*	*174*	*benzene derivative*
α-ionene B	*1497*	-	*159*	*174*	*benzene derivative*
1-sec-butyl-4-methylbenzene	*1755*	-	*148*	*119*	*benzene derivative*
1-methoxy-4-propyl-benzene	*2113*	-	*121*	*150*	*benzene derivative*
acetic acid	1447	1447	60	45	carboxylic acid
4-methyl-2-oxovaleric acid	*1454*	-	*85*	*57*	*carboxylic acid*
2-methyl-propanoic acid	1562	1564	88	73	carboxylic acid
pivalic acid	*1575*	*1527*	*57*	*102*	*carboxylic acid*
3-methyl-butanoic acid	1664	1667	60	87	carboxylic acid
2-methyl-butanoic acid	1664	1652	74	87	carboxylic acid
3-methyl-pentanoic acid	1785	1780	60	87	carboxylic acid
hexanoic acid	1837	1834	60	73	carboxylic acid
2-ethyl-hexanoic acid	1942	1950	88	116	carboxylic acid
heptanoic acid	1947	1953	73	101	carboxylic acid
octanoic acid	2070	2072	73	101	carboxylic acid
nonanoic acid	2213	2194	115	129	carboxylic acid

Table 2. *Cont.*

Compound Name	ri calc	ri rif	Quantifier Ion	Qualifier Ion	Chemical Class
decanoic acid	2333	2316	73	129	carboxylic acid
geranic acid	*2424*	*2356*	*100*	*123*	*carboxylic acid*
methylene chloride	929	937	49	84	chloride
chlorobenzene	1217	1220	114	112	chloride
1-chloro-octane	1245	1257	83	91	chloride
ethyl acetate	890	891	61	70	ester
ethyl propanoate	959	957	57	102	ester
ethyl butanoate	1034	1039	88	71	ester
t-butyl-3-hydroxybutyrate	*1320*	-	*87*	*57*	*ester*
ethyl 2-hydroxy-propanoate	1340	1341	45	75	ester
ethyl decanoate	1638	1637	155	88	ester
diethyl butanedioate	1671	1661	101	129	ester
ethyl benzoate	1679	1675	150	105	ester
ethyl benzeneacetate	1793	1793	164	91	ester
methyl salicylate	1796	1779	152	120	ester
3-hydroxy-2,2,4-trimethylpentyl 2-methyl propanoate	*1871*	-	*89*	*71*	*ester*
2,2,4-trimethyl-1,3-pentanediol diisobutyrate	*1884*	-	*71*	*43*	*ester*
methyl anthranilate	2221	2198	119	151	ester
tetrahydro-2,2,5,5-tetramethyl-furan	*872*	-	*95*	*113*	*furan*
2-methylfuran	878	871	82	81	furan
3-methylfuran	898	901	82	81	furan
2,5-dimethylfuran	955	946	96	81	furan
2-ethyl-5-methylfuran	1030	1013	95	110	furan
2-pentylfuran	1227	1228	138	81	furan
(2R,5S)-2-methyl-5-(prop-1-en-2-yl)-2-vinyltetrahydrofuran	1243	1199	137	110	furan
furan unidentified	*1563*	-	*95*	*110*	*furan*
3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran	*1621*	-	*137*	*152*	*furan*
2-furanmethanol	1650	1650	81	53	furan
1-heptene	756	736	56	98	hydrocarbon
octane	806	800	43	85	hydrocarbon
1-octene	854	837	112	43	hydrocarbon
(*Z*)-2-octene	867	866	112	43	hydrocarbon
nonane	899	900	85	71	hydrocarbon
1-nonene	941	931	56	69	hydrocarbon
decane	999	1000	142	57	hydrocarbon
1,2-dimethyl-cyclopentane	*1793*	-	*98*	*83*	*hydrocarbon*
butan-2-one	904	903	72	43	ketone
2,3-butanedione	978	976	86	43	ketone
2-pentanone	982	980	71	58	ketone
2-methyl-3-pentanone	1002	1003	100	71	ketone
hexan-2-one	1011	1024	100	58	ketone
4-methylpent-3-en-2-one	1128	1131	83	98	ketone
2,3-heptadienone	*1138*	-	*85*	*57*	*ketone*
2-heptanone	1179	1183	114	58	ketone
2,4,4-trimethyl-cyclopentanone	*1225*	-	*126*	*83*	*ketone*
3-hydroxy-2-butanone	1293	1292	88	45	ketone
4-butoxy-2-butanone	*1304*	-	*71*	*72*	*ketone*
6-methylhept-5-en-2-one	1340	1343	108	126	ketone
2-hydroxy-3-pentanone	1364	1361	59	57	ketone
nonan-2-one	1393	1393	142	71	ketone
(E)-1-(3,5,5-trimethyl-2-cyclohexen-1-ylidene)-2-propanone	*1410*	-	*163*	*145*	*ketone*
β-isophorone	1421	1429	138	96	ketone
α-isophorone	1617	1621	138	82	ketone

Table 2. *Cont.*

Compound Name	ri calc	ri rif	Quantifier Ion	Qualifier Ion	Chemical Class
2-hydroxy-isophorone	1676	1675	154	139	ketone
4-oxoisophorone	1708	1710	152	96	ketone
2-(1-methylethylidene)-cyclohexanone	*1808*	-	*138*	*123*	*ketone*
β-damascenone	1839	1827	190	121	ketone
2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl-1H-2-indenone	*1844*	-	*192*	*177*	*ketone*
α-pinene	1025	1024	93	136	monoterpene
β-pinene	1144	1124	93	69	monoterpene
α-phellandrene	1160	1163	136	77	monoterpene
α-terpinene	1178	1174	93	121	monoterpene
2,3-dehydro-1,8-cineole	1190	1195	109	79	monoterpene
limonene	1199	1195	68	93	monoterpene
β-phellandrene	1212	1214	136	93	monoterpene
eucalyptol	1213	1213	154	139	monoterpene
(Z,Z)-cosmene	*1217*	-	*119*	*134*	*monoterpene*
γ-terpinene	1247	1249	136	121	monoterpene
(Z,E)-cosmene	*1255*	-	*119*	*134*	*monoterpene*
2-bornene	*1399*	-	*121*	*93*	*monoterpene*
cis-linaloloxide (furanoid)	1448	1420	111	94	monoterpene
trans-linaloloxide (furanoid)	1477	1478	111	94	monoterpene
3,9-epoxy-p-mentha-3,8-diene	1497	1487	108	150	monoterpene
3,9-epoxy-p-mentha-1,8(10)-diene A	*1534*	-	*135*	*150*	*monoterpene*
linalol	1540	1540	121	93	monoterpene
lilac aldehyde isomer 1	1552	1556	111	153	monoterpene
lilac aldehyde isomer 2	1568	1564	111	153	monoterpene
lilac aldehyde isomer 4	1575	1588	111	153	monoterpene
3,9-epoxy-p-mentha-1,8(10)-diene B	*1580*	-	*135*	*150*	*monoterpene*
lilac aldehyde isomer 3	1600	1588	111	153	monoterpene
hotrienol	1603	1589	71	82	monoterpene
terpinen-4-ol	1610	1612	154	111	monoterpene
1R,4R-*p*-mentha-2,8-dien-1-ol	1631	1640	134	137	monoterpene
α,4-dimethyl-3-cyclohexene-1-acetaldehyde A	1635	1620	94	79	monoterpene
α,4-dimethyl-3-cyclohexene-1-acetaldehyde B	1638	1620	94	79	monoterpene
(1R)-(-)-myrtenal	*1656*	-	*107*	*79*	*monoterpene*
3,9-epoxy-p-mentha-1,8(10)-diene	*1673*	-	*135*	*150*	*monoterpene*
β-citral	1691	1687	69	84	monoterpene
α,4-dimethyl-3-cyclohexene-1-acetaldehyde C	*1696*	*1620*	*94*	*79*	*monoterpene*
α-terpineol	1700	1700	136	59	monoterpene
endo-borneol	1710	1704	95	110	monoterpene
p-mentha-1,5-dien-8-ol	1727	1725	119	94	monoterpene
lilac alcohol A	1733	1736	111	155	monoterpene
phellandral	*1749*	*1700*	*109*	*152*	*monoterpene*
epoxylinalool	*1768*	*1423*	*143*	*127*	*monoterpene*
lilac alcohol C	1791	1796	111	155	monoterpene
2-caren-10-al	*1819*	-	*150*	*121*	*monoterpene*
2,6-dimethyl-3,7-octadiene-2,6-diol	1928	1914	82	71	monoterpene
6-camphenol	*2167*	-	*108*	*93*	*monoterpene*
isobutyronitrile	1008	993	68	42	nitrile
2-methyl-butanenitrile	1083	1094	54	55	nitrile
3-methyl-butanenitrile	1121	1134	68	43	nitrile
nitrile undefined	*1237*	-	*57*	*41*	*nitrile*
benzyl nitrile	1945	1927	117	116	nitrile
dimethyl sulfide	783	760	62	47	other
2,4,5-trimethyl-1,3-dioxolane	*944*	-	*101*	*73*	*other*
2,3-dimethyl-2-norbornene	989	984	94	122	other
dimethyl disulfide	1070	1068	94	79	other
2,5-dimethylpirazine	1340	1329	108	42	other

Table 2. *Cont.*

Compound Name	ri calc	ri rif	Quantifier Ion	Qualifier Ion	Chemical Class
tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2H-pyran	1359	1363	139	154	other
2,3,5-trimethylpyrazine	1411	1411	122	81	other
tetrahydro-2,5-dimethyl-2H-pyranmethanol	*1415*	-	*113*	*43*	*other*
3,5,6,8a-tetrahydro-2,5,5,8a-tetramethyl-trans-2H-1-benzopyran (*trans*-edulan)	1623	1620	177	133	other
5-ethenyldihydro-5-methyl-2(3H)-furanone	1689	1689	111	99	other
methoxy-phenyl-oxime	*1741*	-	*133*	*151*	*other*
1,1,5-trimethyl-1, 2-dihydronaphthalene	*1769*	-	*157*	*172*	*other*
4-methyl-1,2-dihydronaphthalene	*1792*	-	*129*	*144*	*other*
1-methyl-4-(1-methylethenyl)-1,2-cyclohexanediol	2352	2325	108	152	other
3-methyl phenol	2103	2099	108	107	volatile phenol
thymol	2216	2189	135	150	volatile phenol
eugenol	2193	2176	164	103	volatile phenol
carvacrol	2251	2225	135	150	volatile phenol
trimethyl-phenol	*2462*	-	*121*	*136*	*volatile phenol*

RI_{CAL}: non-isothermal Kovats retention indices from temperature programming, using the definition of Van den Dool and Kratz (1963). RI_{REF}: non-isothermal Kovats retention indices from temperature programming from Chemistry WebBook.

For a characterization of honey samples from different origins, the identified VOCs were grouped according to the chemical class, mean and median values were calculated and, using the Kruskal–Wallis test followed by Dunn's test, honey from origins with at least 5 samples were discriminated. The median values of each chemical class for all botanic (A) and geographic origins (B) are reported in Table 3, with letters for significant differences for origins with at least 5 samples. Of course, for those origins with less than 5 samples, the reported data must be considered as preliminary.

Concerning total VOCs, eucalyptus (1192.0 ng/g) and honeydew (830.8 ng/g) samples showed a greater content than acacia honey (529.5 ng/g), and Trentino-Alto Adige (998.5 ng/g) samples showed a greater content than the Tuscan samples (800.1 ng/g). The chemical classes that showed a certain capability of discrimination among the botanic origins were alcohols, benzene derivatives, carboxylic acids, hydrocarbons, aromatic ketones, monoterpenes and volatile phenols; ketones discriminated eucalyptus, while esters and furans discriminated acacia from the other botanic origins (Table 3A). A lower variability occurred in the case of geographic origins: the chemical classes that showed a certain discrimination capability were aldehydes, carboxylic acids, furans, hydrocarbons and monoterpenes (Table 3B).

Table 3. Median ± Median Absolute Deviation values (ng/g) and *p*-values from the Kruskal–Wallis test of different classes of VOCs in: (A) honey samples from different botanical origin; (B) honey samples from different geographical origin. "n°" is the number of samples. For origins with n° = 1 it is not the median but the measurement value. For origins represented by at least 5 samples, different letters in a column indicate significant differences at $p < 0.05$, according to Dunn's test.

A. Botanical Origin	**n°**	**Σ Alcohols**	**Σ Aromatic Alcohols**	**Σ Aldehydes**	**Σ Aromatic Aldehydes**	**Σ Benzene Derivative**	**Σ Carboxylic Acid**	**Σ Chloride**	**Σ Ester**	**Σ Furan**	**Σ Hydrocarbon**	**Σ Ketone**	**Σ Aromatic Ketone**	**Σ Monoterpene**	**Σ Nitrile**	**Σ Volatile Phenol**	**Σ VOCs**
		(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)
Honeydew	19	42.3 ± 14.1 ab	19.7 ± 5.7 a	42.0 ± 10.5 a	157.0 ± 34.8 a	29.5 ± 14.5 ab	116.0 ± 19.5 bc	13.5 ± 3.4 a	9.6 ± 2.7 a	4.3 ± 1.8 b	88.9 ± 39.3 bc	6.0 ± 1.6 a	20.8 ± 12.0 b	47.1 ± 16.6 ab	1.8 ± 0.9 a	6.7 ± 1.2 ab	830.8 ± 126.2 b
Wildflower	15	22.1 ± 7.7 a	53.6 ± 47.3 a	22.3 ± 8.6 a	135.2 ± 67.1 a	70.2 ± 45.1 b	93.0 ± 19.4 ab	11.2 ± 4.6 a	8.2 ± 4.1 a	3.4 ± 2.3 b	23.1 ± 8.0 a	5.5 ± 1.3 a	14.1 ± 9.0 b	66.1 ± 43.4 b	1.7 ± 0.8 a	18.0 ± 10.1 b	703.4 ± 212.5 ab
Chestnut	14	26.3 ± 6.7 ab	17.3 ± 6.2 a	30.8 ± 9.6 a	181.6 ± 47.1 a	36.4 ± 16.3 ab	123.7 ± 15.8 abc	10.9 ± 3.8 a	10.2 ± 2.2 a	3.9 ± 1.5 b	38.0 ± 19.6 ab	4.6 ± 0.7 a	91.2 ± 49.1 c	29.0 ± 9.4 a	2.5 ± 1.0 a	3.2 ± 1.8 a	871.5 ± 128.8 ab
Acacia	12	19.8 ± 5.2 ab	9.6 ± 2.5 a	21.3 ± 5.1 a	103.6 ± 22.4 a	16.3 ± 9.4 a	81.6 ± 11.9 a	15.5 ± 5.2 a	14.1 ± 2.1 b	1.0 ± 0.2 a	13.4 ± 1.4 a	3.7 ± 0.9 a	3.2 ± 0.6 a	44.6 ± 5.3 ab	0.7 ± 0.1 a	2.9 ± 0.8 a	529.5 ± 77.6 a
Eucalyptus	5	64.6 ± 34.7 b	17.2 ± 1.9 a	39.0 ± 8.7 a	78.5 ± 17.2 a	67.9 ± 4.5 b	279.1 ± 48.1 c	20.9 ± 3.2 a	8.9 ± 0.8 a	4.4 ± 0.9 b	271.8 ± 36.3 c	29.1 ± 16.0 b	11.0 ± 2.1 ab	75.9 ± 9.2 b	1.3 ± 0.2 a	36.9 ± 20.8 b	1192.0 ± 58.7 b
Linden	3	25.7 ± 7.5	293.0 ± 18.4	35.1 ± 7.1	561.6 ± 9.3	614.5 ± 94.6	146.0 ± 1.3	31.9 ± 7.7	19.6 ± 4.4	31.3 ± 4.5	15.3 ± 2.0	9.2 ± 1.2	28.3 ± 8.0	540.4 ± 11.9	1.6 ± 0.3	151.2 ± 23.2	2672.5 ± 392.5
Ivy	3	54.3 ± 38.0	34.3 ± 23.9	30.8 ± 15.7	223.6 ± 42.3	36.3 ± 7.6	116.5 ± 3.8	7.7 ± 1.1	13.3 ± 6.2	3.1 ± 0.5	47.7 ± 2.1	42.8 ± 3.3	52.7 ± 2.3	91.5 ± 0.5	56.2 ± 21.4	84.4 ± 35.0	1529.1 ± 698.4
French honeysuckle	3	41.9 ± 4.8	5.9 ± 0.6	30.0 ± 0.3	95.8 ± 1.2	10.6 ± 8.1	90.1 ± 5.9	5.7 ± 4.1	10.2 ± 0.2	0.9 ± 0.1	16.5 ± 6.8	3.1 ± 0.6	6.4 ± 2.3	55.1 ± 16.7	1.2 ± 0.1	2.3 ± 0.5	621.1 ± 10.2
Strawberry tree	2	68.0 ± 46.3	16.9 ± 3.7	30.7 ± 16.3	112.8 ± 75.7	14.0 ± 4.2	168.2 ± 17.1	18.4 ± 6.0	69.4 ± 39.5	77.8 ± 50.2	55.5 ± 39.8	2431.9 ± 567.3	241.8 ± 57.2	66.0 ± 20.8	47.4 ± 43.8	817.0 ± 34.4	4829.2 ± 347.6
Sunflowers	2	39.2 ± 0.6	23.8 ± 16.5	42.4 ± 1.4	145.5 ± 51.4	33.6 ± 20.9	115.0 ± 10.0	17.0 ± 1.6	6.0 ± 1.2	6.3 ± 1.7	34.0 ± 8.7	3.2 ± 0.3	6.8 ± 0.1	166.8 ± 32.0	1.7 ± 0.6	8.5 ± 4.8	787.0 ± 44.9
Coriander	2	87.0 ± 2.5	29.4 ± 15.6	184.4 ± 10.7	456.1 ± 69.1	48.5 ± 9.1	191.5 ± 3.7	16.0 ± 1.0	10.9 ± 0.2	28.2 ± 5.4	167.2 ± 28.6	9.9 ± 0.2	15.5 ± 1.4	1543.8 ± 154.9	4.6 ± 1.2	14.0 ± 0.4	3180.6 ± 239.7
Heather	2	21.2 ± 2.1	17.0 ± 8.3	45.0 ± 12.1	447.8 ± 83.0	267.0 ± 63.8	106.1 ± 6.1	24.3 ± 5.9	9.9 ± 0.7	35.1 ± 15.5	14.4 ± 0.4	28.3 ± 14.4	16.3 ± 0.3	55.7 ± 9.4	1.8 ± 0.9	58.2 ± 38.2	1334.6 ± 216.3
Clover	2	37.6 ± 7.1	6.5 ± 1.1	52.7 ± 3.1	150.6 ± 25.0	14.7 ± 4.2	95.6 ± 11.8	18.8 ± 7.3	8.0 ± 1.7	4.1 ± 2.8	33.4 ± 10.7	4.7 ± 0.7	5.7 ± 0.7	91.3 ± 65.3	3.0 ± 2.1	4.7 ± 2.3	671.2 ± 105.4
Orange Tree	2	22.4 ± 1.1	7.2 ± 1.6	26.3 ± 6.7	81.7 ± 8.5	15.0 ± 0.8	117.3 ± 0.6	21.3 ± 3.1	46.3 ± 0.3	10.3 ± 0.3	31.0 ± 6.5	4.4 ± 0.5	3.4 ± 0.3	377.5 ± 30.3	4.2 ± 3.0	3.6 ± 2.0	941.8 ± 37.5
Bitter	1	40.2	5.6	12.6	56.5	23.0	79.0	20.6	7.4	4.5	47.3	9.2	12.1	77.4	7.2	18.1	624.8
Fir	1	46.9	18.2	35.3	109.5	24.2	84.0	13.0	6.7	5.4	101.9	4.4	6.8	43.3	0.8	5.7	680.7
Marruca	1	25.0	3.0	41.4	103.7	20.2	120.1	21.4	8.3	3.0	125.3	6.2	6.4	337.1	0.9	1.2	998.5
Lavander	1	338.4	11.7	276.6	378.7	22.5	131.8	35.6	21.7	4.9	13.1	4.4	4.1	250.2	1.2	3.1	1681.9
Alps flower	1	24.4	75.2	30.5	267.6	365.7	126.3	12.9	13.1	13.4	35.0	9.3	25.6	263.0	4.0	76.2	1501.3
Forest honey	1	96.6	14.6	53.5	192.2	13.0	147.5	18.1	13.4	6.4	59.9	4.9	8.1	35.5	1.5	5.1	829.9
Alfalfa	1	34.4	44.1	38.3	182.2	52.4	77.2	17.5	11.4	2.0	20.3	3.1	7.8	41.2	1.3	13.7	697.3
Paradise Tree	1	42.3	136.3	52.6	236.9	144.3	179.5	24.4	10.5	11.8	144.8	20.7	16.4	967.8	1.9	43.5	2233.3
Thyme	1	21.8	28.2	33.3	226.0	73.3	88.3	15.9	9.7	4.4	47.6	5.1	6.8	80.6	10.7	21.7	851.7
Apple	1	41.9	46.7	45.7	1017.4	52.0	396.1	13.9	16.9	6.5	20.5	20.1	15.0	175.9	749.7	68.4	2937.2
Ailanthus	1	30.9	23.8	52.2	96.7	45.9	128.1	23.4	10.3	7.1	70.3	12.0	7.9	837.4	10.8	19.6	1544.5
Sweet clover	1	112.5	13.0	26.0	88.5	52.7	125.2	8.8	107.7	2.0	13.5	5.0	6.8	52.4	1.5	17.9	1081.5

B. Geographic origin	**n°**	**Σ alcohols**	**Σ aromatic alcohols**	**Σ aldehydes**	**Σ aromatic aldehydes**	**Σ benzene derivative**	**Σ carboxylic acid**	**Σ chloride**	**Σ ester**	**Σ furan**	**Σ hydrocarbon**	**Σ ketone**	**Σ aromatic ketone**	**Σ monoterpene**	**Σ nitrile**	**Σ volatile phenol**	**Σ VOCs**
		(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)	(ng/g)
Tuscany	55	28.6 ± 12.4 a	14.1 ± 6.9 a	29.2 ± 9.8 a	147.4 ± 64.8 a	32.9 ± 17.6 a	100.4 ± 22.7 a	13.6 ± 5.1 a	10.8 ± 2.9 a	3.4 ± 2.4 a	25.5 ± 12.5 a	5.3 ± 1.9 a	15.3 ± 10.3 a	46.3 ± 16.0 a	1.6 ± 0.9 a	6.6 ± 4.0 a	800.1 ± 253.0 a
Trentino-Alto Adige	25	41.9 ± 17.5 a	25.3 ± 15.8 a	41.4 ± 8.5 b	191.3 ± 87.6 a	52.4 ± 32.2 a	120.3 ± 30.2 b	15.9 ± 3.0 a	10.6 ± 2.7 a	5.8 ± 3.2 b	35.0 ± 20.2 a	5.1 ± 1.9 a	11.0 ± 5.4 a	92.0 ± 50.8 b	1.9 ± 1.2 a	14.4 ± 12.6 a	998.5 ± 367.2 b
Veneto	5	29.9 ± 3.2 a	15.3 ± 6.5 a	36.4 ± 13.8 ab	147.8 ± 44.6 a	34.3 ± 20.1 a	135.2 ± 17.4 b	17.7 ± 3.1 a	9.8 ± 1.6 a	4.2 ± 0.5 ab	98.5 ± 74.0 b	6.4 ± 1.8 a	13.1 ± 1.3 a	175.0 ± 97.2 c	4.0 ± 1.4 a	16.1 ± 0.1 a	996.8 ± 116.8 ab
Greece	4	28.1 ± 12.9	4.0 ± 1.6	14.0 ± 0.9	62.3 ± 13.2	37.0 ± 17.4	100.2 ± 18.3	13.7 ± 2.2	7.7 ± 0.2	3.2 ± 1.5	37.5 ± 12.1	7.9 ± 2.0	8.3 ± 3.8	36.6 ± 14.1	2.9 ± 1.1	14.3 ± 3.9	573.7 ± 62.8
Emilia Romagna	3	35.7 ± 5.0	18.3 ± 4.3	42.0 ± 3.3	154.5 ± 27.3	14.9 ± 1.9	116.3 ±16.4	11.9 ± 5.3	11.3 ± 0.3	1.8 ± 0.6	13.7 ± 12.1	4.5 ± 1.5	32.2 ± 20.0	46.6 ± 3.0	2.7 ± 0.8	5.5 ± 1.8	644.4 ± 32.7
Sicily	2	37.5 ± 15.4	9.5 ± 6.0	44.8 ± 0.1	139.3 ± 41.1	43.1 ± 26.3	196.4 ± 81.6	16.7 ± 6.8	10.2 ± 0.6	2.8 ± 1.4	227.3 ± 104.1	24.2 ± 18.0	36.8 ± 27.1	69.9 ± 44.5	6.5 ± 4.0	13.9 ± 10.4	1072.2 ± 416.9
Piemonte	1	79.5	20.7	42.2	126.6	10.9	185.3	12.4	14.9	12.2	100.5	5.3	6.9	63.7	1.2	8.6	818.6
Calabria	1	50.5	2.9	31.4	105.4	10.0	121.7	20.5	12.3	1.8	138.0	4.5	8.8	15.8	1.6	2.3	733.6
Lombardia	1	98.8	21.8	46.6	127.2	13.5	238.0	17.3	15.4	16.9	134.1	6.0	6.9	68.1	1.8	7.4	994.2
Sardinia	1	113.1	15.6	22.2	24.6	66.4	279.1	24.4	8.9	5.0	271.8	29.1	3.7	42.6	1.1	36.9	1133.3

Considering all the honey samples, a greater variability in volatile profiles was surely pointed out for botanic than for geographic origins. Acacia honey was the one with the lowest total VOCs content; in the literature, this type of honey is generally characterized by the presence of benzaldehyde and *cis*-linalool oxide [16,58,59], which in our samples were the first (53.9 ng/g) and the sixth (21.54 ng/g) most abundant VOCs out of the 212; other VOCs reported in the literature are the alcohol 3-methyl-3-buten-1-ol and the aldehyde heptanal, but in our samples they were present in low amounts in the acacia samples and in greater amounts in samples from other botanical origins. Wildflower honey showed a total VOCs content of 703.4 ng/g and was mainly represented by aromatic aldehydes (135.2 ng/g), carboxylic acids (93.0 ng/g) and benzene derivatives (70.2 ng/g). Of course, given the multifloral origin of this type of honey. It is less investigated in the literature than monofloral honeys and it is more difficult to identify the specific VOCs characterizing it. In the samples of this research, hotrienol (58.9 ng/g), benzaldehyde (58.4 ng/g), *p*-cymen-8-ol (53.6 ng/g) and *p*-cymenene (51.5 ng/g) were the most abundant VOCs (values of all VOCs in wildflower honey are reported in Supplementary Table S1), and VOCs such as (*E*)-(3,3-dimethylcyclohexylidene)-acetaldehyde, *p*-cymenene, *p*-cymen-8-ol, ethylbenzene, (1-methelethyl)-benzene, α-methylstyrene seemed to be those most capable of discriminating wildflower honey from the other four main represented honey types of this research. Chestnut honey showed a total VOC content of 871.5 ng/g and was mainly represented by aromatic aldehydes (181.6 ng/g), while aromatic ketones (91.2 ng/g) best discriminate it from the other main honeys, in agreement with the literature [16,60–62]. In fact, although benzaldehyde was the VOC present in the greater amount in this type of honey, it was in much greater amounts in other types of honey samples (87.0 ng/g while ranging 13.0–883.1 ng/g in all samples), in partial agreement with Machado (2020), who reported this molecule as the most that characterizes the chestnut honey [16]. On the contrary, although acetophenone was found in lower amounts (10.7 ng/g), it was in much greater amounts than in the other main honeys and second only in comparison to the less represented ivy honey, in agreement with the previous literature [60]. Eucalyptus honey samples showed the greatest total VOC content (1192.0 ng/g) among the five most represented types of honey, even if some minor types showed about 4.5-fold greater total VOCs content; it was mainly represented by hydrocarbons and carboxylic acids, mainly thanks to the contribute of the hydrocarbon octane (the most abundant VOC with 263.5 ng/g) and the acids nonanoic acid (the second most abundant VOC with 49.5 ng/g), 2-ethyl-hexanoic acid (46.7 ng/g) and 3-methyl-butanoic acid (46.6 ng/g). The prevalence of octane and nonanoic acid is in agreement with the literature [34,63]. At the same time, hydroxyketones such as 3-hydroxy-2-butanone and 2-hydroxy-3-pentanone showed the highest amounts in the eucalyptus samples, in agreement with D'Arcy et al. (1997) [64], while norisoprenoids were present in low amounts, in disagreement with these authors [64]. Honeydew honey showed a total VOCs content of 830.8 ng/g and was mainly represented by aromatic aldehydes (157.0 ng/g) and carboxylic acids (116.0 ng/g); the main aromatic aldehydes were benzaldehyde (69.5 ng/g) and furfural (50.0 ng/g). Among carboxylic acids, 2-methyl butanoic acid (55.6 ng/g) was present in a significantly great concentration in honeydew honey, and honeydew honey also showed quite a high content of acetic acid in comparison to the other types of honey, in agreement with the literature [65].

However, the greater variability in honey samples from different botanical origins was even more evident when the less represented origins were considered. The most obvious example in that sense was provided by strawberry tree honey, which showed the highest total VOC content (i.e., 4829.2 ng/g). Such a honey showed ketones and volatile phenols contents 2–3 and 1–2 orders of magnitude greater than all the other origins, respectively. Despite two being a low number of samples to obtain a conclusion, this finding appears quite robust considering that the sum of ketones in the two strawberry tree honey samples was 2999.3 and 1864.5 ng/g while in all the other 96 samples it ranged from 1.4 to 85.0 ng/g, and that the sum of volatile phenols in the two strawberry tree honey samples was 851.4 and 782.5 ng/g while in all the other 96 samples it ranged from 0.9

to 174.4 ng/g (Supplementary Table S2). In particular, the very high ketones content in strawberry tree honey was due to norisoprenoids such as isophorones (mainly α-isophorone (average content 1897.3 ng/g), followed by 2-hydroxyisophorone (230.5 ng/g), 4-oxoisophorone (177.6 ng/g) with minor amounts of β-isophorone (14.3 ng/g)) and 2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl-1H-2-indenone (61.9 ng/g) (Supplementary Table S3). Isophorones have already been reported in the literature as characteristic VOCs of strawberry tree honey [55,66,67]. Isophorones were also reported as characteristics of VOCs in heather [40] and thyme honeys [68]; our results only partially agreed, in that the isophorones were found in medium amounts in heather honey (10.6 ng/g) and in very low amounts in thyme honey (<1 ng/g). Other types of monofloral honey with great total VOC contents were coriander honey (3180.6 ng/g) and apple honey (2937.2 ng/g). Apple honey was mainly represented by aromatic aldehydes and nitriles, the latter of about two orders of magnitude greater than almost all other honeys. Coriander is mainly represented by monoterpenes (i.e., 1543.8 ng/g, which is half of the total VOCs content), among which α,4-dimethyl-3-cyclohexene-1-acetaldehyde isomers A and B (244.1 ng/g and 212.4 ng/g, respectively), lilac aldehyde isomers 1, 2 and 4 (191.5 ng/g, 163.8 ng/g and 112.9 ng/g, respectively) and linalool (65.5 ng/g) largely prevailed over the other single monoterpenes. These molecules were also largely prevalent in coriander (followed by orange tree honey) in comparison to the other monofloral honey samples (Supplementary Table S4). Lilac aldehydes were reported in the literature as markers of citrus honey [69] and were reported among the most abundant compounds in coriander honey [70,71]); according to such literature studies, data from this research pointed out a great presence of lilac aldehydes in honey from orange tree (a citrus plant) and coriander. Orange tree honey was also by far the richest honey in the ester methyl anthranilate (32.6 ng/g vs. a range of 0–2.3 ng/g of all other honey samples, Supplementary Table S4), together with lilac aldehydes, and methyl anthranilate is reported as a marker of citrus honey [69,72,73], in agreement with our data.

3.2. Botanical and Geographic Authentication of Honey by Using Genetic Algorithm (GA)

To achieve the goal of proposing a new chemical statistical approach able to classify honey samples based on their botanic and geographic origins using the relative concentrations of volatile compounds (VOCs), a genetic algorithm (GA) was employed to find the best combination of VOCs capable of reaching this aim. The genetic algorithm is an optimization technique inspired by natural selection and genetic principles, and since it is usually used to find approximate solutions to complex problems, it perfectly fits with the scope of this research study, given the very complex volatile composition of honey with different botanical and geographic origins [9]. A GA has never been used in combination with the data of volatile compounds for honey authentication purposes before.

During the execution of the GA, a population of solutions to the problem are randomly generated. Each solution leads to the definition of a "chromosome" (combination of variables), in turn made up of several elements called "genes" (variables). A selected "fitness function" evaluates each solution in the population, assigning a "score" so that the solutions with the best "scores" are selected. Then, a crossover procedure generates hybrid solutions (offspring), starting from pairs of the selected solutions (parents), while a random mutation step introduces random changes to the "genes", aimed at preserving diversity within the population. A new generation is created introducing new offspring in the population that replace some of the old ones, and all the whole procedure is re-executed. The obtained solutions are represented by a combination of variables (V1, V2, V3, Vn).

The parameters used for the GA were the following, both for botanical and geographic authentication purposes: number of variables (n1) = 5; number of initial combination (n2) = 150; number of the best combinations selected for the "crossover" (n3) = 50; number of generations = 25, meaning a total of 1400 combination tested. For each combination of variables, the fitness function (i.e., the LDA) gave a confusion matrix and the relevant score. The algorithm gave the best 10 variable combinations (i.e., the 10 variable combinations with the best scores) and the frequency with which each variable was present in the

10 variable combinations. Since the algorithm was random, it was run two times, both for geographic and botanical authentication purposes, in order to confirm the reproducibility and reliability of the data. The obtained results were similar in the two runs, in terms of both most frequent variables and error rates. The most frequent variables were as follows:

- Botanical origin run 1: octane (90%), 1-nonene (90%), 5-ethenyldihydro-5-methyl-2(3H)-furanone (80%).
- Botanical origin run 2: octane (100%), 1-nonene (80%), 5-ethenyldihydro-5-methyl-2(3H)-furanone (80%).
- Geographic origin run 1: butanal (100%), eucalyptol (100%).
- Geographic origin run 2: butanal (100%), eucalyptol (90%).

The content of these molecules in the samples of the different botanical and geographic origins are reported in the box plot in Figure 3. First, it is important to underline that different VOCs were mainly responsible for the botanical and geographic differentiations of the honey samples, confirming that these two factors differently affect the volatile composition of honey. As for the botanical origin, octane strongly characterized the eucalyptus honey sample for the high content and the acacia honey for the very low content, in agreement with the literature [34]; 1-nonene characterized the honeydew honey for the greatest content, and acacia and wildflower honey for the lowest content, while 5-ethenyldihydro-5-methyl-2(3H)-furanone characterized acacia honey for the greatest content and chestnut honey for the lowest content. As for the geographic origin, eucalyptol clearly differentiated the samples from Veneto, while butanal clearly differentiated the samples from Trentino-Alto Adige for the greatest content.

Concerning honey classification in terms of geographic and botanical origins, the results are reported in the confusion matrices in Table 4. The genetic algorithm, using LDA as the fitness function, well-classified all acacia and eucalyptus samples (100% in both the two runs), followed by chestnut honey 82.1%. Slightly worst results were achieved for the honeydew honey, likely because of the wide variability in composition (and sensory) properties among samples from the same source, and because of the frequent existence of the honey resulting from a blend of nectar and honeydew. Finally, the honey type with the worst rate of correct classification was the wildflower honey, though the result was not surprising since this type of honey can be characterized by the presence of diverse types of botanic origins. Concerning the geographic origin, the best results were for the Tuscan samples with 90.9% and 94.4% of correct classification in the two runs, respectively, while samples from Trentino-Alto Adige were more difficult to classify since they were from a lot of different botanical origins (72.0% and 76.0% of correct classification in the two runs, respectively).

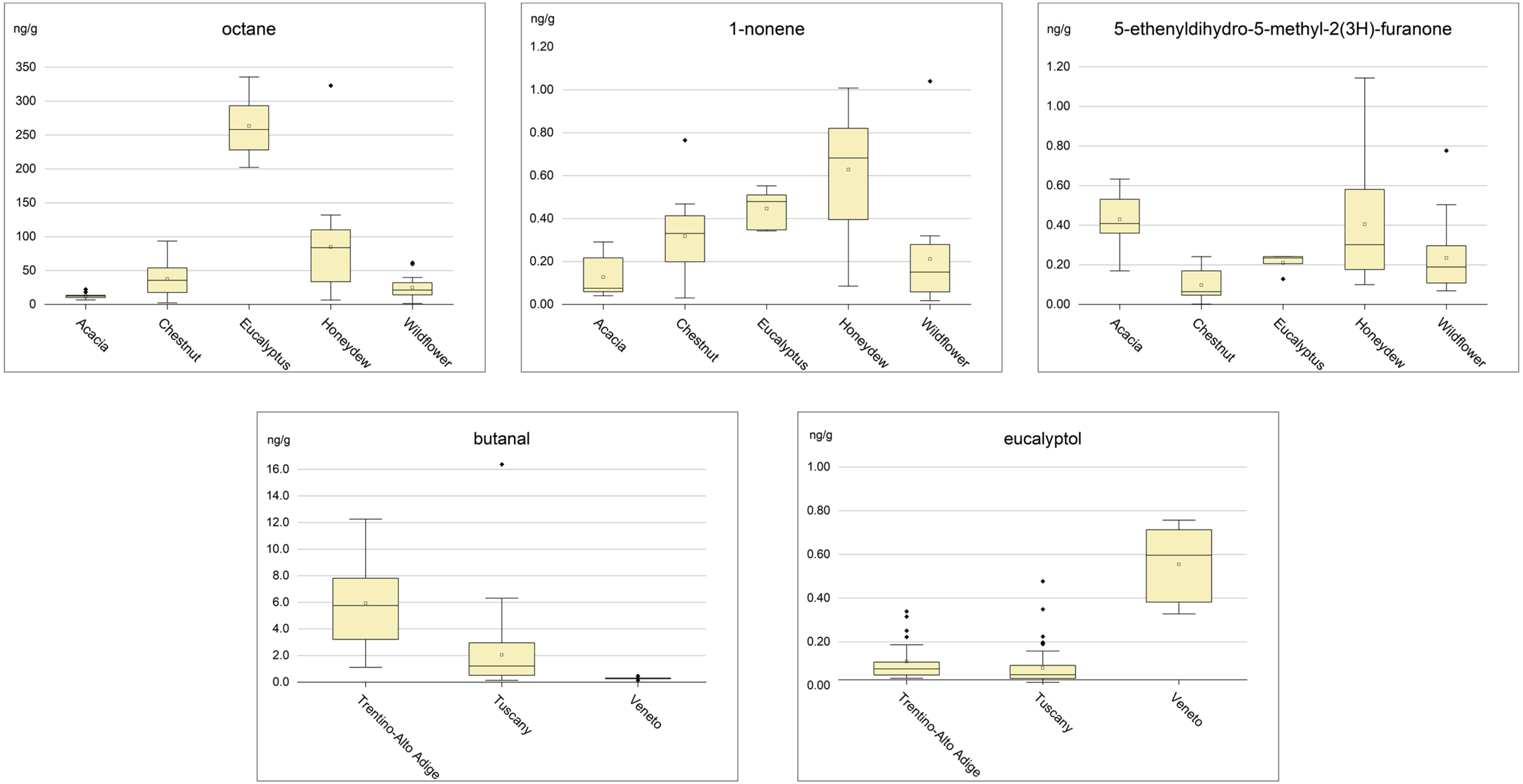

Figure 3. Box plot of the volatile organic compounds identified by the genetic algorithm as those more capable of differentiating the botanical (octane, 1-nonene and 5-ethenyldihydro-5-methyl-2(3H)-furanone) and geographic (butanal and eucalyptol) origin of the honey samples. Empty square is the mean; full black rhombuses are outliers.

Table 4. Confusion matrix of the results obtained by applying the genetic algorithm (GA) concerning the botanical origin (A and B) and the geographic origin (C and D).

A. Botanical origin—GA run 1

	Samples	Acacia	Chestnut	Eucalyptus	Wildflower	Honeydew
Acacia	12	**12 (100%)**	-	-	-	-
Chestnut	14	-	**11 (78.6%)**	-	2 (14.3%)	1 (7.1%)
Eucalyptus	5	-	-	**5 (100%)**	-	-
Wildflower	15	4 (26.6%)	1 (6.7%)	-	**9 (60.0%)**	1 (6.7%)
Honeydew	19	2 (10.5%)	1 (5.3%)	1 (5.3%)	-	**15 (78.9%)**

Average error rate = 16.5%

B. Botanical origin—GA run 2

	Samples	Acacia	Chestnut	Eucalyptus	Wildflower	Honeydew
Acacia	12	**12 (100%)**	-	-	-	-
Chestnut	14	-	**12 (85.7%)**	-	2 (14.3%)	-
Eucalyptus	5	-	-	**5 (100%)**	-	-
Wildflower	15	3 (20.0%)	2 (13.3%)	-	**9 (60.0%)**	1 (6.7%)
Honeydew	19	2 (10.5%)	1 (5.3%)	1 (5.3%)	1 (5.3%)	**14 (73.7%)**

Average error rate = 16.1%

C. Geographic origin—GA run 2

	Samples	Tuscany	Trentino-Alto Adige	Veneto
Tuscany	55	**50 (90.9%)**	3 (5.5%)	2 (3.6%)
Trentino-Alto Adige	25	7 (28.0%)	**18 (72.0%)**	-
Veneto	5	1 (20.0.%)	-	**4 (80.0%)**

Average error rate = 19.0%

D. Geographic origin—GA run 1

	Samples	Tuscany	Trentino-Alto Adige	Veneto
Tuscany	55	**52 (94.4%)**	2 (3.6%)	1 (1.8%)
Trentino-Alto Adige	25	6 (24.0%)	**19 (76.0%)**	-
Veneto	5	1 (20.0%)	-	**4 (80.0%)**

Average error rate = 16.5%

4. Conclusions

This research proposed an original chemometric approach for authentication of the botanical and geographic origins of honey samples. The genetic algorithm was used here for the first time for that purpose. It was applied to the semi-quantitative data of volatile compounds of Italian honey samples with different origins analyzed using HS-SPME-GC-MS, which was confirmed as a powerful tool for rapid analysis of a high number of volatile molecules.

The combination of HS-SPME-GC-MS data and the genetic algorithm has showed a very good potential for the simultaneous authentication of both the geographic and botanical origins of honey.

Considering that the characteristics of the honey, including the volatile composition, is affected by several factors in addition to the botanical and/or geographic origins, the results obtained using the GA can be considered satisfactory. Further research is required for collecting greater numbers of honey samples of several origins (both botanic and geographic) to confirm the reliability of the combination of HS-SPME-GC-MS and the genetic algorithm for honey authentication purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11090266/s1, Table S1: Content in ng/g of the 212 identified VOCs in wildflower honey samples; Table S2: Total content in ng/g of ketones and volatile phenols in all samples as a function of the botanical origin; Table S3: Content in ng/g of α-isophorone, 2-hydroxyisophorone, 4-oxoisophorone, β-isophorone and 2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl-1H-2-indenone in honey samples from different botanical origins; Table S4: Content in ng/g of lilac aldehyde isomers 1, 2 and 4, α,4-dimethyl-3-cyclohexene-1-acetaldehyde A, α,4-dimethyl-3-cyclohexene-1-acetaldehyde B, linalool and methyl anthranilate in honey samples from different botanical origins.

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