Journal of Chromatography B, 877 (2009) 3776–3780

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Gas chromatographic and mass spectrometric characterization of the organic acids extracted from some preparations containing lyophilized royal jelly

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ARTICLE INFO

Article history:
Received 16 May 2009
Accepted 13 September 2009
Available online 17 September 2009

Keywords:
Royal jelly
Hydroxy fatty acids
Retention indices
Mass spectra

ABSTRACT

Thirty-five aliphatic acids were identified by gas chromatography–mass spectrometry from the ether extract of commercial preparations containing lyophilized royal jelly. The article presents linear-programmed retention indices on capillary columns with non-polar and low-polar stationary phases and mass spectra for identified compounds which were not characterized earlier by these parameters. Nine compounds are reported for the first time as royal jelly constituents.

1. Introduction

Royal jelly (RJ) is a secretion from the hypopharyngeal and mandibular glands of young worker bees (*Apis mellifera* L.) and is used to feed the larvae [1]. The unique feature of RJ is the set of short-chained hydroxy fatty acids [2,3]. 10-Hydroxy-2-decenoic acid (10-HDA), which is believed to be present only in RJ, is known for having various pharmacological effects [4–16].

Chemically, fresh royal jelly comprises water (50–70%), proteins (9–18%), carbohydrates (7–18%), fatty acids and lipids (3–8%), mineral salts (ca. 1.5%), and small amounts of polyphenols and vitamins. The lyophilized product contains <5% of water, 27–41% of proteins, 22–31% of carbohydrates and 15–30% of fats [11,12]. It was elucidated by Lercker et al. [3] that the major fatty acids of RJ consisted of 10-HDA and 10-hydroxydecanoic (10-HDAA) acids. Of particular interest has been the occurrence of 10-HDA, which appears to be an acid specific to the RJ [17]. For this reason, the 10-HDA content has been adopted as a marker for RJ and is used for royal jelly quality analysis [12,18]. However, on the whole the composition of the acid fraction of RJ has not been studied sufficiently up to now. In the available literature we managed to find only two publications, the authors of which have investigated with the aid of GC–MS the composition of fresh [5] and lyophilized [19] RJ CH_2Cl_2 and methanol extracts.

The objective of this study was the qualitative characterization of RJ acids by their gas chromatographic retention indices and mass spectra.

2. Experimental

2.1. Materials

Six samples of commercial preparations containing the lyophilized RJ were purchased from drug-stores in Poland, Latvia and Estonia (Table 1).

Pyridine, bis(trimethylsilyl)trifluoroacetamide (BSTFA) with addition of 1% trimethylchlorosilane and 12-hydroxydodecanoic acid were purchased from Sigma–Aldrich (Poznań, Poland). Extractions were carried out by diethyl ether and methanol (POCH SA, Gliwice, Poland).

2.2. Sample preparation and analysis

The friable contents of three capsules produced by Apipol–Farma with the total mass 790 ± 7 mg was transferred into a retort of 25 mL in volume and extracted, constantly stirred, by three portions of 15 mL of diethyl ether. After this, the non-soluble material was extracted with methanol (3× 15 mL). The duration of each extraction cycle at room temperature was 15 min. The joint extracts were filtered through a paper filter and the solvent was removed at 50 °C. The residue left on the walls was washed out (after its mass was determined) by 2 mL of ether or methanol, and 0.5 mL of this solution was put into a vial of 2 mL in

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doi:10.1016/j.jchromb.2009.09.016

volume. After evaporation of solvent, 220 μL of pyridine and 80 μL of BSTFA were added. The reaction mixture was sealed and heated during 0.5 h at 60 °C.

Three tablets of Apilac (produced in Latvia and Estonia) with the total mass 460 ± 15 mg were ground to dust state; the material obtained was subjected to the procedure described above. All experiments were performed in triplicates.

Solutions of trimethylsilyl (TMS) derivatives were analysed by GC–MS on a HP 6890 gas chromatograph with mass selective detector MSD 5973 (Agilent Technologies, USA). This device was fitted with a HP-5ms (low-polar) and HP-1ms (non-polar) fused silica columns (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium flow rate through the column was 1 mL/min. Injection of 1 μL of the sample was performed with the aid of HP 7673 autosampler. The injector (250 °C) worked in splitless mode for 5 min. The initial column temperature was 50 °C rising to 300 °C at 5 °C/min. The EIMS spectra were obtained at 70 eV of ionization energy. Detection was performed in the full scan mode from 41 to 600 a.m.u.

Hexane solution of C_{10}–C_{28} *n*-alkanes were separated under the above conditions. Linear temperature programmed retention indices (LTPRI) were calculated from the results of the separation of silylated RJ extracts.

To calibrate MSD, a series of five solutions of 12-hydroxydodecanoic acids in methanol covering the concentration range 20–2000 mg/L was prepared. 1 mL of calibration solution was transferred to the vial of 2 mL in volume. After evaporation of methanol, 220 μL of pyridine and 80 μL of BSTFA were added into the vial and heated at 60 °C during 0.5 h. TMS derivatives were subjected to GC–MS analysis in the conditions described above. On the basis of the analysis results, regression equation was calculated.

3. Results and discussion

3.1. Analytical procedure and chemical composition of extracts

In this investigation, the sample procedure includes successive extraction of RJ preparations with diethyl ether and methanol. Carboxylic acids are well soluble in slightly polar ether. In turn, methanol dissolves highly polar carbohydrate compounds: mono- and disaccharides and carbohydrate acids. The used procedure does not required expensive solvents and special equipment.

Table 1 contains (together with characteristics of the investigated commercial preparations) data on the average fractional composition of extracts. About 8.3% of the mass of RJ preparation produced by Apipol–Farma was extracted by ether and about 20.5% was transferred in methanol extract.

Fig. 1 presents part of the chromatogram of ether and methanol extracts from one of the investigated samples (RJ1). As it can be seen, the main part of the acid fraction of the preparation is identified in the ether extract. The results of six analyses of preparations RJ1–RJ3 showed that this part is 97.7 ± 2.1%. On the other hand, only small part of monosaccharides (3.0 ± 2.9%, $n = 6$) transferred into the ether solution. Consequently, triple ether extraction of lyophilized RJ allows sufficiently full extraction of aliphatic acids and exclusion of detrimental effects of monosaccharides, which are the main extractive components of RJ.

The described procedure turned out to be much less effective in the case of preparations produced in Latvia and Estonia (RJ4–RJ6): the fullness of extracting acids by ether from the tablets after their thorough grinding was only 71.4 ± 20.1% and did not get higher even when ultrasonic extraction was used. It is probably connected with the fact that the tablets contain such components as talc and potato starch, which form quite strong complexes with polar hydroxy acids.

Table 2 presents the list of 35 TMS derivatives of C_8, C_{10} and C_{12} aliphatic acids extracted by ether from preparations under the investigation. Eight of these acids were previously identified in extracts of mandibular glands taken from queen bees *A. mellifera carnica* [20], and 21 compounds was reported as RJ constituents by authors [2–5,19]. Despite our scrupulous search, we failed to identify 10-acetoxydecanoic, 10-acetoxydec-2-enoic, and 11-oxododecanoic acids, which were first found by authors [5] in lyophilized RJ from Greece. Nine components, which we detected by us for the first time, are homologues of the earlier identified in RJ mono- and dihydroxy acids and dicarboxylic acids. The complete list of compounds identified in ether and methanol extract from RJ1–RJ6 preparations is presented in Table 3 in Supplementary information in the WEB-edition of Journal. For comparison, the table also presents the chemical composition of ether extracts from two samples of fresh royal jelly.

In accordance with literature data [3,5], among the compounds listed in Table 2 the most abundant ones were 10-HDA, 10-HDAA, sebacic acid, 3,10-DDA, and additionally, 2-decene-1,10-dioic acid. Quantitative analysis with 12-hydroxydodecanoic acid being used as a standard shows average content of 10-HDA and 10-HDAA 21.3 ± 2.5 and 4.6 ± 0.6 mg/g ($n = 6$), respectively, in RJ1–RJ3 samples. These values are comparable to the ones obtained in work [5] for lyophilized RJ from Greece: 36 and 2.6 mg/g of 10-HDA and 10-HDAA, respectively.

In addition to the aliphatic acids, trace amounts of 4-hydroxybenzoic acid and 4-hydroxy methyl benzoate (HOB) were detected in all extracts. It is noteworthy that HOB and 9-hydroxy-2-decenoic acid, which was found in all the preparations, are ranked among the signal components (pheromone) of honey bee queen [20,23–25]. Besides, free aminoacids serine, β-alanine, proline, aspartic and pyroglutamic acids, glutamine and histidine were identified only in one of the preparations produced by Grindex Co.

Table 1
Characteristics of the investigated commercial preparations containing lyophilized RJ (according to information claimed on the container label) and fractional composition of extracts ($n = 3$).

Trade name (abbreviation)	Manufacturer, city, country (lot no.)	Content of lyophilized RJ and other ingredients	Fractional composition of extracts, mg (%)	
			Ether	Methanol
Mleczko pszczele (RJ1)	Apipol–Farma, Myślenice, Poland (130607)	100 mg per 1 capsule; glucose	60 ± 1 (28)	158 ± 2 (72)
Mleczko pszczele (RJ2)	The same (160108)	The same	66 ± 2 (28)	166 ± 2 (72)
Mleczko pszczele (RJ3)	The same (211107)	The same	70 ± 2 (30)	162 ± 2 (70)
Apilac Grindex (RJ4)	Grindex Co., Riga, Latvia (6941007)	10 mg per 1 tablet; lactose, calcium stearate, talc, potato starch	17 ± 3 (25)	52 ± 2 (75)
Apilac Grindex (RJ5)	The same (4880508)	The same	14 ± 1 (21)	54 ± 1 (79)
Apilac (RJ6)	Tallinn Pharmaceutical Factory, Tallinn, Estonia (50 10807)	The same	27 ± 2 (24)	86 ± 4 (76)

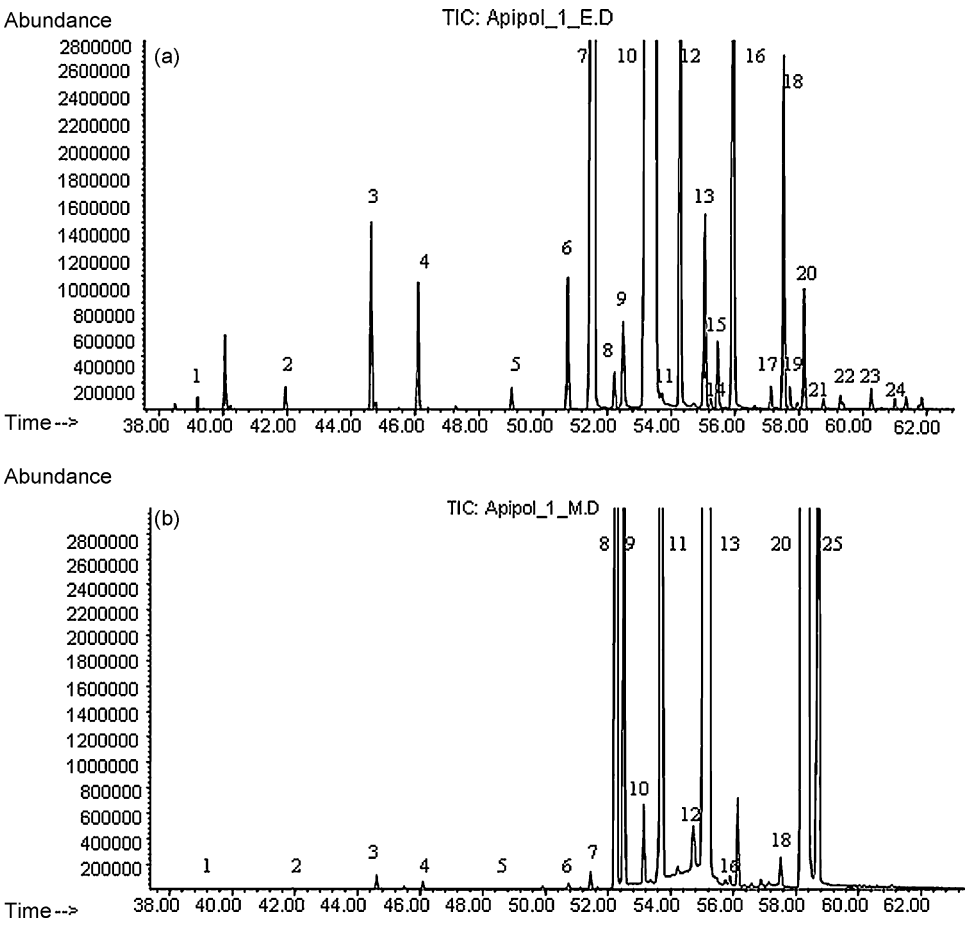

Fig. 1. Typical gas chromatograms of the TMS derivatives of compounds extracted from sample RJ1 by ether (a) and methanol (b): 1, 3-hydroxyoctanoic acid; 2, 7-hydroxyoctanoic acid; 3, 8-hydroxyoctanoic acid; 4, 3-hydroxydecanoic acid; 5, 9-hydroxydecanoic acid; 6, 9-hydroxy-2-decenoic acid; 7, 10-hydroxydecanoic acid (10-HDAA); 8, α-fructofuranose; 9, β-fructofuranose; 10, 10-hydroxy-2-decenoic acid (10-HDA); 11, α-glucofuranose; 12, 1,10-decanedioic acid; 13, α-glucopyranose; 14, 3,9-dihydroxydecanoic acid; 15, 11-hydroxydodecanoic acid; 16, 2-decene-1,10-dioic acid; 17, 11-hydroxy-2-dodecenoic acid; 18, 3,10-dihydroxydecanoic acid; 19, 12-hydroxydodecanoic acid; 20, β-glucopyranose; 21, hexadecanoic acid; 22, 12-hydroxy-2-dodecenoic acid; 23, 1,12-dodecanedioic acid; 24, 3,11-dihydroxydodecanoic acid; 25, gluconic acid.

Their content in the extracts was between 0.01–0.2% of total ion current (TIC).

3.2. Gas chromatographic and mass spectrometric characteristics of RJ acids

In our experiments, the components of ether extracts were identified by their fragmentation patterns and by comparison with available mass spectra (the NIST MS Library contains the spectra of 3- and 7-hydroxyoctanoic, 9-hydroxydecanoic, sebacic, 4-decene-1,10-dioic, 3-hydroxydecanedioic and dodecanedioic acids; mass spectra of 2-octene-1,8-dioic and some other unsaturated dioic acids are published in Ref. [22]). The mass spectra of the sample components agreed with those given in literature, when considering normal variability.

Molecular ion, or intensive ion $[M^+-15]$, which corresponds to the loss of one CH_3 group, were found in the spectra of all the compounds apart from the compound with LTPRI = 2173. Ions at *m*/*z* 73 and intensive ion peaks at *m*/*z* 147, which are characteristic of the molecules containing more than one TMS group, appeared to be common for all spectra. In the low-mass region of spectra there are also ions characteristic of different types of substitution. In the case of terminal (ω) substitution this is ion at *m*/*z* 103 ($[^+CH_2–O–Si(CH_3)_3]$, for ω − 1 acids this is very intensive ion at *m*/*z* 117, and for ω − 2 acids this is ion at *m*/*z* 131. In the high-mass region of spectra, ω hydroxy acids had an additional characteristic [M−31] ions; while the spectra of ω − 1 acids had [M−44] ions. Ion at *m*/*z* 233 (cleavage between C-3 and C-4 carbons) is characteristic of the spectra of linear mono- and dihydroxy acids, the third carbon atom of which has a OTMS group. The mass spectra of TMS derivatives of acids, the peaks of which had sufficient intensity on the chromatographs of extracts (not less than 0.5% of TIC) are presented in Supplementary information.

The presence of homologues and isomers of aliphatic acids in the samples also allows the determination of certain regularities of gas chromatographic behaviour of their TMS derivatives during the separation on a column with a stationary phase of low polarity. In each of the homologous series, the highest values LTPRI correspond to ω-substituted isomers of hydroxy acids. Displacement of a hydroxyl group by one carbon atom (ω − 1 acids) leads to a decrease of LTPRI by 72 ± 2 i.u. ($n = 8$). For ω − 2 acids this value is 87 ± 3 i.u. ($n = 5$). The appearance of one C=C bond in the molecules of both mono hydroxy acids and dicarboxylic acids increases LTPRI by 54 ± 2 i.u. ($n = 8$) in comparison with the corresponding saturated acids.

On non-polar stationary phase (like the polydimethylsilicone in HP-1 column), retention is due to the dispersion forces. For 5% phenylpolydimethyl silicone phase, dipole and induced dipole interactions of double bond with the phenyl group add to the dispersion forces. As a consequence, some difference could be expected in the values of LTPRI of unsaturated acids on these columns. This was indeed observed for C_8, C_{10} and C_{12} unsaturated

Table 2
Identification parameters of TMS derivatives of aliphatic acids extracted from RJ preparations.

TMS derivative of acid	Formula	MW	LTPRI ± δ		LTPRI[Lit]	Target ions, m/z (I)[a]
			HP-5ms	HP-1ms		
3-Hydroxyoctanoic[b,c]	$C_{14}H_{32}O_3Si_2$	304	1486 ± 3 (n = 12)	1486 ± 1 (n = 2)	1483 [21]	147(100), 73(43), 173(38), 233(37), 289(25)
7-Hydroxyoctanoic[b]	$C_{14}H_{32}O_3Si_2$	304	1555 ± 3 (n = 14)	1553 ± 1 (n = 3)	1555 [21]	117(100), 73(81), 75(48), 217(25), 147(18)
7-Hydroxy-2-octenoic[b]	$C_{14}H_{30}O_3Si_2$	302	1604 ± 3 (n = 14)	–	–	117(100), 147(95), 73(90), 75(60), 81(29)
8-Hydroxyoctanoic[c,d]	$C_{14}H_{32}O_3Si_2$	304	1624 ± 5 (n = 18)	1621 ± 2 (n = 3)	–	147(100), 289(98), 73(79), 75(66), 199(47)
3-Hydroxydecanoic[c,d]	$C_{16}H_{36}O_3Si_2$	332	1667 ± 4 (n = 18)	1663 ± 1 (n = 3)	–	73(100), 147(52), 233(32), 201(25), 317(12)
8-Hydroxy-2-octenoic	$C_{14}H_{30}O_3Si_2$	302	1675 ± 3 (n = 4)	1660 (n = 2)	–	147(100), 73(50), 287(32), 95(31), 81(23)
9-Hydroxydecanoic[b]	$C_{16}H_{36}O_3Si_2$	332	1750 ± 4 (n = 14)	1746 ± 2 (n = 3)	–	117(100), 73(71), 217(15), 317(14), 147(13)
2-Octene-1,8-dioic	$C_{14}H_{28}O_4Si_2$	316	1759 ± 4 (n = 9)	1740 ± 3 (n = 3)	1738 [22]	136(100), 75(65), 73(60), 147(51), 81(30)
8-Hydroxy-2-decenoic	$C_{16}H_{34}O_3Si_2$	330	1784 ± 3 (n = 3)	-	–	73 (100), 131(80), 211(51), 315(24), 81(23)
9-Hydroxy-2-decenoic[b], 9-HDA	$C_{16}H_{34}O_3Si_2$	330	1801 ± 2 (n = 12)	1788 ± 1 (n = 3)	–	117(100), 73(51), 147(34), 286(23), 81(18)
10-Hydroxydecanoic[b,c,d], 10-HDAA	$C_{16}H_{36}O_3Si_2$	332	1820 ± 4 (n = 20)	1815 ± 3 (n = 5)	–	317(100), 73(70), 75(60), 147(55), 227(32)
10-Hydroxy-2-decenoic[b,c,d], 10-HDA	$C_{16}H_{34}O_3Si_2$	330	1875 ± 3 (n = 17)	1858 ± 3 (n = 5)	–	147(100), 315(99), 73(61), 75(48), 81(46)
1,10-Decanedioic (sebacic)[d]	$C_{16}H_{34}O_4Si_2$	346	1904 ± 2 (n = 17)	1890 ± 1 (n = 5)	1904 [21]	73(100), 75(75), 331(33), 315(23), 129(21)
10-Hydroxydodecanoic[c]	$C_{18}H_{40}O_3Si_2$	360	1931 ± 2 (n = 10)	1926 ± 1 (n = 3)	–	331(100), 131(99), 73(76), 75(38), 217(29)
3,9-Dihydroxydecanoic[d]	$C_{19}H_{44}O_4Si_3$	420	1936 ± 2 (n = 8)	–	–	73(100), 117(86), 147(83), 305(46), 233(41)
11-Hydroxydodecanoic[c,d]	$C_{18}H_{40}O_3Si_2$	360	1944 ± 2 (n = 10)	–	–	117(100), 73(56), 345(20), 329(17), 147(16)
2-Decene-1,10-dioic[c]	$C_{16}H_{32}O_4Si_2$	344	1958 ± 2 (n = 14)	1934 ± 2 (n = 5)	–	73(100), 75(94), 136(82), 164(65), 119(53)
10-Hydroxy-2-dodecenoic	$C_{18}H_{38}O_3Si_2$	358	1982 ± 3 (n = 5)	1986 ± 2 (n = 3)	–	131(100), 329(89), 73(64), 147(29), 253(28)
11-Hydroxy-2-dodecenoic	$C_{18}H_{38}O_3Si_2$	358	1997 ± 3 (n = 5)	1991 ± 1 (n = 3)	–	117(100), 73(55), 253(37), 75(34), 343(28)
3,10-Dihydroxydecanoic[d], 3,10-DDA	$C_{19}H_{44}O_3Si_3$	420	2011 ± 2 (n = 19)	2008 ± 1 (n = 3)	–	73(100), 147(99), 233(69), 405(51), 75(29)
12-Hydroxydodecanoic[c]	$C_{18}H_{40}O_3Si_2$	360	2015 ± 2 (n = 8)	–	–	345(100), 73(81), 147(64), 255(46), 329(37)
8,9-Dihydroxydecanoic[d]	$C_{19}H_{44}O_4Si_3$	420	2025 (n = 2)	–	–	317(100), 73(37), 318(26), 147(20), 129(12)
12-Hydroxy-2-dodecenoic[c]	$C_{18}H_{38}O_3Si_2$	358	2070 ± 3 (n = 5)	2054 ± 3 (n = 3)	-	343(100), 147(77), 253(68), 73(56), 81(16)
3-Hydroxydecanedioic	$C_{19}H_{42}O_5Si_3$	434	2088 ± 4 (n = 3)	–	–	73(100), 147(85), 233(36), 217(29), 303(24)
1,12-Dodecanedioic[c]	$C_{18}H_{38}O_4Si_2$	374	2099 ± 2 (n = 6)	2087 ± 2 (n = 3)	2102 [21]	73(100), 75(60), 359(23), 217(19), 243(18)
3,10-Dihydroxydodecanoic	$C_{21}H_{48}O_4Si_3$	448	2111 ± 2 (n =6)	–	–	73(100), 131(96), 147(79), 233(35), 305(31)
3,11-Dihydroxydodecanoic[d]	$C_{21}H_{48}O_4Si_3$	448	2125 ± 1 (n = 6)	2127 ± 1 (n = 3)	–	73(100), 117(85), 147(77), 305(61), 233(50)
13-Hydroxytetradecanoic[c]	$C_{20}H_{44}O_3Si_2$	388	2140 ± 2 (n = 7)	2136	–	117(100), 73(51), 75(36), 373(15), 357(13)
10,11-Dihydroxydodecanoic[c]	$C_{21}H_{48}O_4Si_3$	448	2151 ± 1 (n = 6)	–	–	331(100), 73(36), 332(28), 147(24), 117(21)
2-Dodecene-1,12-dioic (traumatic)[c]	$C_{18}H_{36}O_4Si_2$	372	2154 ± 1 (n = 3)	2131	–	73(100), 192(57), 164(47), 357(47), 81(36)
11,12-Dihydroxydodecanoic[d]?	$C_{21}H_{48}O_4Si_3$	448	2172 ± 2 (n = 3)	–	–	73(100), 117(35)103(18), 271(12), 129(11)
3,12-Dihydroxydodecanoic	$C_{21}H_{48}O_4Si_3$	448	2198 ± 1 (n = 6)	–	–	147(100), 73(90), 233(67), 433(59), 217(25)
10,12-Dihydroxydodecanoic	$C_{21}H_{48}O_4Si_3$	448	2218 ± 2 (n = 7)	–	–	345(100), 73(57), 147(26), 129(16), 433(9)
3-Hydroxydodecanedioic[d]	$C_{21}H_{46}O_5Si_3$	462	2290 ± 2 (n = 3)	–	2274 [21]	75(100), 73(80), 147(75), 233(18), 305(11)
3,13-Dihydroxytetradecanoic	$C_{23}H_{52}O_4Si_3$	476	2314 ± 2 (n = 3)	–	–	117(100), 73(85), 147(67), 233(37), 461(11)

[a] In the order of decreasing of ion intensity.
[b] Compound identified in extracts of mandibular glands taken from adult queens of the honeybee [20].
[c] Compound was reported as fresh RJ constituent by authors [19].
[d] Compound was reported as lyophilized RJ constituent by authors [5].

acids: the appearance of double bond increases LTPRI on the HP-5 column by 15 ± 2 i.u. (n = 7) in comparison with HP-1 column.

It was established that close relationships exist between the chromatographic behaviour of solutes and their structure (Quantitative Structure–Chromatographic Retention Relationship, QS–CRR) [26]. These relationships may be useful for both calculating retention indices and confirmation of the structure of the compounds identified by MS. The linear temperature programmed retention indices of the identified acids, which belong to different homologous series, are connected by linear dependence with the number of carbon atoms in a molecule (Fig. 2). To construct graphs for ω, ω − 1 and 3-hydroxy acids we used LTPRI values from Table 2 and literature data [21] for homologues with less than eight carbon atoms.

The obtained correlating equations can be successfully used to calculate the indices of odd-numbered homologues with a satisfactory accuracy as well as to confirm the correctness of MS identification. For instance, the LTPRI value 1265.2, which was calculated for TMS derivative of 4-hydroxypentanoic acid, coincides with the one, which was found experimentally in the work [21], i.e. 1265 (this value was not used in calculating the coefficients in corresponding equation for ω − 1 acids).

Chromatograms of extracts RJ1–RJ3 reveal a peak of the component with the retention index 2137 ± 1. The mass spectrum was of a low quality (due to low intensity of a peak) that is why only main ions could be registered there. Among them, the most intensive ion was ion at m/z 117, which is characteristic of ω − 1 acid. In the high-mass region was a low-intensity ion at m/z 373 (probably ion [M+ −15]). On the basis of this limited data, the compound was tentatively identified as 13-hydroxytetradecanoic acid. The retention index value (2138.2) calculated on the basis of regression equation

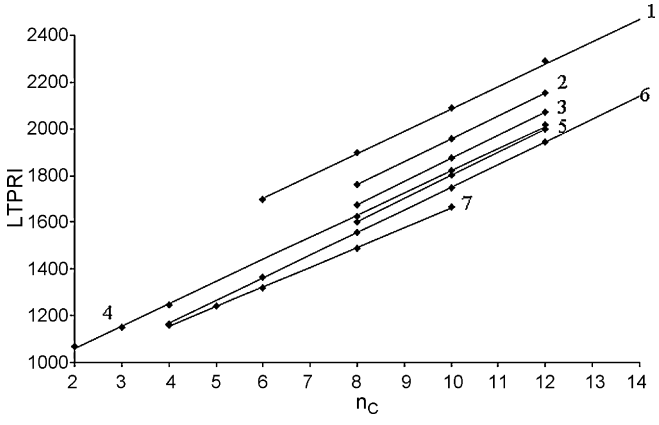

Fig. 2. Plot of LTPRI (HP-5MS column) against the number of carbon atoms in the carboxylic acid residues: 1, 3-hydroxydioic acids ($y = 98.25x + 1109.2$; $R^2 = 0.9997$); 2, unsaturated dioic acids ($y = 98.25x + 975.17$; $R^2 = 1$); 3, unsaturated ω-hydroxy acids ($y = 98.75x + 885.83$; $R^2 = 0.9999$); 4, saturated ω-hydroxy acids ($y = 94.928x + 870.8$; $R^2 = 0.9998$); 5, unsaturated (ω − 1)-hydroxy acids ($y = 98.25x + 818.17$; $R^2 = 1$); 6, saturated (ω − 1)-hydroxy acids ($y = 97x + 780.2$; $R^2 = 0.9997$); 7, saturated 3-hydroxy acids ($y = 84.397x + 816.98$; $R^2 = 0.9991$).

coincided with the experimental value, which can be viewed as a confirmation of the correct identification of this compound.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.09.016.

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