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Assessment of gas chromatography time-of-flight accurate mass spectrometry for identification of volatile and semi-volatile compounds in honey

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ABSTRACT

The performance of gas chromatography (GC) combined with a hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) system for the determination of volatile and semi-volatile compounds in honey samples is evaluated. After headspace (HS) solid-phase microextraction (SPME) of samples, the accurate mass capabilities of the above system were evaluated for compounds identification. Accurate scan electron impact (EI) MS spectra allowed discriminating compounds displaying the same nominal masses, but having different empirical formulae. Moreover, the use of a mass window with a width of 0.005 Da provided highly specific chromatograms for selected ions, avoiding the contribution of interferences to their peak areas. Additional information derived from positive chemical ionization (PCI) MS spectra and ion product scan MS/MS spectra permitted confirming the identity of novel compounds. The above possibilities are illustrated with examples of honey aroma compounds, belonging to different chemical classes and containing different elements in their molecules. Examples of compounds whose structures could not be described are also provided. Overall, 84 compounds, from a total of 89 species, could be identified in 19 honey samples from 3 different geographic areas in the world. The suitability of responses measured for selected ions, corresponding to above species, for authentication purposes is assessed through principal components analysis.

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1. Introduction

Honey is a complex natural product which is largely consumed worldwide. It is elaborated by honey bees from carbohydrate-containing exudates produced by plants. In addition to sugars, honey contains moisture and other valuable nutrients such as vitamins, minerals, enzymes, free amino acids and numerous volatile as well as semi-volatile species [1,2]. The two latter groups of compounds are responsible for the aromatic profile, which is one of the most distinctive features of honey [1]. Thus, profiles of volatile compounds can be used for honey authentication purposes, since they tend to differ based on the floral origin as well as the processing type [3–5]. Furthermore, volatile and semi-volatile compounds can be also correlated with antimicrobial and other medicinal properties of honey. Consequently, many efforts, in the field of sample preparation and analytes determination, have been conducted for the characterization and the identification of new compounds in this foodstuff. Even though gas chromatography combined with mass spectrometry (GC–MS) is the reference technique for volatile and semi-volatile compounds analysis [6], the obtained information is conditioned by several parameters, such as the sample preparation technique, the efficiency and selectivity of the capillary column, and the particular features of the different types of mass analyzers usually implemented in bench-top GC–MS instruments.

With regards to sample preparation, purge and trap (P&T) and headspace (HS) solid-phase microextraction (SPME) are the preferred techniques for the concentration of honey volatiles [7–11]. Nowadays, it is recognized that HS-SPME covers a higher number of species than P&T (particularly semi-volatile compounds), it is more versatile because different fiber coatings are commercially available, and it does not require any modification in the GC instrument at the level of sample introduction. As regards to the type of column, semi-polar and polar coatings offer (1) a higher

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selectivity than non-polar ones, reducing the number of co-eluted peaks; and (2) maximum operational temperatures high enough to deal with species concentrated by HS-SPME [12–14].

The differences between the information provided by MS spectrometers are related to their sensitivity and mass resolution. Quadrupole instruments present a limited sensitivity when operated in the scan mode; therefore, they are unsuitable for minor compounds (or those displaying a limited affinity for the SPME fiber) detection. Ion-trap (IT) and time-of-flight (TOF) MS systems provide an enhanced sensitivity for the screening of unknown compounds. Furthermore, the latter offers mass resolutions in the range from 3500 to 8000 for ions with *m/z* ratios from 69 to 500 units. High resolution MS spectra could be useful for the identification of novel compounds in honey samples and/or for discerning between species with the same nominal mass, but having different empirical formulae. The suitability of hybrid QTOF systems, providing accurate MS and MS/MS spectral information, for the screening of anthropogenic compounds (e.g. pesticides) in foodstuff vegetable samples [15–17] and antimicrobial, volatile species in medicinal plants [18,19] has been already demonstrated. However, their applicability for honey aroma compounds identification has not been assessed yet.

Multivariate analysis strategies (i.e. principal component analysis, PCA) employed to investigate the authenticity and origin of honey, usually deal with profiles corresponding to total ion current (TIC) GC–MS chromatograms. However, due to the complexity of these chromatograms, the possibility of having several compounds under the same peak is not negligible, particularly, when chromatographic deconvolution is not performed. This fact introduces an additional variability which turns more difficult honey classification. The selection of responses for specific ions, isolated with mass windows in the region of the low milidaltons (mDa), limits the risk that signals are the contribution of several compounds; thus, they could be employed for classification purposes, even when the molecular species responsible for the selected ion has not been identified.

The aim of this study is to assess the possibilities of GC–MS, based on the use of a hybrid QTOF instrument, for the identification of volatile and semi-volatile compounds in honey samples from three different geographical areas of the world, using single MS, tandem MS/MS and different ionization modes capabilities of that system. HS-SPME was considered as an extraction technique, adapting previously reported sample preparation conditions. Responses measured for characteristic ions were employed to discriminate the three groups of samples, attending to their geographic origins and independently of their mono- or multi-floral character. Possibilities, and limitations, of the GC–QTOF–MS for the unambiguous identification of chromatographic peaks are discussed by selecting compounds from different chemical families. Also, the presence of new compounds in honey is reported.

2. Experimental

2.1. Chemicals and reagents

Standards of 1,4-dichlorobenzene, toluene, 4-quinolinecarboxaldehyde, 3-quinolinecarbonitrile, 1-isoquinolinecarbonitrile, 2,4,6-trichlorobiphenyl (PCB-30) and 3-octanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The latter two species were considered as possible internal surrogates (IS) during HS-SPME, although only 3-octanol was used under the final conditions. Individual stock solutions of the above compounds were prepared in acetone. Further dilutions were made in the same solvent (case of IS), and in ethyl acetate for direct injection of the resulting standards in the GC–QTOF–MS system. A mixture of n-alkanes (C_8–C_{40}) in dichloromethane was provided by Supelco (Bellefonte, PA, USA). This solution was employed to calculate the linear retention index (LRI) of compounds identified in honey samples. Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA).

A manual SPME holder, poly(dimethylsiloxane)–divinylbenzene (PDMS/DVB, 65 μm film thickness, 1 cm length) and DVB–carboxen–PDMS (DVB/CAR/PDMS, 50/30 μm film thickness, 2 cm length) coated fibers were obtained from Supelco (Bellefone, PA, USA). Before being used for the first time, fibers were thermally conditioned at the temperatures recommended by the supplier.

2.2. Samples and sample preparation conditions

A total of 19 samples, obtained in three different geographic areas of the world (Galician: Northwest of Spain, Malaysia and Bangladesh) and corresponding to mono- and multi-floral honeys, were employed in this work. Samples were provided by bee keepers and the respective Ministries of Agriculture (case of some honeys from Malaysia and Bangladesh). Codes corresponding to each sample and information regarding the collection year, and their mono- or multi-floral character are summarized in Table 1.

Table 1
Characteristics and geographic origin of honey samples.

Code	Type	Region	Color	Harvest year
GAL-8	Multi-floral	Galician	Light-brown	2012
GAL-14	Multi-floral	Galician	Dark	2012
GAL-15	Multi-floral	Galician	Dark	2012
GAL-16	Multi-floral	Galician	Dark	2012
GAL-17	Multi-floral	Galician	Dark	2012
GAL-18	Multi-floral	Galician	Dark	2012
GAL-19	Multi-floral	Galician	Dark	2012
MY-2	Mono-floral (Pineapple)	Malaysia	Light-brown	2011
MY-3	Mono-floral (Gelam)	Malaysia	Dark	2011
MY-4	Mono-floral (Longan)	Malaysia	Dark	2011
MY-5	Mono-floral (Mangium tree)	Malaysia	Light	2011
MY-6	Mono-floral (Rubber tree)	Malaysia	Light	2011
MY-7	Mono-floral (Sourwood tree)	Malaysia	Dark	2011
MY-8	Multi-floral (Tualang)	Malaysia	Dark	2011
BD-1	Mono-floral (Mustard flower)	Bangladesh	Dark	2012
BD-4	Mono-floral (Kalizira)	Bangladesh	Dark	2012
BD-5	Mono-floral (Padmo flower)	Bangladesh	Light	2012
BD-12	Mono-floral (Mustard flower)	Bangladesh	Dark	2012
BD-21	Multi-floral	Bangladesh	Dark	2012

All samples were maintained at room temperature in amber glass vessels until they were processed. Sample code MY8 was obtained from the honeycombs hanging from Tualang trees in Malaysia forests. The rest of samples were from honeycombs hosted in commercial beehives.

SPME experiments were carried out in 22 mL volume glass vessels furnished with a Teflon-layered silicon septum and an aluminum crimp cap. A sample amount of 2.0 ± 0.1 g was introduced in each vessel and spiked with 0.1 mL of a mixture standard solution, containing 3-octanol (10 μg mL^{-1}) and PCB-30 (1 μg mL^{-1}). Vessels were vortexed and equilibrated in dark for 30 min. Thereafter, 2 mL of ultrapure water and a Teflon-covered magnetic stir bar were added. After closing, extraction vessels were maintained at 50 °C for 10 min before extraction. Microextraction conditions were adopted from elsewhere [12]. In brief, the PDMS–DVB fiber was exposed to the HS of the sample, at 50 °C, for 40 min while stirring at 600 rpm. Thereafter, the fiber was retracted and the outlet surface of the metallic needle was dried using a lint free tissue. Fibers were desorbed in the injector of the GC–QTOF–MS system for 2 min. Before being exposed to the next sample, they were additionally heated at 250 °C, for 5 min, in presence of a flow (5 mL min^{-1}) of dry nitrogen.

2.3. Determination conditions

An Agilent (Wilmington, DE, USA) GC–QTOF–MS system consisting of a 7890A gas chromatograph connected to a hybrid QTOF mass analyzer (Agilent model7200) was employed for identification of compounds desorbed from SPME fibers. Usually, the system was operated in the electron impact (EI) mode, using an energy of 70 eV, moreover, some injections were made using positive chemical ionization (PCI), with methane as the ionization gas. The system was furnished with DB-WAXETR type polar column (30 m × 0.25 mm i.d., 0.50 μm film thickness) provided by Agilent. Helium (99.999) was used as carrier gas at a constant flow of 1.2 mL min^{-1}. The injector was maintained at 260 °C and fibers were desorbed in the splitless mode, with the solenoid valve changing to the split position after 2 min (split flow 60 mL min^{-1}). Direct injections of the alkanes mixture and individual standards of other compounds were made in the same conditions, using an injection volume of 1 μL. The temperature of the column was programmed as follows: 60 °C (1 min), rated at 5 °C min^{-1} to 240 °C (20 min). The TOF mass analyzer was operated in the extended dynamic range 2 GHz mode (mass resolution from 3700, at m/z 69, to 8200, at m/z 414). Although mass resolution can be increased by operating the system in the 4 GHz mode, a wider linear response range and better mass accuracy have been reported for the 2 GHz mode [20,21]. Source and quadrupole temperatures were set at 230 (300 °C for PCI) and 150 °C, respectively. The transfer line between the GC and the MS was maintained at 250 °C.

Usually, the QTOF system was operated in the single MS mode, with scan spectra recorded every 0.2 s between 40 and 650 m/z units. Under these conditions, each spectrum corresponded to the combination of 2700 transients. In order to obtain additional information regarding the identity of some chromatographic peaks, their accurate ion product scan (MS/MS) spectra were also recorded. Precursor ions were isolated by the quadrupole mass analyzer within a window of 1.2 Da and MS/MS spectra recorded (40–400 Da) every 0.2 s. The mass axis of the TOF mass analyzer was re-calibrated every 3 injections with a commercial solution of perfluorotributylamine (PFTBA).

Responses (peak areas) for identified compounds were measured for a selected m/z value (if present the molecular ion, or any other intense ion) within a mass window of 5 mDa [20]. The use of a narrow window guarantees that the obtained signal corresponds to a single species, even when its identity remains unclear, as it was the case of a few compounds.

2.4. Compounds identification

Identification of peaks observed in the EI-MS chromatograms from honey samples was made considering the following items: (1) search against the low (nominal) resolution National Institute of Standards and Technology (NIST) data base (version 2.0), and (2) comparison of the experimental m/z value for the molecular ion (when available) with the calculated ones for candidate compounds resulting from NIST data base search. Positive identifications required a match above 70% in the NIST data base search plus a difference between the empirical and the theoretical mass of the molecular ion lower than 5 mDa. If the molecular ion was not observed, the differences between the masses of two intense fragments and those corresponding to the candidate compound must remain below 5 mDa. The exact masses for fragments observed in NIST spectra were calculated using the Mass Interpreter function, which is implemented in the Mass Hunter software. When several candidates fulfilled the above conditions, the chromatographic peak was assigned to that presenting the highest scores (best match against the NIST data base and lowest difference between empirical and theoretical masses), considering also the previous report of such species in honey. Additionally, the LRI of identified compounds were calculated and compared with those reported for the same column [12]. In a few cases, further information was obtained with accurate MS/MS spectra, PCI spectra and/or by injection of pure standards of candidate species.

2.5. Principal component analysis

Principal component analysis (PCA) was performed using The Unscrambler (Camo Software As, Oslo, Norway) statistical software. Responses measured for selected ions of each compound, extracted using a mass window of 5 mDa, were first corrected with the peak area of 3-octanol (measured for ion at 83.0681 Da), to compensate for the variability of the HS SPME sample preparation process, and further normalized to the average peak area for compounds detected in each sample.

3. Results and discussion

3.1. HS SPME conditions

GC profiles of honey samples obtained after HS SPME highly depend on extraction conditions, with temperature and fiber coating displaying the most important effects in the amount and type of semi-volatile species concentrated in the SPME fiber [22]. Systematic comparison among the extraction efficiencies obtained for five different SPME coatings have ended in the adoption of DVB/CAR/PDMS as the most suitable fiber for honey characterization [10,12]; nevertheless, the PDMS/DVB fiber was not considered in the above comparison studies. On the other hand, other authors have proposed the use of this latter fiber for honey aroma compounds extraction, since it provides higher responses than PDMS and CAR/PDMS [4,7]. Thus, we have performed a comparison between PDMS/DVB and DVB/CAR/PDMS relative extraction efficiencies with a multi-floral honey sample. Obtained responses were compound dependant. As a general rule, the so-called triple fiber was superior to the PDMS/DVB one for earlier eluting compounds, whereas the second one provided higher intensities for peaks in the second half of the chromatogram (see Fig. S1). Taking into account that the amount of coating in the PDMS/DVB fiber is half of that in the DVB/CAR/PDMS one, the former one is

Table 2
Summary of compounds identified in honey samples.

	Code	Retention time (min)	LRI	CAS no.	Exact mass (Da)	Quantification ion (Da)	GAL	MY	BD
Benzene derivatives									
Toluene	B1	4.421	–	108-88-3	92.0626	92.0626	X	X	X
Styrene	B2	8.925	1277	100-42-5	104.0626	104.0626	X	X	
Benzene, 1-methoxy-4-methyl-	B3	13.425	1466	104-93-8	122.0732	122.0732			
Benzene, 1,4-dichloro-	B4	13.600	1473	106-46-7	145.969	145.9690		X	X
Ethanone, 2-(formyloxy)-1-phenyl-	B5	18.662	1686	55153-12-3	164.0474	105.0323	X		
Benzoic acid, ethyl ester	B6	18.930	1693	93-89-0	150.0681	105.0336			
Estragole	B7	19.000	1700	140-67-0	148.0888	148.0888		X	
Anethole	B8	20.966	1788	104-46-1	148.0888	148.0888			
Benzeneacetic acid, ethyl ester	B9	21.602	1816	101-97-3	164.0837	164.0837		X	
Acetic acid, 2-phenylethyl ester	B10	22.272	1849	103-45-7	164.0837	104.0640			X
Phenylethyl alcohol	B11	24.368	1949	60-12-8	122.0732	122.0732	X	X	X
Benzaldehyde, 4-methoxy-	B12	26.794	2070	123-11-5	136.0524	135.0439	X		
2-Propenal, 3-phenyl-	B13	27.060	2083	104-55-2	132.0575	131.0488	X		
2-Propenoic acid, 3-phenyl-, ethyl ester	B14	28.704	2170	103-36-6	176.0837	131.0505			
Thymol	B15	29.637	2220	89-83-8	150.1045	135.0800	X		
Ethanone, 1-(2,4,5-triethylphenyl)-	B16	31.424	2320	2715-54-0	204.1514	189.0914	X		
Phenol, 2,4-bis(1,1-dimethylethyl)-	B17	31.826	2343	96-76-4	206.1671	191.1467	X	X	X
4,4′-Dimethoxybenzil	B18	32.009	2353	1226-42-2	270.0892	135.0433			
Phenol, 2,4,6-trimethyl-	B19	33.082	2415	527-60-6	136.0888	121.0633	X		
Benzoic acid, 3,5-dimethoxy-, methyl ester	B20	33.604	2447	2150-37-0	196.0736	196.0733			
Benzoic acid	B21	34.250	2485	65-85-0	122.0368	122.0368	X		
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	B22	35.107	2538	1620-98-0	234.1619	219.1403	X	X	X
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	B23	35.775	2579	84-69-5	278.1518	149.0247	X	X	X
Benzeneacetic acid	B24	36.305	2612	103-82-2	136.0524	91.0532	X		
1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester	B25	38.353	2736	84-64-0	304.1675	149.0257	X	X	
Linear carboxylic acids									
Acetic acid	A1	13.938	1487	64-19-7	60.0211	60.0211	X	X	X
Octanoic (caprylic) acid	A2	27.196	2090	124-07-2	144.115	60.0207	X		
Dodecanoic (lauric) acid	A3	34.779	2517	143-07-7	200.1776	73.0295		X	X
Linoleic acid	A4	37.737	2702	60-33-3	280.2402	67.0534	X		
Esters									
Octanoic acid, ethyl ester	E1	13.018	1449	106-32-1	172.1463	61.0291		X	
Nonanoic acid, methyl ester	E2	14.490	1510	1731-84-6	172.1463	74.0360	X		
Nonanoic acid, ethyl ester	E3	15.504	1552	123-29-5	186.1619	61.0293	X	X	
Decanoic acid, methyl ester	E4	16.982	1614	110-42-9	186.1619	74.0357	X		
Decanoic acid, ethyl ester	E5	17.922	1654	110-38-3	200.1776	61.0291	X	X	
2-Nonenoic acid, ethyl ester	E6	18.443	1676	17463-01-3	184.1463	73.0299		X	
Butanedioic acid, diethyl ester	E7	18.986	1700	123-25-1	174.0892	101.0229		X	
Linolenic acid, methyl ester	E8	21.900	1831	301-00-8	292.2402	79.0537	X		
Dodecanoic acid, ethyl ester	E9	22.493	1859	106-33-2	228.2089	61.0280		X	X
Butanoic acid, 4-hexen-1-yl ester	E10	24.758	1968	53398-84-8	170.1307	67.0541	X		
Tetradecanoic acid, methyl ester	E11	25.963	2027	124-10-7	242.2246	74.0363	X	X	
Tetradecanoic acid, isopropyl ester	E12	26.388	2049	110-27-0	270.2559	60.0210	X	X	X
Tetradecanoic acid, ethyl ester	E13	26.689	2064	124-06-1	256.2402	73.0296		X	
Hexadecanoic acid, methyl ester	E14	29.908	2235	112-39-0	270.2559	87.0437		X	
Hexadecanoic acid, isopropyl ester	E15	30.229	2253	142-91-6	298.2872	60.0204			X
Hexadecanoic acid, ethyl ester	E16	30.556	2271	628-97-7	284.2715	73.0290		X	
Ethyl oleate	E17	34.484	2499	111-62-6	310.2872	310.2872		X	
Ethyl linoleate	E18	35.322	2551	544-35-4	308.2715	67.0546		X	
Linolenic acid, ethyl ester	E19	36.441	2621	1191-41-9	306.2559	79.0548			

Non-aromatics ketones and aldehydes									
3-Hexen-2-one	K1	14.238	1499	763-93-9	98.0732	83.0481			
Lilac aldehyde B	K2	16.426	1590	53447-45-3	168.1150	93.0687			
Isophorone	K3	17.246	1625	78-59-1	138.1045	82.0408	X		
3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-(B-ionone)	K4	17.438	1633	14901-07-6	192.1514	177.1276	X	X	
1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl- (Safranal)	K5	18.466	1677	116-26-7	150.1045	150.1045	X	X	
2-Hydroxyl-3,5,5-trimethyl-cyclohex-2-enone	K6	18.823	1693	4883-60-7	154.0994	154.0994			
2,6,6-Trimethyl-2-cyclohexene-1,4-dione (Oxoisophorone)	K7	19.574	1726	1125-21-9	152.0837	152.0837			
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)- (B-Damascenone)	K8	22.332	1852	23726-93-4	190.1358	69.0330	X	X	
1H-Indene-4-carboxaldehyde, 2,3-dihydro-	K9	24.806	1970	51932-70-8	146.0731	146.0731			X
2,6-Dimethyl-2,5-heptadiene-4-one (Phorone)	K10	25.142	1986	504-20-1	138.1045	123.0794	X		
Megastigmatrienone	K11	30.009	2241	38818-55-2	190.1357	190.1357			
Terpineols									
Cis-linalool oxide	T1	13.332	1462	5989-33-3	170.1307	59.0492	X	X	
Trans-linalool oxide	T2	14.067	1492	34995-77-2	170.1307	59.0497	X	X	
1,6-Octadien-3-ol, 3,7-dimethyl- (linalool)	T3	15.819	1565	78-70-6	154.1358	71.0491	X		
1,5,7-Octatrien-3-ol, 3,7-dimethyl- (hotrienol)	T4	17.391	1631	29957-43-5	152.1201	67.0543	X		
α-Terpineol	T5	19.490	1722	98-55-5	154.1378	59.0488			
Furanes									
Furan, 3-phenyl-	F1	23.181	1891	13679-41-9	144.0575	115.0534	X		
2,5-Furandicarboxaldehyde	F2	25.901	2024	823-82-5	124.016	124.0160		X	X
Benzofuran, 2,3-dihydro-	F3	33.511	2441	496-16-2	120.0575	120.0575	X		
5-Hydroxymethyl furfural	F4	35.402	2556	67-47-0	126.0317	126.0317			
Nitrogenated compounds									
Benzenamine, N-ethyl	Ni1	20.385	1762	103-69-5	121.0892	106.0665	X	X	X
Benzyl nitrile	Ni2	24.826	1971	140-29-4	117.0579	117.0579	X		
Benzothiazole	Ni3	25.423	1999	95-16-9	135.0143	135.0143	X	X	X
Quinolinecarbonitrile	Ni4	32.780	2397	–	154.0531	154.0531	X		
4-Quinolinecarboxaldehyde	Ni5	34.339	2491	4363-93-3	157.0528	129.0566			
Napthalenes and hydronaphthalene derivatives									
α-Ionene	N1	14.372	1505	475-03-6	174.1409	159.1167	X		
1,1,5-Trimethyl-1,2-dihydronaphthalene	N2	20.732	1778	NIST357258	172.1252	157.1009	X	X	
Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	N3	22.568	1863	483-77-2	202.1722	159.1170	X	X	
1-Naphthalenol, 4-methyl-	N4	24.418	1951	10240-08-1	158.0732	158.0725	X		
γ-Eudesmol	N5	29.302	2201	1209-71-8	222.1984	161.1305	X		
α-Eudesmol	N6	30.303	2257	473-16-5	222.1984	161.1304			
Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	N7	30.449	2265	483-78-3	198.1409	183.1167	X	X	
β-Eudesmol	N8	30.482	2267	473-15-4	222.1984	161.1304			
Naphthalene, 2,3-dimethoxy-	N9	31.806	2342	10103-06-7	188.0838	188.0836			
Other cyclic hydrocarbons									
Cycloprop[a]indene, 1,1a,6,6a-tetrahydro-	Ch1	16.562	1596	15677-15-3	130.0783	130.0783	X		
α-Gurjunene	Ch2	30.883	2289	489-40-7	204.1878	189.1273	X		
Isoledene	Ch3	20.847	1783	95910-36-4	204.1878	161.1321	X		

considered more efficient for aroma compounds extraction and was utilized in this work. The sampling time was maintained in 40 min, as in previous studies [10,12], and the extraction temperature was slightly reduced (from 60 to 50 °C), in order to limit the potential formation of furfural-like compounds during sample preparation. Operating under above conditions, the repeatability of the HS SPME method was evaluated for replicate (n=5) extractions of a multi-floral and a mono-floral honey, from two different geographical regions. A total of 16 compounds, with retention times between 4.4 and 38.4 min, belonging to different chemical classes and rendering peaks with different intensities, were considered for repeatability assessment. The relative standard deviations (RSDs, %) of their peak areas, measured for the most intense ion in their spectra, varied from 4% to 15%. Responses for 3-octanol displayed RSDs in the same order of magnitude and those measured for PCB-30 stayed around 30%. Thus, the first species was selected as IS. After IS correction, RSDs ranged from 1% to 11% (see Table S1).

3.2. Compounds identification

Table 2 summarizes the list of species identified in the processed samples. They are grouped according to their chemical classes, assuming that, in some cases, the same species could be included in different groups. Retention times, LRI and CAS numbers for each compound are also included in Table 2, together with their monoisotopic molecular weights (calculated values), and the experimental mass of the ion whose response was used for classification purposes. The last three columns of Table 2 include a mark for these species detected in all specimens from each group of samples. Fig. 1 shows the total ion current (TIC) GC–EI-MS chromatograms for selected samples belonging to the three considered geographic areas. In general, honeys from Bangladesh (BD) displayed a lower chromatographic complexity than the ones from Galician (GAL) and Malaysia (MY).

The first group of compounds in Table 2 corresponds to benzene derivatives. Their EI-MS spectra contained a reduced number of signals and, in most cases, the molecular ion is visible. Thus, their identification was normally a simple task. Most of the benzene species identified in honey samples are natural flavors, being previously described in honey and other foodstuffs and/or essential oils. In other cases, their origin is more difficult to be established. An example is compound B4 (1,4-dichlorobenzene), whose identity was confirmed by injection of a pure standard. This chlorinated aromatic species was detected in all tropical honey samples, including the one from Tualang forest (code MY-8), but not in those harvested in Galician. Previously, this compound has been reported in honeys from different geographic areas, e.g. Madeira Islands [10]; however, it is mostly accepted that 1,4-dichlorobenzene is not a natural compound, but comes from insecticide treatments applied to honeycombs against the Wax moth [23]. Two phthalates, B23 and B25, were also identified in all specimens from Galician and Malaysia, but not in those from Bangladesh. The first has been recently reported as a constituent of buckwheat honey samples collected in Italy [6]. Likely, phthalates are the result of honey contamination with wax from the honeycombs.

Within the group of benzene compounds, Bangladesh samples contained significantly higher levels of toluene (B1) than the rest of samples. This species turned to be a key variable for their discrimination from other honeys, particularly those from Malaysia.

A few benzene derivatives co-eluted with other compounds; therefore, their identification was not straightforward, particularly when the co-eluting species are present at similar levels. An example of the above situation corresponds to benzoic acid ethyl ester (B6) and estragole (B7), whose chromatographic peaks are partially overlapped when present in the same sample; thus, their

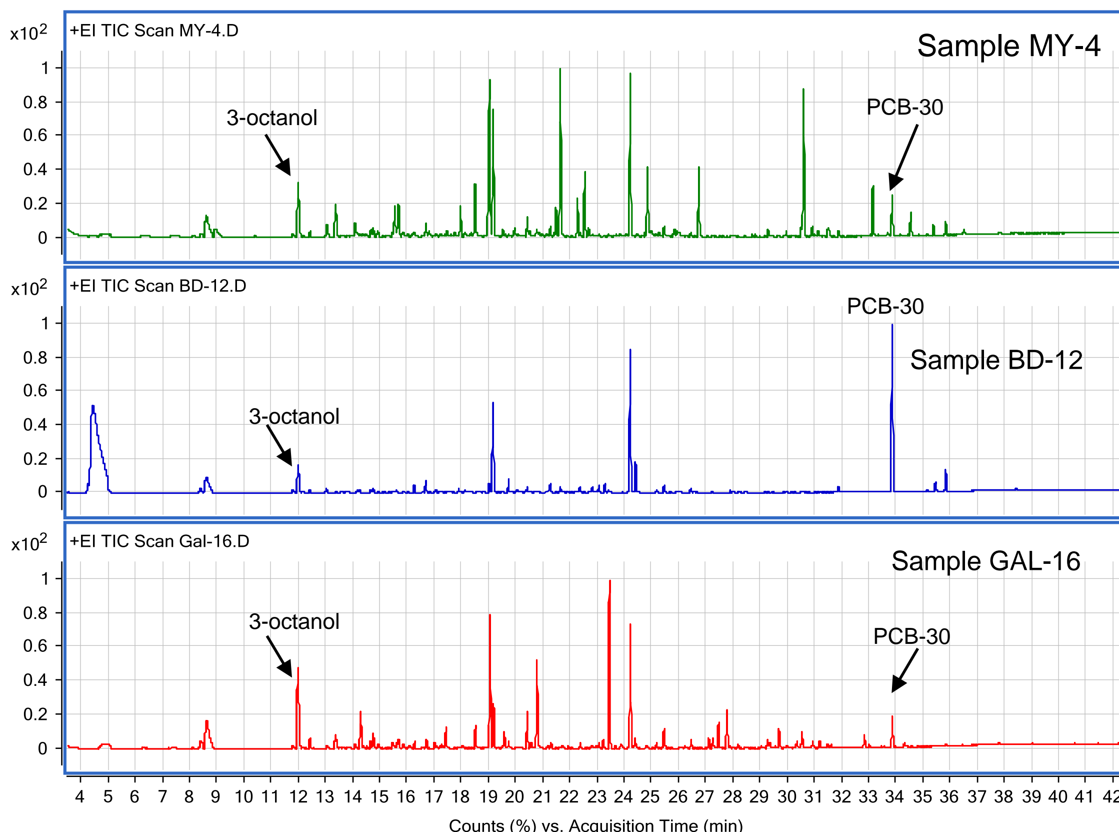

Fig. 1. TIC chromatograms for selected samples of Bangladesh (blue), Galician (red) and Malaysia (green) honeys. Normalized responses to the highest peak in each chromatogram. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

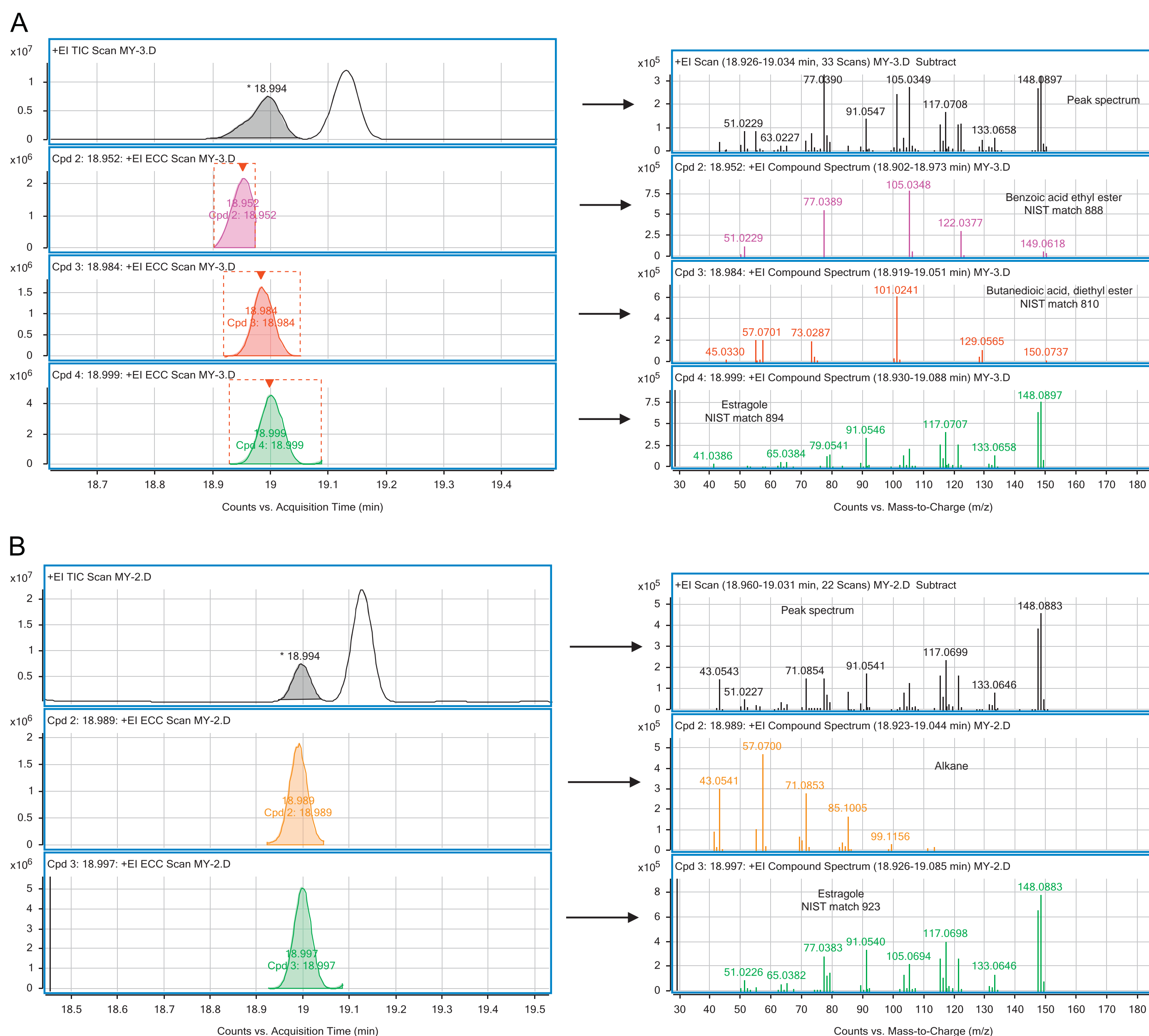

Fig. 2. TIC, extracted compound chromatograms (ECC) and spectra for peak at retention time 18.9–19.0 min in honey samples MY-3 (A) and MY-2 (B).

pure spectra can be obtained only after deconvolution. Fig. 2 shows the results (chromatograms and spectra) obtained following deconvolution of a chromatographic region around retention times of the above species in two different honey samples. In case of sample MY-3 (Fig. 2A), a third compound (identified as a non-aromatic ester) was detected under the same peak as species B6 and B7. Sample MY-2 (Fig. 2B) did not contain the ethyl ester of benzoic acid (B6); however, a linear hydrocarbon (heptadecane) was detected co-eluting with estragole (B7). Obviously, systematic chromatographic deconvolution will improve the purity of EI-MS spectra. On the other hand, comprehensive deconvolution throughout chromatograms turned to be extremely demanding in terms of computing time (particularly if a low response threshold is defined for minor peaks detection); thus, the deconvolution function, implemented in the Mass Hunter software, was only considered when coelution problems (e.g. NIST search does not report any satisfactory identification) are suspected.

With regards to linear carboxylic acids, the most abundant species were acetic (A1), caprylic (A2), lauric (A3) and linoleic acids (A4), Table 2. The former was present in all samples, caprylic and linoleic acids were only found in Galician samples and lauric acid was detected in both groups of tropical samples. The total number of identified esters of non-aromatic carboxylic acids was 19. They are mainly methyl, ethyl and isopropyl derivatives, with only compound E12 (tetradecanoic acid isopropyl ester) detected in all samples. Overall, MY honeys displayed the larger variety of esters, with only four congeners (E2, E4, E8 and E10) being absent in all samples obtained from this geographic area. In case of tetra- and hexadecanoic acids, the three esters were noticed, with the highest abundance corresponding to the ethyl forms. The EI-MS spectra of these compounds displayed a low, although detectable, signal for the molecular ion, which facilitates the unambiguous confirmation of their identities. Chromatograms corresponding to methyl, isopropyl and ethyl derivatives of hexadecanoic acid and the spectrum for ethyl hexadecanoate are provided as Supplementary information (see Fig. S2). The differences between experimental and calculated masses for different ions in this spectrum remained below 1 mDa. Ethyl esters of linear fatty acids with 9, 10, 12, 14

and 16 carbon atoms are among the most intense peaks in the TIC chromatograms for MY samples. These compounds have been reported as strongly correlated with the sweetness of honey [10].

Ketones and aldehydes species are characterized by their intense flavor, even when present at low concentrations. Most of the 11 compounds classified within this group (from K3 to K11, Table 2) may be generated from degradation of carotenoids [24]. Compounds K1, K2, K6, K7 and K10 showed very low levels, remaining undetected in most honeys. K3 (Isophorone) was only found in Galician samples and K9 (1H-indene-4-carboxaldehyde, 2,3-dihydro-) only in those from Bangladesh. β-Ionone (K4) stayed at higher levels in Malaysia samples than in Galician ones and the opposite trend was observed in case of β-Damascenone (K8). Finally, the range of values (peak areas) for Safranal (K5) was similar in honey samples from both regions. Following the low resolution NIST data base search, K10 was initially identified as a derivative of fluorobenzoic acid, with the base peak in the NIST data base spectrum corresponding to the aromatic ring bonded to a carbonyl moiety and one atom of fluorine ($[C_7H_4OF]^+$ ion, nominal mass 123 Da). However, the calculated mass for this ion (123.0246 Da, $[C_7H_4OF]^+$) differed in 56 mDa from that observed in the experimental spectrum (123.0803 Da), Fig. 3. Taking into account the list of NIST candidates with match values above 700, the chromatographic signal was attributed to phorone (a possible precursor of isophorone). The calculated mass for the $[M\text{-}CH_3]^+$ ion of phorone (123.0810 Da) differed in less than 1 mDa of that observed in the experimental spectrum (Fig. 3). Obviously, the use of a low resolution MS spectrometer, e.g. quadrupole or ion trap, would not permit to elucidate whether the ion at 123 Da contains fluorine, or not. It is worthy to note that, Soria et al. [13] also reported the presence of a compound with a base peak at 123 Da and a LRI of 1972 (measured with a Carbowax type column) in honey samples; however, these authors were not able to identify this species on the basis of its low resolution EI-MS spectra. Likely, this peak corresponded to phorone with a LRI of 1986 in this study.

Five terpineols were found in the processed samples (Table 2). Together with ketone K2, they are related to the structure of linalool (T3). T3 and hotrienol (T4) were only present in samples from Galician. Identification of compounds T1 and T2 was made by comparison of their EI-MS spectra with the NIST data base and using also the LRI data published by Plutowska et al. [12] for the same column. The highest match, resulting from NIST search, for both compounds corresponded to ethyl 2-(5-methyl-5vinyltetrahydro-furan-2-yl) propan-2yl carbonate, ($C_{13}H_{22}O_4$, molecular weight 242.1518) followed by linalool oxide ($C_{10}H_{18}O_2$, molecular weight 170.1307) isomers. In fact, the former species is an acetylated derivative of the latter ones and their NIST spectra are practically identical as shown in Supplementary information, Fig. S3A–C. The calculated LRI values for T1 and T2 were 1462 and 1492, very close to

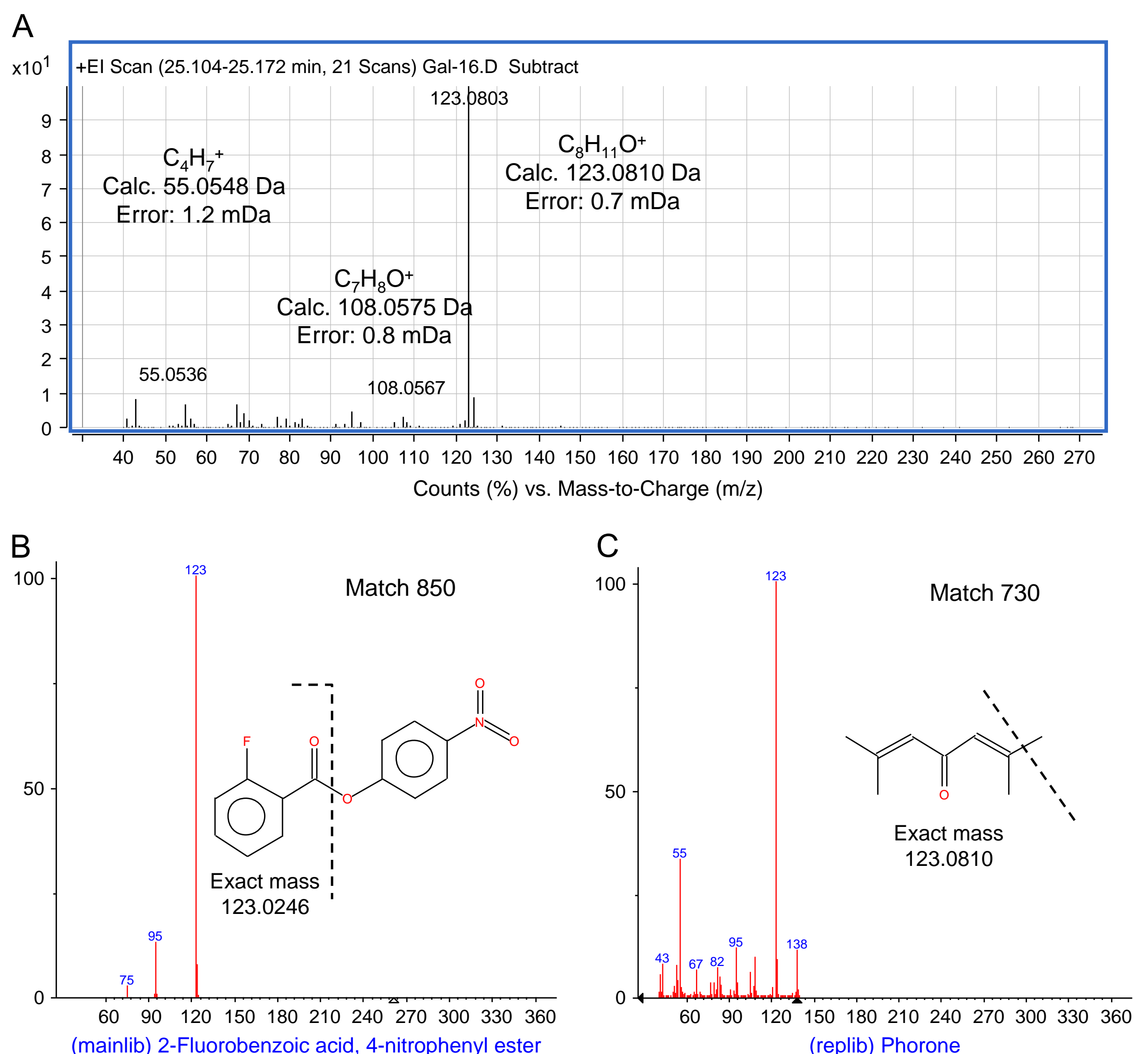

Fig. 3. EI-MS spectrum for peak K10 (A) and NIST data base spectra of 2-fluorobenzoic acid 4-nitrophenyl ester (B) and phorone (C).

those published for cis- and trans-linalool oxide isomers (1461 and 1490, respectively) [12]. Therefore, the identities of T1 and T2 species were assigned to linalool oxide isomers. The importance derived from a narrow *m/z* window for the selective extraction of a given compound is illustrated in the case of compound T2. The extracted ion current (EIC) chromatogram for ion $[C_3H_7O]^+$ (exact mass 59.0497 Da) using a mass window of 1 Da contains an interfering peak at shorter retention time, which is partially overlapped to that of trans-linalool oxide (T2), Fig. S3D. The interfering peak was filtered by reducing the extraction window from 1 to 0.005 Da (Fig. S3E).

Another group of oxygen containing species compiled in Table 2 corresponds to furan derivatives. Again, tropical honey samples displayed a different profile to those from Galician. Species F1 and F3 were predominant in the latter group of honeys; whereas, F2 was only found in the tropical honeys. F4 (5-hydroxymethyl furfural) was the less often detected compound. Its presence in honey is recognized to be related with storage conditions more than with the botanic origin of honey [13].

Five nitrogenated compounds were found in the processed samples(Table 2). Benzenamine N-ethyl (Ni1) and benzothiazole (Ni3) were ubiquitous in all samples, whereas the other three species were only detected in Galician honeys. Although the presence of nitrogen compounds in honey samples has been recognized in several works [9,10,13], to the best of our knowledge, the species Ni4 and Ni5 have not been previously reported, thus, their identity was further investigated. The EI-MS spectrum and the retention time of Ni5 were identical to those corresponding to a standard of 4-quinolinecarboxaldehyde (Fig. S4). This alkaloid has been previously identified in some plants, e.g. *Ruta chalepensis*, and it has been proven to be active against human intestinal bacteria [25], and also useful for the treatment of certain liver diseases [26]. Ni4 (retention time 32.78 min) showed the same EI-MS spectrum, although different retention times, as those obtained for the standards of two quinolinecarbonitrile derivatives (3-quinolinecarbonitrile, retention time 35.83 min; and 1-isoquinolinecarbonitrile, retention time 36.61 min) (Fig. 4). Accurate scan MS–MS spectra for the three species are also similar (Fig. S5); furthermore, their PCI-MS spectra contained the same base peak corresponding to the protonated species ($[C_{10}H_6N_2+H]^+$, calculated mass 155.0602 Da) and the adduct with methanol ($[C_{10}H_6N_2+C_2H_5]^+$, calculated mass 183.0917 Da) (figure not shown). Probably, Ni4 is a positional isomer of quinolinecarbonitrile with empirical formula $C_{10}H_6N_2$. Certain quinoline alkaloids (e.g. kynurenic acid and 4-quinolone-2-carboxylic acid) have been proposed as markers of chestnut honey [27], a common tree in Galician, and correlated with the wound-healing properties of honey [28]. However, the quinoline species identified in this study are different to those described in previous publications [27,28].

Samples from Galician and Malaysia contained nine naphthalene-like compounds (naphthalene and hydronapthalenes labeled as N1–N9) attached to hydroxyl and/or methyl moieties. They displayed very rich EI-MS spectra containing, in most cases, the molecular ion; thus, identification of their empirical formula was straightforward. On the other hand, the NIST data base contains several forms (usually positional isomers) for some of these compounds (e.g. in case of trimethyl dihydronaphthalene); thus, the correct identification of chromatographic peaks will require injection of pure standards for the different possible isomers. Peak assignations compiled in Table 2, for this family of compounds, correspond to the highest match provided by the NIST data base search, although the difference with the following candidates is rather small in most cases. Some compounds included in the above family (case of Eudesmol isomers) are sesquiterpenoids with empirical formula $C_{15}H_{26}O$. Thus, they are also susceptible of being classified within the group of terpineols. In fact, their EI-MS spectra display intense ions corresponding to the hydrogenated naphthalene ring, and also to the $C_3H_7O^+$ moiety (*m/z* 59.0497 Da), which is characteristic of terpineols (figure not shown). In addition to hydronaphthalene derivatives, other tricyclic, non-aromatic hydrocarbons were identified in honey samples. They have been labeled as Ch1 to Ch3 in Table 2, corresponding in some cases, e.g. α-Grujenene (Ch2) and isoledene (Ch3), to sesquiterpenes.

Linear hydrocarbons, such as C_{15} and C_{17}, (not included in Table 2) were ubiquitous in all samples. They can be identified by comparison of their retention times with those corresponding to species in the mixture of alkanes used for LRI calculation. On the other hand, their EI-MS spectra were similar, independently of their empirical formula, with the most intense signal corresponding to the $C_4H_9^+$ ion (calculated *m/z* 57.0699 Da). Identification of

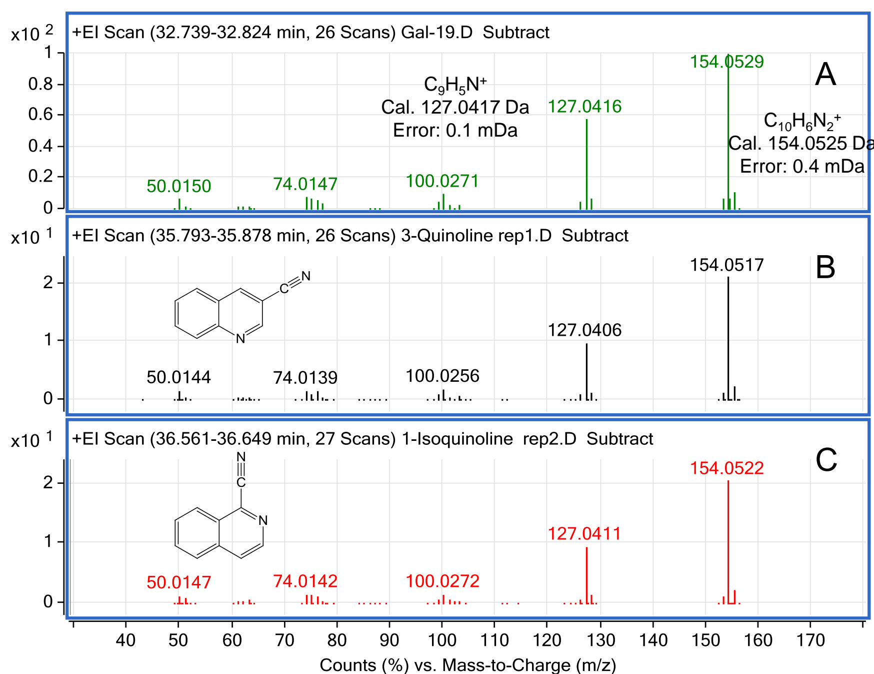

Fig. 4. EI-MS spectra obtained for compound Ni4 (A), and standards of 3-quinolinecarbonitrile (B) and 1-isoquinolinecarbonitrile (C).

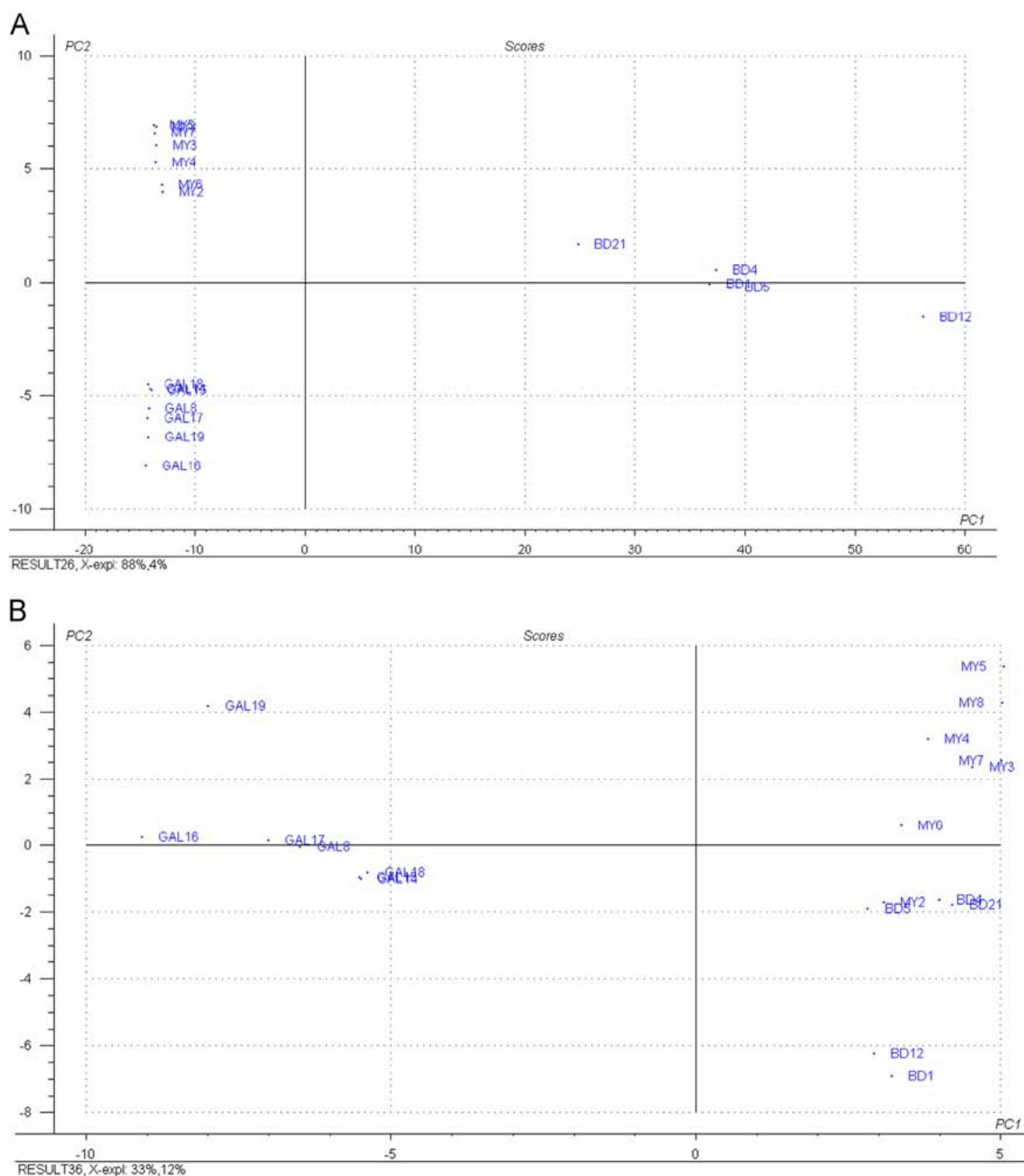

Fig. 5. PCA two-dimensional plots considering responses for all compounds (A), and after excluding toluene (B).

other hydrocarbons, containing a low number of unsaturated bonds, or cyclic structures, was more difficult. In some cases, their EI-MS spectra displayed only ions at low *m/z* values and their concentrations were not high enough to be detected using PCI ionization. In other situations, even though the molecular ion was observed, the NIST data base search did not result in any candidate with a match above 700. An example of this latter situation is provided as Supplementary information (Fig. S6). The compound responsible for this spectrum (Fig. S6) displayed a retention time of 19.126 min and stayed within the 10 most intense peaks in all chromatograms (see Fig. 1). Its EI-MS spectra suggest an empirical formula of $C_{21}H_{40}$ (calculated mass for the radical molecular ion $[M^{+}]$ 292.3125 Da) and 2 double bond equivalents (Fig. S6). Spectral deconvolution proved that this compound did not co-elute with other species (figure not shown); however, it could not be identified using the NIST library. Table S2 compiles some features of the 4 compounds detected in most samples, whose chemical structures could not be identified. In some cases, an empirical formula is proposed.

4. Principal components analysis

The three groups of honey samples involved in this study differed not only in their geographic origin, but also in their mono- or multi-floral character (Table 1); therefore, they did not represent well-defined categories, since large differences are expected within each group. Despite this assumption, the feasibility of using the responses corresponding to quantification ions compiled in Tables 2 and S2 for discrimination purposes was considered. PCA of all above variables reflected that two factors were enough to explain 92% of the total variance. As shown in Fig. 5A, a clear separation among samples from the 3 geographic areas was attained, with specimens from Galician and Malaysia

representing more homogeneous groups than those from Bangladesh. In this two-dimensional representation, the first component (PC1, 88% of explained variance) was mainly associated with toluene (compound B1, Table 2), and it allowed the discrimination of BD honeys from the rest. The second component (PC2, 4%) was useful to separate the latter two groups of samples (Fig. 5A). A second PCA model was built after removing the responses measured for toluene. In this case, the first two principal components justified only 45% of the total variance (Fig. 5B). The PC1 discriminated GAL from both groups of tropical samples. On the other hand, using two principal components, samples with the same geographic origin occupied rather different positions in the projection plot (i.e. likely to depend on their botanic origin) while sample MY-2 appeared in the same quadrant as BD honeys. Thus, selection of response variables represents a major issue for discrimination among different groups of samples.

5. Conclusions

GC–QTOF–MS analysis following HS SPME provides enough sensitivity for the identification of a relevant number of volatile and semi-volatile compounds in honey samples, with mass errors usually remaining below 1 mDa. Identified compounds can be classified in four major classes: benzene derivatives, non-aromatic carboxylic acids and esters, terpenes and nitrogenated compounds, all of which are known to be related with honey aroma. The accurate scan EI-MS spectra are of high usefulness to discriminate between compounds rendering ions with the same nominal masses, but having different empirical formulae. When combined with ion product accurate MS/MS and PCI spectra capabilities of the GC–QTOF–MS system, additional information is available for the identification of novel compounds, or at least for elucidation of their empirical formula, as demonstrated for two quinoline alkaloids in this study. Exploiting the huge amount of information provided by the GC–QTOF–MS system, particularly in the EI mode, requires the use of categorized data mining strategies. A suggested approach for compounds detection and identification, derived from experience acquired in this study, is integration of chromatographic peaks above a given threshold, followed by automated search in the NIST data base, and final comparison of accurate masses measured for ions in experimental spectra and those calculated for identified fragments in the NIST data base of candidate matches. For non-identified compounds, signal deconvolution and search of extracted compounds spectra are recommended.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.06.019.

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