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Investigation of combwax of honeybees with high-temperature gas chromatography and high-temperature gas chromatography–chemical ionization mass spectrometry
II: High-temperature gas chromatography–chemical ionization mass spectrometry

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Abstract

Crude combwax of six various honey bee species have been analyzed by high-temperature gas chromatography (HTGC)–chemical ionization mass spectrometry after a two-step silylation procedure. An optimized chromatographic procedure, described previously, enables the separation of high-molecular mass lipid compounds resulting in a characteristic fingerprint of the combwaxes of different honeybee species. The coupling of HTGC to mass spectrometry requires appropriate instrumentation in order to achieve sufficient sensitivity at high elution temperatures and avoid loss of chromatographic resolution. Chemical ionization was carried out using methane as reagent gas in order to determine the molecular mass of the individual compounds by means of abundant quasi molecular ions. To confirm the presence of unsaturated wax esters, ammonia was used as reagent gas. More than 80 lipid constituents were separated and characterized by their mass spectra. Representative chemical ionization mass spectra of individual compounds are presented. Both, HTGC–flame ionization detection data and the results of the HTGC–mass spectrometric investigations enabled a rapid profiling of the individual classes of compounds in crude combwaxes.

Keywords: Beeswax; *Apis* spp.; Interfaces, GC–MS; Instrumentation; Mass spectrometry; Lipids

1. Introduction

The use of capillary gas chromatography offers a wide application field for the investigation of neutral lipids [1]. Due to the selectivity of various stationary phases not only compounds with a different chemical structure but also isomers, e.g., *cis*/*trans* and regioselective isomers, were separated sufficiently. The investigation of thermally stable compounds by gas chromatography is advantageous because of the high peak capacity of the method. High-temperature gas chromatography (HTGC) with an upper temperature limit of approximately 400°C, results in a remarkable extension of the application range. This analytical tool requires appropriate technical equipment, e.g., a

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high-temperature gas chromatograph and high-temperature stable capillary columns [2].

The coupling of wall-coated open tubular capillary columns to a chemical ionization source of a mass spectrometer was first reported by Arsenault et al. [3] and Blum and Richter [4–7]. The later authors introduced the separate introduction of carrier and reagent gas (dual gas arrangement) and the coaxial interface as the basis of the coupling of HTGC to an ion source which enables both, electron impact ionization (EI) and chemical ionization (CI).

The coupling of HTGC with EI/CI mass spectrometry (MS) requires a temperature stable interface which can be kept on constant high temperatures along the entire interface line, and an additional heater assembly between the very end of the interface capillary and the ion source in order to avoid cold spots and re-condensation of high-molecular mass compounds [8].

Chemical ionization is well known for the characterization and identification of neutral lipids, e.g., fatty acids, triacylglycerols, eicosanoids [9] and steroids [10]. The main advantage of this soft ionization method is the production of dominant quasi molecular ions.

This study reports on the investigation of crude combwax of various honeybees like *Apis mellifera* (western honeybee), *Apis cerana* (eastern honeybee), *Apis dorsata* (giant honeybee), *Apis laboriosa* (rock honeybee), *Apis florea* (dwarf honeybee), and *Apis andreniformis* (bush honeybee) by means of HTGC–MS using methane and ammonia as reagent gas.

Beeswax mainly consists of complex mixtures of hydrocarbons, free fatty acids, monoesters, diesters, triesters, hydroxy monoesters, hydroxy polyesters, fatty acid polyesters and some unidentified compounds. Each class of compounds consists of a series of homologues differing in chain length by two carbon atoms. With respect to the compounds containing exchangeable hydrogen atoms, e.g., free fatty acids and hydroxy esters, a two-step derivatization method was developed previously [11].

The HTGC–MS analyses were carried under CI conditions using methane (CI/CH_4) or ammonia (CI/NH_3) as reagent gas. To our knowledge, the CI mass spectra of some of the bees wax constituents, e.g., hydroxy monoester, diester and hydroxy diester, are reported for the first time.

2. Material and methods

2.1. Gas chromatography–mass spectrometry

A Carlo Erba Mega 5160 (Milan, Italy), equipped with an on-column injector, was coupled to a Finnigan TSQ-700 mass spectrometer (San Jose, CA, USA) via a specially designed high-temperature interface [8]. The gas chromatographic separation was achieved using a 10 m×0.2 mm Duran glass capillary column coated with poly(50% diphenyl/50% 1H,1H,2H,2H-perfluorodecylmethyl)siloxane (SOP-50-PFD, film thickness 0.10 μm) [12], which was connected to the interface capillary. The interface capillary consists of a Duran glass capillary (length, 60 cm; inner diameter, 0.2 mm; outer diameter, 0.80 mm), which was pretreated according to the procedure described in Ref. [13] using a mixture of hexamethyldisilazane and 1,3-diphenyltetramethyldisilazane (1:1, v/v) for persilylation. A high-temperature stable connection of the analytical column to the interfacing glass capillary was achieved by a wrap glass capillary (length, 4 cm; inner diameter, 0.82 mm) with widened ends. The expanding of the wrap capillary ends was carried out by a conical graphite needle obtained from a refill lead using the technique described by Grob [14]. The capillary was cleaned in an ultrasonic bath using 1 *M* aqueous sodium hydroxide, water and acetone, subsequently. Finally the coupling of the analytical column and interface was performed and sealed carefully with silver chloride, which was melted by a micro torch under reduced pressure of the running mass spectrometer (see Fig. 1) (J. Roeraade, private communication). The vacuum system of TSQ-700 mass spectrometer consists of two corrosive gas stable turbo molecular pumps (Type 360C, Balzers) at the analyzer chamber (bottom and right flange) and one turbo molecular pump (Type 360, Balzers) at the analyzer chamber of the manifold of the mass spectrometer, respectively.

Hydrogen was employed as carrier gas with a linear velocity of 0.5 m/s. The oven temperature was programmed from 80 to 380°C at a rate of 6°C/min. Chemical ionization was carried out using methane or ammonia. The ion source of the mass spectrometer was heated to a temperature of 180°C, filament current was 20 mA, electron multiplier voltage 1400

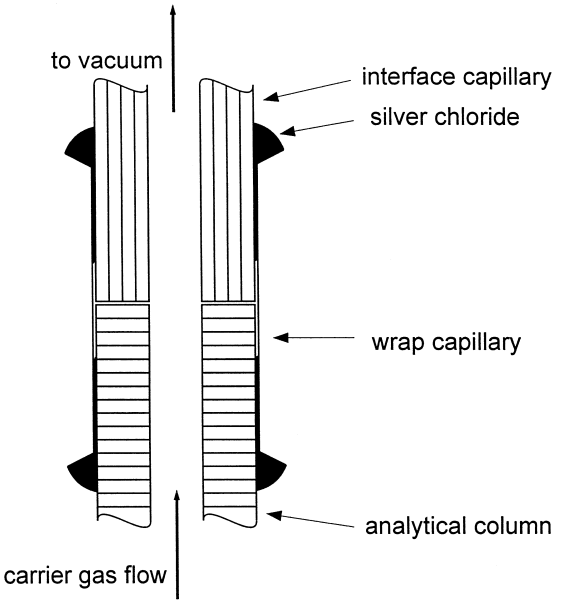

Fig. 1. High-temperature connection of analytical column and interface capillary for HTGC–MS.

V, the conversion dynode was set to 15 kV, and the acquisition mass range was 200–1100 u in 2 s.

2.2. Chemicals, samples and supplies

N-Methyl-*N*-*tert*.-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), used for derivatization of the combwax [11], and all solvents were purchased from Fluka (Buchs, Switzerland). The combwax of the following honey bees species was investigated: *Apis mellifera* (Kathmandu, Nepal), *Apis cerana* (Kathmandu, Nepal), *Apis dorsata* (Kathmandu, Nepal), *Apis laboriosa* (Kodari, Nepal), *Apis florea* (Chiang Rai, Thailand) and *Apis andreniformis* (Kalimantan, Indonesia). The collection of the wax samples was supported by local apiarists and/or honey hunters.

3. Results and discussion

3.1. Remarks with regard to the coupling of HTGC and MS

The coupling of HTGC to a MS requires not only adequate chromatographic equipment [2], but also a high-temperature stable coaxial interface which does not affect the chromatographic resolution, in particular at high column temperatures. The temperature gradient should be negligible, even at the upper temperature limit of the GC column, and the system should be heatable from the connection of column and interface to the entrance into the ion source. This can only be achieved by a supplementary heater assembly between the very end of the interface and the ion source in order to avoid recondensation of high-boiling compounds [8].

Hydrogen is the preferred carrier gas for HTGC [15]. Therefore the mass spectrometer should be equipped with a vacuum system that can pump off hydrogen sufficiently. In order to increase the pumping capacity, an additional turbo molecular pump was installed at the source chamber of the mass spectrometer. Moreover, the use of corrosive reagent gases, like ammonia, requires dedicated corrosion-resistant turbo molecular pumps (see description in Section 2).

A further critical point is the interface capillary between the gas chromatograph and the mass spectrometer. The use of corrosive reagent gases leads to a rapid decomposition of the outside coating of the fused-silica, leaving an uncoated and fragile silica capillary. Therefore, a deactivated glass capillary was favored as interface line, which was coupled to the analytical column by a wrap capillary. This chromatographic system can be heated up to 430°C without any loss of chemical and mechanical stability.

3.2. Chemical ionization of combwax constituents

Methane as reagent gas leads to a low response of alkanes, alkenes and dienes compared to the response of the fatty acids and ester components. Due to this systematic discrimination of these compounds it is not possible to compare the peak pattern of combwaxes of various honeybee species on the basis of the total ion current (TIC) chromatograms. Nevertheless, the dominant quasi molecular ions of the investigated combwax compounds obtained by chemical ionization are advantageous for the characterization of the individual classes of compounds. In

practice, the analysis of the composition of bees waxes requires both, the peak areas of the HTGC–flame ionization detection (FID) analyses for pattern recognition and the mass spectra of the HTGC–MS analyses for characterization of the individual compounds. In order to avoid confusion, the peak numbering of the FID chromatograms of Ref. [11] was used on the TIC chromatograms.

3.2.1. Alkanes and alkenes

The CH_4 chemical ionization mass spectra of alkanes, alkenes and alkadienes are described elsewhere, e.g., Ref. [16]. The spectra of unbranched alkanes are characterized by abundant $[M-H]^+$ ions, which are originated by hydride abstraction. The fragmentation of the quasi molecular ion yields in lower mass alkyl ions of minor intensity.

Abundant quasi molecular ions ($[MH]^+$ and $[M-H]^-$) of both, monounsaturated alkenes and diunsaturated alkenes are formed during the methane chemical ionization process. Fragment ions are only of minor intensity and can neither be used for the localization of double bonds nor for the differentiation between *cis*/*trans* isomers. Consequently, the characterization of unsaturated hydrocarbons with the same number of carbons, which could not be separated, is not possible.

Odd chain alkanes (C_{23}–C_{33}) and mono unsaturated alkenes (C_{27}–C_{39}) were detected in all investigated bees wax samples. The wax of *Apis andreniformis* contains diunsaturated alkenes (C_{35}–C_{41}). The broadened chromatographic peaks indicate the presence of different individual compounds with the same carbon number. The mass values and relative ion intensities of the molecular ions of the alkanes, alkenes and dienes are shown in Table 1.

Table 1
Observed mass values and abundances of molecular ions of alkanes, alkenes and dienes derived from HTGC-CI–MS analyses of combwax using methane as reagent gas[a]

	Peak[b]	MH^+	$[M-H]^+$
Alkanes			
C_{23}	1	–	323 (100)
C_{25}	3	–	351 (100)
C_{27}	10	–	379 (100)
C_{29}	17	–	407 (100)
C_{31}	22	–	435 (100)
C_{33}	26	–	463 (100)
C_{35}	30	–	491 (100)
Alkenes			
$C_{27:1}$	8	379(100)	377(60)
$C_{29:1}$	16	407(100)	405(63)
$C_{31:1}$, different isomers	21	435(100)	433(64)
$C_{33:1}$, different isomers	25	463(100)	461(83)
$C_{35:1}$, different isomers	29	491(100)	489(79)
$C_{37:1}$	34	519(100)	517(61)
$C_{39:1}$	38	547(100)	545(63)
$C_{41:1}$	41	575(100)	573(60)
Dienes			
$C_{35:2}$, different isomers	28	489(100)	487(95)
$C_{37:2}$, different isomers	33	517(100)	515(90)
$C_{39:2}$, different isomers	39	545(100)	543(93)
$C_{41:2}$, different isomers	40	573(100)	571(97)

[a] The ion abundances (%) are given in parentheses and are relative to the base peak.
[b] Peak nos. correspond to Figs. 3–8 and Table 2 in Ref. [11].

3.2.2. Free fatty acids

The homologous series of saturated, unbranched free fatty acids with even carbon numbers are well known as constituents of beeswax [17]. The applied two-step derivatization leads to *tert*.-butylmethylsilyl (TBDMS) derivatives of the fatty acids and carbon numbers between C_{20} and C_{36} were identified. Fig. 2 shows the CI/CH_4 mass spectrum of hexacosanoic acid TBDMS ester (peak 27) and is representative for this class of compounds (see Table 2). Characteristic features are the abundant quasi molecular ions MH^+ (m/z 623) and $[M-H]^+$ (m/z 621). The dominant fragment ions are insignificant for structure elucidation, because they are formed by fragmentation of the derivatizing group itself. The ions at m/z 607 and m/z 565 correspond to the loss of a methyl group and the *tert*.-butyl group, respectively, from the silyl moiety. Both types of ions are generated from odd electron molecular ions $[M^{+\cdot}]$ by charge exchange. The mass spectra of the dominant chromatographic peaks show characteristic ions of minor intensity, corresponding to the loss of *tert*.-butyldimethylsilanol $[MH\text{-}TBDMSOH]^+$.

3.2.3. Monoesters

The monoesters are important components of the beeswax lipids. The wax esters, identified by Tulloch [18] consist mainly of saturated wax esters, (alkyl palmitates; C_{38}–C_{52}, Fig. 3a), and unsaturated wax esters (alkyl oleates; C_{46}–C_{54}, Fig. 3b).

Methane and isobutane chemical ionization mass spectra of saturated wax esters were reported by

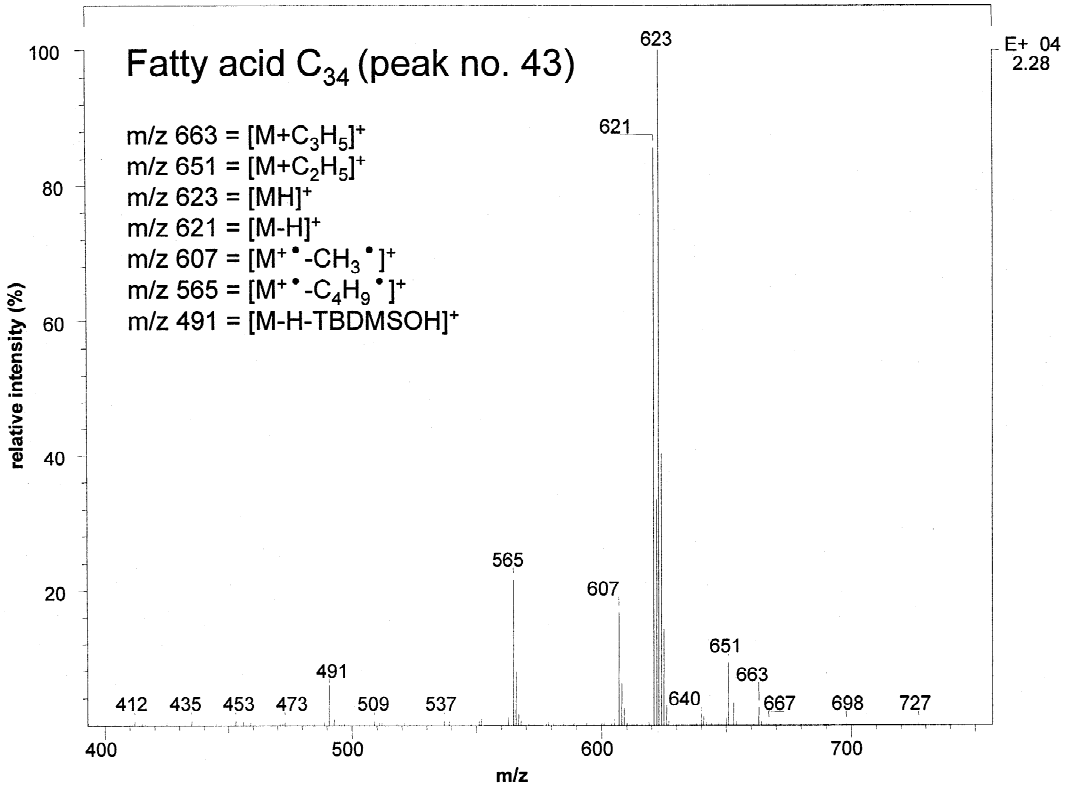

Fig. 2. Positive methane chemical ionization mass spectrum of fatty acid C_{34} acid *tert*.-butyldimethylsilylester (peak 43). The peak number corresponds to Table 2 in Ref. [11].

Table 2
Observed mass values and abundances of principle ions of fatty acids and fatty alcohols derived from HTGC–CI-MS analyses of combwax using methane as reagent gas[a]

	Peak[d]	MH^+	$[M-H]^+$	$[M^{+\cdot}-CH_3^{\cdot}]^+$	$[M^{+\cdot}-C_4H_9^{\cdot}]^+$	$[MH-TBDMSOH]^+$
Fatty acids[b]						
C_{20}	13	427 (87)	425 (100)	–	–	–
C_{22}	19	455 (83)	453 (100)	439 (37)	395 (49)	–
C_{24}	24	483 (93)	481 (100)	467 (33)	425 (47)	351 (12)
C_{26}	27	511 (100)	509 (84)	495 (41)	453 (54)	379 (9)
C_{28}	31	539 (83)	537 (100)	523 (29)	481 (50)	407 (8)
C_{30}	35	567 (80)	565 (100)	551 (29)	509 (60)	435 (7)
C_{32}	39	595 (92)	593 (100)	581 (26)	535 (53)	463 (10)
C_{34}	43	623 (87)	621 (100)	607 (28)	565 (44)	491 (6)
C_{36}	46	651 (80)	649 (100)	635 (9)	593 (22)	–
Fatty alcohols[c]						
C_{33}	32	553 (100)	551 (80)	539 (20)		
C_{35}	36	581 (100)	579 (85)	565(18)		

[a] The ion abundances (%) are given in parentheses and are relative to the base peak.
[b] Fatty acids were analyzed as TBDMS ester.
[c] Fatty alcohols were analyzed as TMS ether.
[d] Peak nos. correspond to Figs. 3–8 and Table 2 in Ref. [11].

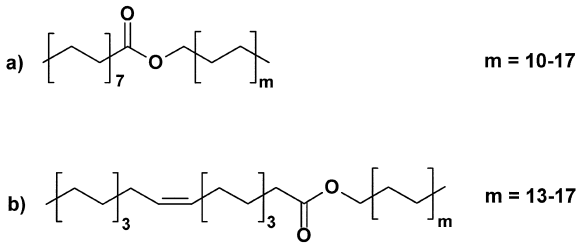

Fig. 3. Structure of alkyl esters of (a) palmitic acid and (b) oleic acid.

Plattner and Spencer [19]. The CI/CH_4 mass spectra of the saturated wax esters show intensive protonated quasi molecular ions MH^+. The base peaks correspond to the hydride-extracted molecular ions $[M-H]^+$. Adduct ions $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ are of low intensities, as are the ions $[MH-RCOOH]^+$ and $[M-H-RCOOH]^+$ formed by loss of the fatty acid. Structure-relevant acylium ions RCO^+ (m/z 239) of the palmitic acid moiety and the protonated palmitic acid (m/z 257) are of minor intensity (see Fig. 4 and Table 3).

The identification of monoenoic wax esters is difficult since the saturated and monoenoic wax esters could not be separated on the chosen stationary phase SOP-50-PFD. CI/CH_4 mass spectra of monoenoic wax esters, obtained at the front side of the respective GC peaks, show abundant quasi molecular ions MH^+ and $[M-H]^+$. In contrast to the saturated wax esters, protonated quasi molecular ions are the base peaks and only little fragmentation was observed. Due to the low concentration, the characterization of the monoenoic wax esters is hampered by the ion intensity. Therefore, CI/NH_3 as reagent gas was used to verify the CI/CH_4 results. The CI/NH_3 mass spectra of saturated and unsaturated wax esters show abundant MH^+ and $[M+NH_4]^+$ quasi molecular ions (Table 3). Fig. 5 shows the

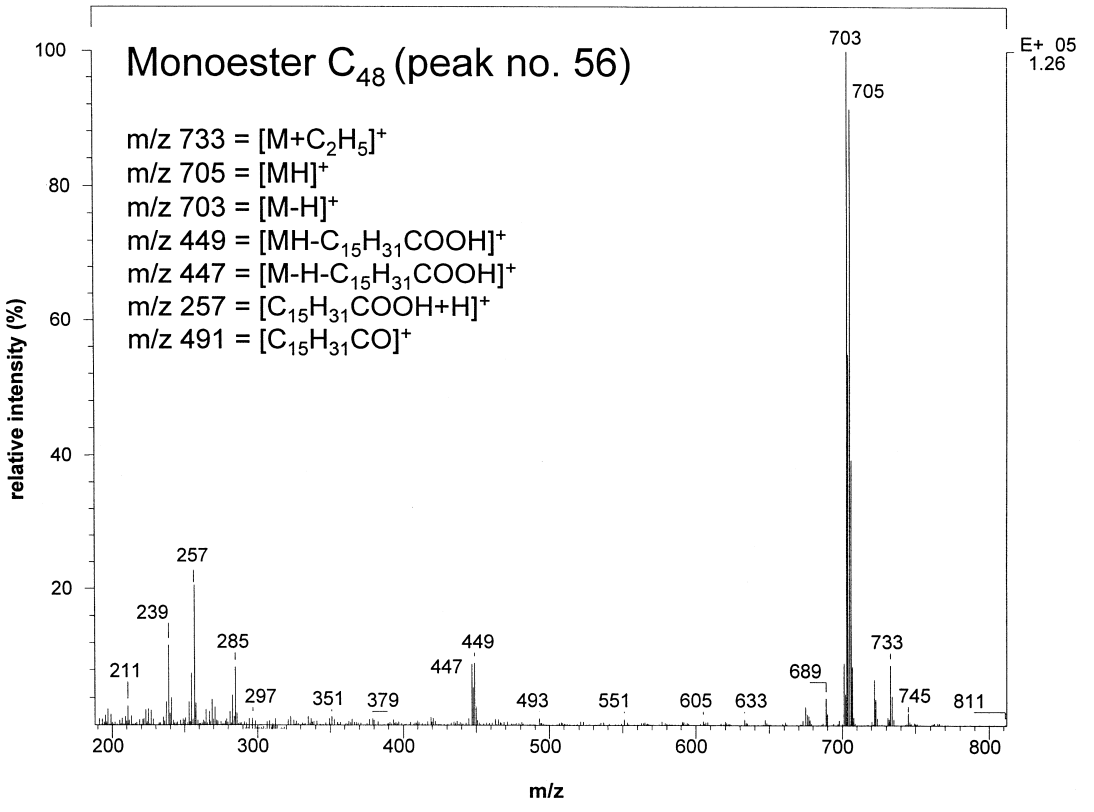

Fig. 4. Positive methane chemical ionization mass spectrum of monoester C_{48} (peak 56). The peak number corresponds to Table 2 in Ref. [11].

Table 3
Observed mass values and abundances of principle ions of monesters derived from HTGC–CI-MS analyses of combwax using methane and ammonia as reagent gas[a]

	Peak[b]	CI/CH_4				CI/NH_3
		$MH^+/[M-H]^+$	$[MH-RCOOH]^+/[M-H-RCOOH]^+$	$[RCOOH_2]^+$	$[RCO]^+$	$[M+NH_4]^+/MH^+$
Monoesters						
$C_{38:0}$	42	565(93)/563(100)	309(25)/–	257(10)	–	582(100)/565(45)
$C_{40:0}$	44	593(89)/591(100)	337(27)/335(14)	257(34)	239(18)	610(100)/593(45)
$C_{42:0}$	47	621(78)/619(100)	365(24)/363(13)	257(18)	239(15)	638(100)/621(44)
$C_{44:1}$	50	–	–	–	–	664(100)/647(43)
$C_{44:0}$		649(66)/647(100)	393(26)/391(10)	257(24)	239(18)	666(100)/649(43)
$C_{46:1}$	53	675(100)/673(47)	–	–	–	692(100)/675(42)
$C_{46:0}$		677(89)/675(100)	421(15)/419(9)	257(22)	239(8)	694(100)/674(44)
$C_{48:1}$	56	703(100)/701(48)	–	–	–	720(100)/703(94)
$C_{48:0}$		705(92)/703(100)	449(10)/447(8)	257(22)	239(15)	722(100)/705(45)
$C_{50:1}$	60	731(100)/729(57)	–	–	–	748(100)/731(90)
$C_{50:0}$		733(90)/731(100)	477(10)/475(10)	–	–	750(100)/733(40)
$C_{52:1}$	66	759(100)/757(52)	–	–	–	776(100)/759(85)

[a] The ion abundances (%) are given in parentheses and are relative to the base peak.
[b] Peak nos. correspond to Figs. 3–8 and Table 2 in Ref. [11].

partial CI/NH_4 mass spectra of the saturated ($C_{48:0}$) and unsaturated ($C_{48:1}$) wax ester. The ratio of unsaturated wax esters to the respective saturated wax esters becomes greater with increasing chain length. This finding is in agreement to the earlier investigation of Tulloch [17].

3.2.4. Hydroxy monoesters

Two types of hydroxy monoesters were characterized by Tulloch [18]. The first group consists of long-chain alcohols with different chain length, esterified by a hydroxy acid, mainly 15-hydroxypalmitic acid (Fig. 6a). The second group consists of long-chain diols with different chain length, esterified at the primary hydroxy group leaving the secondary hydroxyl group free (Fig. 6b). The predominant fatty acid of this type of hydroxy esters is palmitic acid. In the present study this class of compounds was determined by HTGC–MS as trimethylsilyl (TMS) ether with carbon numbers between C_{40} and C_{54}.

The molecular mass of the hydroxy esters was readily identified in all cases via the dominant $[MH]^+$ and $[M-H]^+$ quasi molecular ions obtained by chemical ionization using methane as reagent gas. The additional adduct ions $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ are of little intensity and could be observed only for dominant chromatographic peaks. The charge exchange fragment ion $[M^{+\cdot}-{}^{\cdot}CH_3]^+$ has only moderate intensity. The elimination of trimethylsilanol (TMSOH) from the molecular ions leads to abundant ions and is characteristic for trimethylsiloxy group. Weak ions which were observed only at intensive chromatographic peaks correspond to the elimination of trimethylsiloxy palmitic acid (e.g., m/z 337 of peak 49). Fig. 7 shows the CI/CH_4 spectrum of the trimethylsilylated hydroxy monoester C_{40} (peak 49, Table 2 of Ref. [11]).

Two isomers of hydroxy monoesters with the same carbon number (e.g., peaks 51 and 52, Table 2 of Ref. [11]) were observed as shown in Fig. 8. The isomers could not be distinguished by the respective CI mass spectra. Mass values and ion abundances of the hydroxy monoesters obtained by CI/CH_4-MS are shown in Table 4.

3.2.5. Diesters

The diesters in beeswax have a chain length of C_{54}–C_{64}, and consist of two types: diol diesters (Fig. 9a) and acylated hydroxy esters (Fig. 9b) [18].

The CI/CH_4 mass spectra of the diesters show abundant $[MH]^+$ and $[M-H]^+$ quasi molecular ions (Table 5). Intensive fragment ions are formed by the

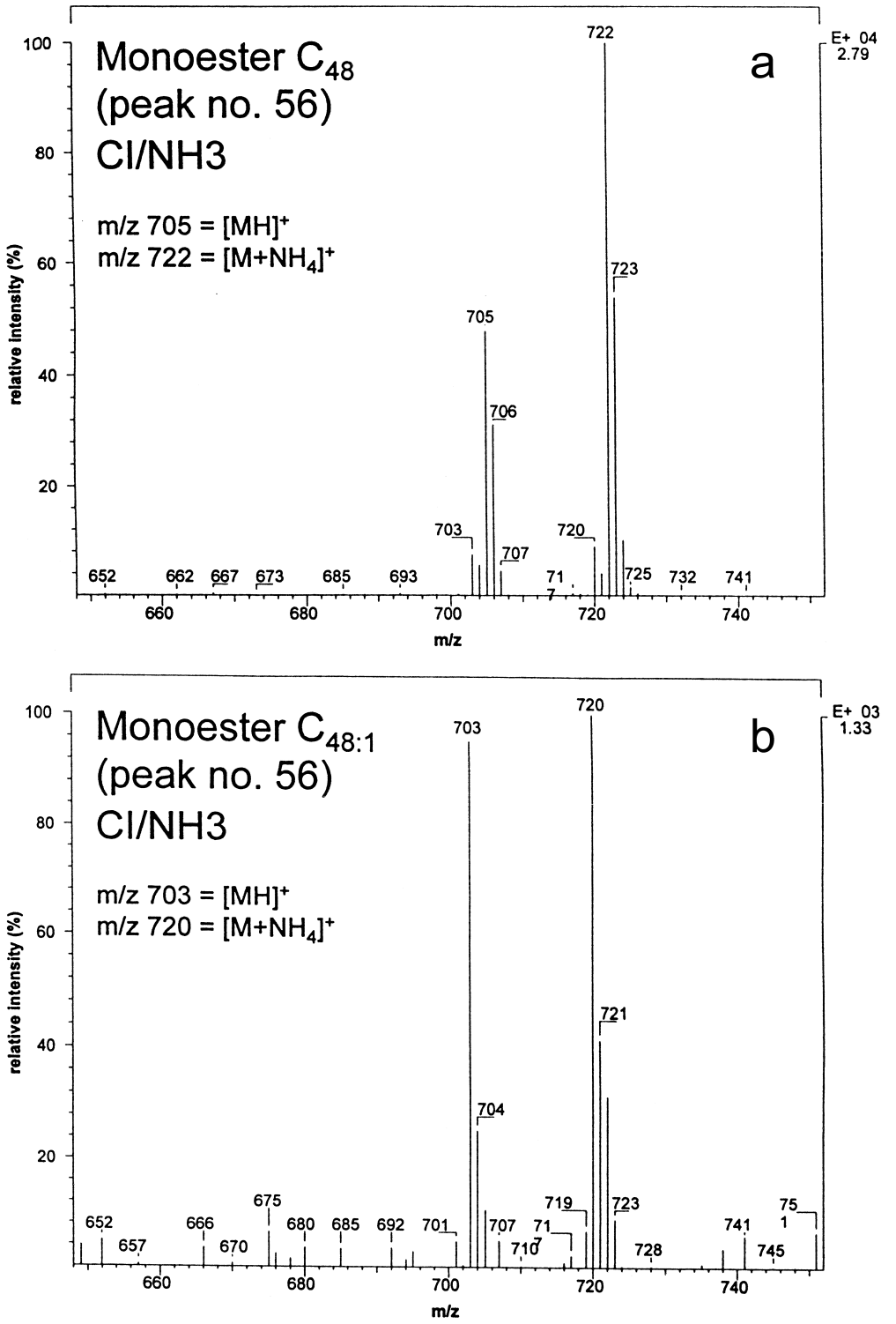

Fig. 5. Partial mass spectra (positive ammonia chemical ionization) of (a) saturated monoester $C_{48:0}$ and (b) unsaturated monoester $C_{48:1}$ (peak 56). The peak number corresponds to Table 2 in Ref. [11].

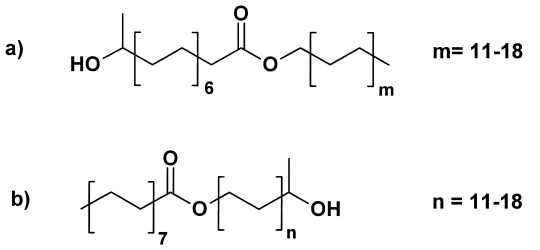

Fig. 6. Structures of hydroxy monoesters: (a) hydroxypalmitic acid esters, (b) palmitic acid diol esters.

loss of palmitic acid $[MH–C_{16}H_{31}COOH]^+$ and by the protonated palmitic acid $[C_{16}H_{31}COOH_2]^+$. The alcohol moiety is indicated by an ion equivalent to $[C_nH_{2n+1}]^+$, e.g., m/z 337 as shown in Fig. 10.

The HTGC–MS analyses of the combwax enables the chromatographic distinction of two isomers with nearly identical mass spectra (e.g., peaks 75 and 76, Fig. 11, see also Table 2 of Ref. [11]. The reconstructed ion chromatograms (RICs) over the molecular mass range of the diesters (m/z 900–1000), of the combwaxes of *Apis mellifera* and *Apis andreniformis* is shown in Fig. 11. The comparison of RIC and FID chromatograms (see Ref. [11]) shows nearly identical peak pattern. This allows an unambiguous assignment of the peaks and subsequent characterization by HTGC–MS of the individual compounds even at high elution temperatures.

3.2.6. Hydroxy diesters

Hydroxy diesters are detected only in minor quantities (1–4%). These complex esters were analyzed as TMS-ether derivatives and were never reported before. The homologous hydroxy diesters are detected in the same retention range as the diester homologues and a chain length of C_{50}–C_{58} could be determined by the HTGC–MS analyses. The pro-

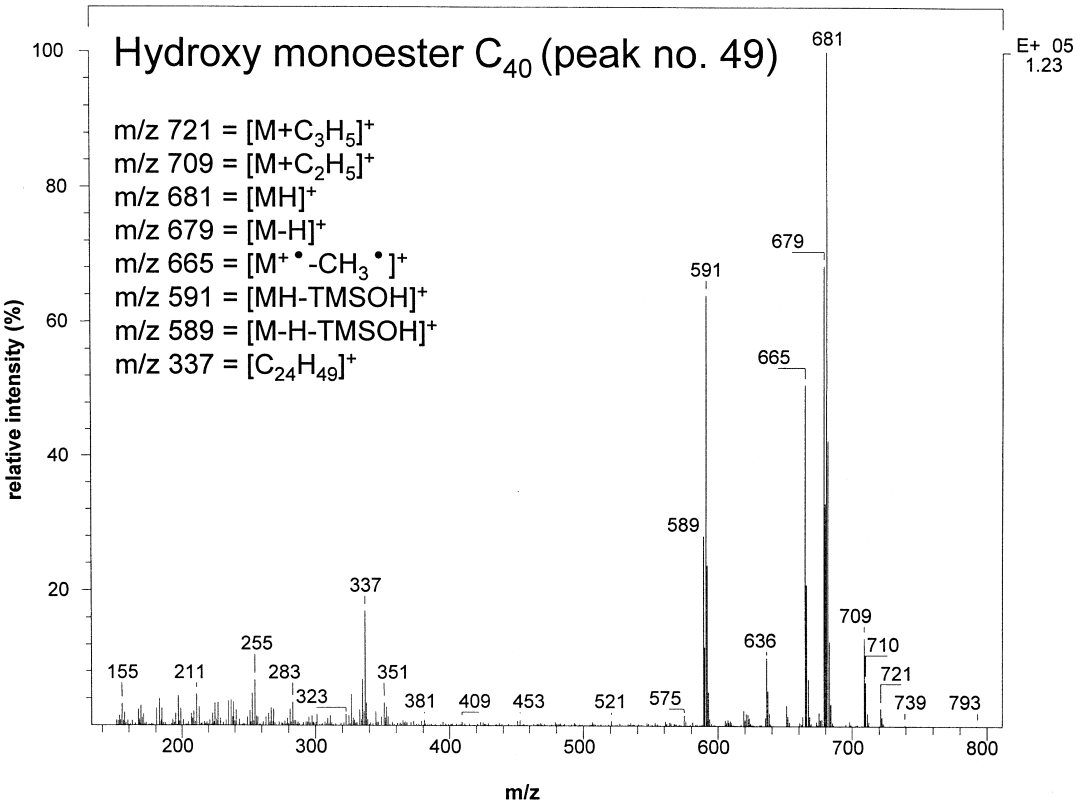

Fig. 7. Positive methane chemical ionization mass spectrum of trimethylsilylated hydroxymonoester C_{40} (peak 49). The peak number corresponds to Table 2 in Ref. [11].

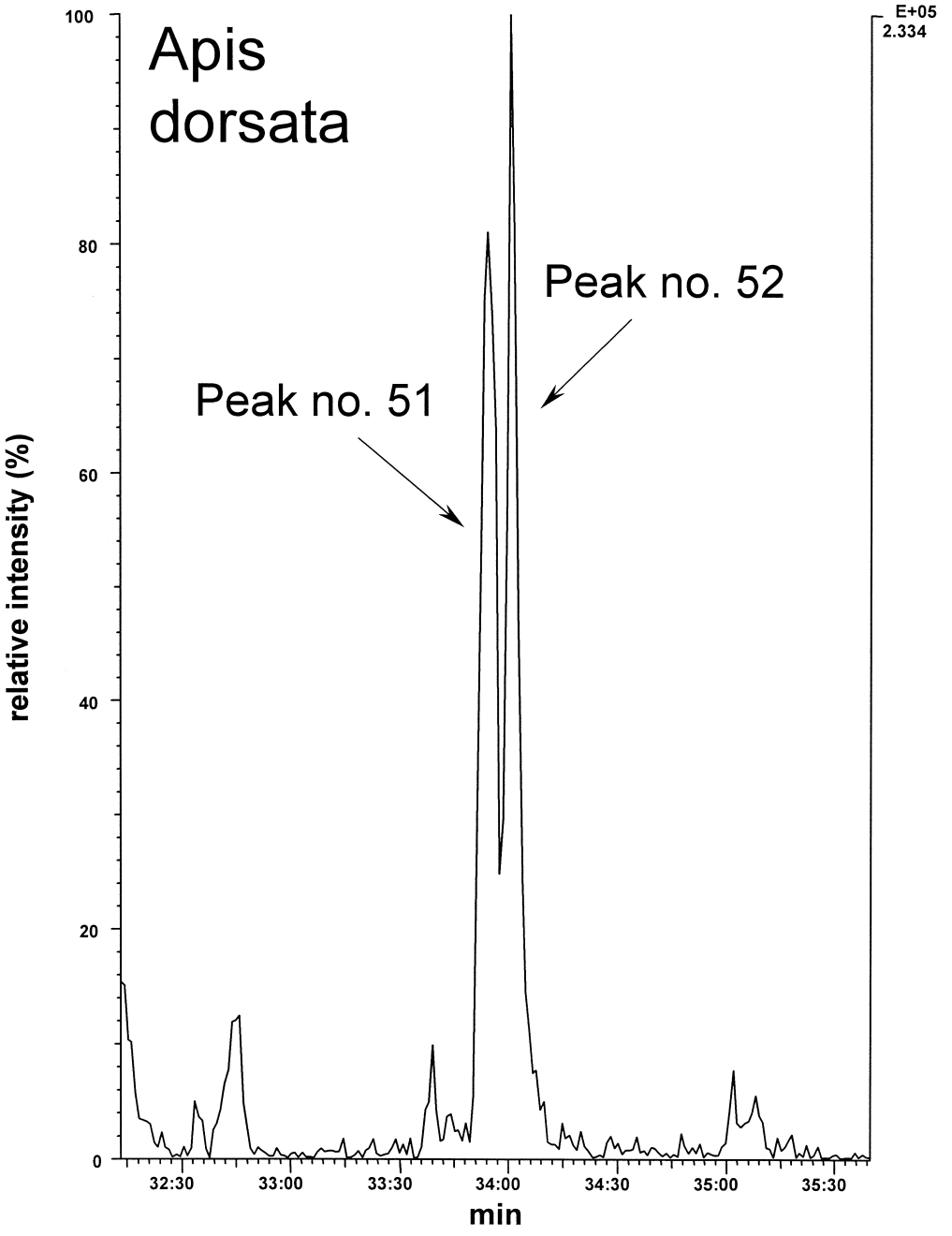

Fig. 8. Selected ion chromatogram over m/z 709 ($[MH]^+$), m/z 707 ($[M-H]^+$), m/z 693 ($[M^{+\cdot}-{}^{\cdot}CH_3]^+$), and m/z 619 ($[MH-TMSOH]^+$ for detection of the isomers of hydroxy monoester C_{42} in the combwax of *Apis dorsata* (peaks 51 and 52). The peak numbers correspond to Table 2 in Ref. [11].

Table 4
Observed mass values and abundances of molecular ions of hydroxy monoesters and hydroxy diesters derived from HTGC–CI-MS analyses of combwax using methane as reagent gas[a]

	Peak[c]	$MH^+/[M-H]^+$	$[M^{+\cdot}-CH_3^{\cdot}]^+$	$[MH-TMSOH]^+/[M-H-TMSOH]^+$
Hydroxy monoesters[b]				
$C_{40,}$ two isomers	48/49	681(100)/679(72)	665(54)	591(68)/589(35)
$C_{42,}$ two isomers	51/52	709(88)/707(100)	693(60)	619(85)/617(37)
C_{44}, two isomers	54/55	709(93)/707(100)	721(93)	647(85)/645(38)
C_{46}, two iosmers	57/58	737(100)/735(65)	749(65)	675(53)/673(22)
C_{48}, two isomers	61/62	765(100)/763(75)	777(53)	703(74)/701(34)
C_{50}, two isomers	64/65	793(100)/791(99)	805(57)	731(71)/729(53)
C_{52}	66	821(100)/819(98)	833(56)	759(60)/757(48)
Hydroxy diesters[b]				
C_{50}	71	935(100)/933(52)	919(36)	845(71)/843(54)
C_{52}	74	963(100)/961(48)	947(14)	873(67)/871(28)
C_{54}	77	991(100)/989(55)	975(24)	901(87)/899(48)
C_{56}	80	1019(82)/1017(61)	1003(11)	929(100)/(62)
C_{58}	82	1047(63)/1045(47)	1031(22)	957(100)/955(96)

[a] The ion abundances (%) are given in parentheses and are relative to the base peak.
[b] Hydroxy monoesters and hydroxy diesters were analyzed as TMS ethers.
[c] Peak nos. correspond to Figs. 3–8 and Table 2 in Ref. [11].

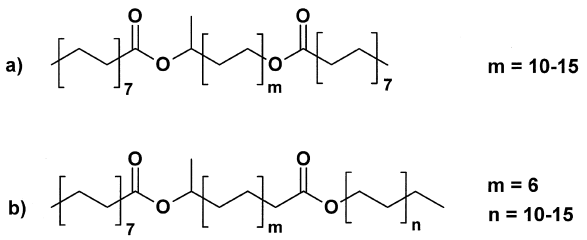

Fig. 9. Structures of diesters: (a) diesters of diols, (b) acylated hydroxy acid esters.

posed structures are hydroxypalmitic acid ester, acylated by hydroxypalmitic acid (Fig. 12b), or palmitic acid diolesters (Fig. 12b), acylated by hydroxypalmitic acid.

Due to the low concentration of this class of compounds the quality of related mass spectra is reduced. The CI/CH_4 mass spectra of the trimethylsilylated hydroxy diesters show abundant $[MH]^+$ and $[M-H]^+$ quasi molecular ions. Adduct ions $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ are of less intensity. The

Table 5
Observed mass values and abundances of molecular ions of diesters derived from HTGC–CI-MS analyses of combwax using methane as reagent gas[a]

Diesters	Peak[b]	$MH^+/[M-H]^+$	$[MH-RCOOH]^+/[M-H-RCOOH]^+$	$[RCOOH_2]^+$
C_{54}, two isomers	67/68	847(100)/845(75)	591(33)/589(8)	257(25)
C_{56}, two isomers	69/70	875(85)/873(100)	619(35)/617(9)	257(23)
C_{58}, two isomers	72/73	903(100)/901(90)	647(33)/645(6)	257(24)
C_{60}, two isomers	75/76	931(100)/929(98)	675(31)/673(5)	257(27)
C_{62}, two isomers	78/79	959(84)/957(100)	703(35)/701(6)	257(25)
C_{64}	81	987(62)/985(100)	731(33)/731(6)	257(23)

[a] The ion abundances (%) are given in parentheses and are relative to the base peak.
[b] Peak nos. correspond to Figs. 3–8 and Table 2 in Ref. [11].

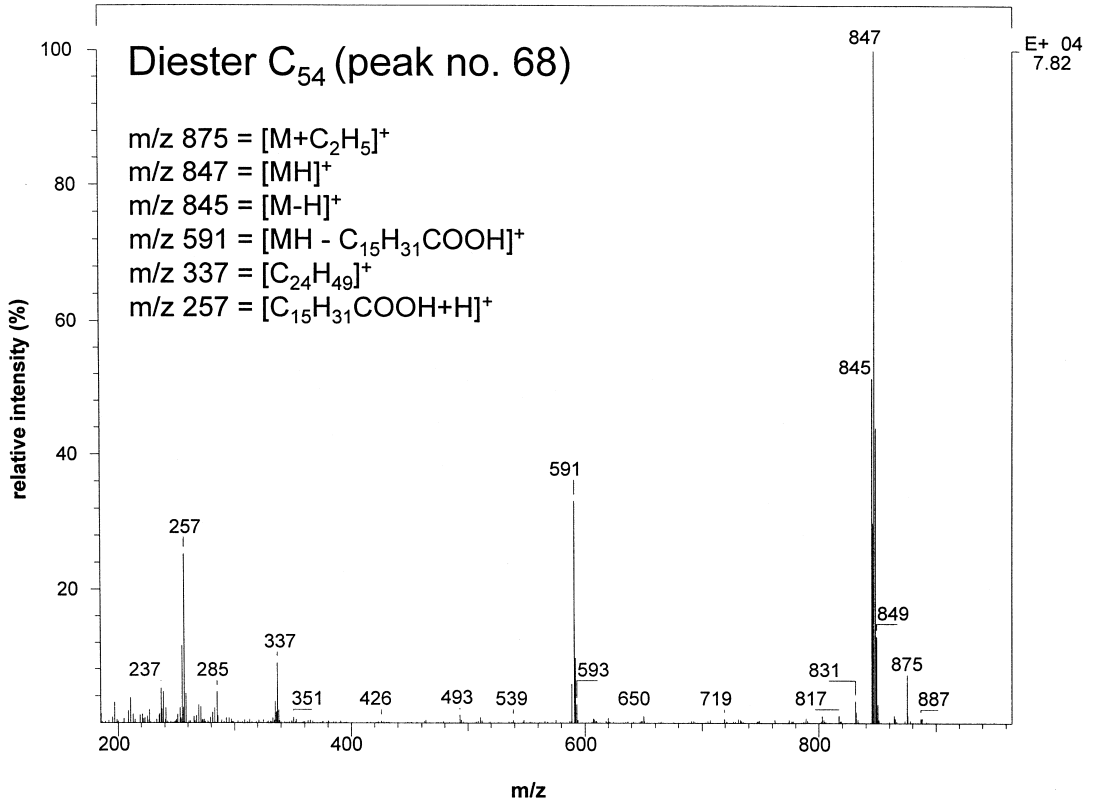

Fig. 10. Positive methane chemical ionization mass spectrum of diester C_{54} (peak 68). The peak number corresponds to Table 2 in Ref. [11].

predominant ions are $[M^{+\cdot}-{}^{\cdot}CH_3]^+$, $[MH-TMSOH]^+$ and $[M-H-TMSOH]^+$ (see also Table 4). The CI/CH_4 mass spectrum of the hydroxy diester C_{54} (peak 77), shown in Fig. 13, is representative for these compounds. For full structure elucidation, subsequent investigations with complementary spectroscopic methods are indispensable.

3.2.7. Fatty alcohols

Free fatty alcohols are minor ingredients of bees waxes. Two odd chain homologous compounds were identified in the wax of *A. mellifera* with a chain length of C_{33} (0.3%) and C_{35} (0.3%). The fatty alcohol C_{33} was also detected in *A. cerana* (1.8) and *A. florea* (0.4%). The mass spectra of both compounds show intensive $[MH]^+$ and $[M-H]^+$ quasi molecular ions and charge exchange fragments of the type $[M^{+\cdot}-{}^{\cdot}CH_3]^+$ with minor intensity. The relative ion abundances are shown in Table 2.

4. Conclusions

HTGC–CI-MS was used for the analysis of crude bees waxes. The mass spectral data obtained by methane and ammonia chemical ionization were used to characterize the individual series of homologous lipid compounds of bees wax. Methane as reagent gas leads to abundant quasi molecular ions and some characteristic fragmentation of the respective molecular species. The separation of unsaturated and saturated wax esters cannot be sufficiently achieved by the chosen stationary phase SOP-50-PFD. In order to confirm the presence of the unsaturated wax esters ammonia was used as reagent gas.

Regarding the complex composition of combwax, a final structure elucidation of each molecular species cannot be attained by HTGC–MS. However, in context with HTGC–FID analyses, the characterization of the molecular species by HTGC–CI-MS

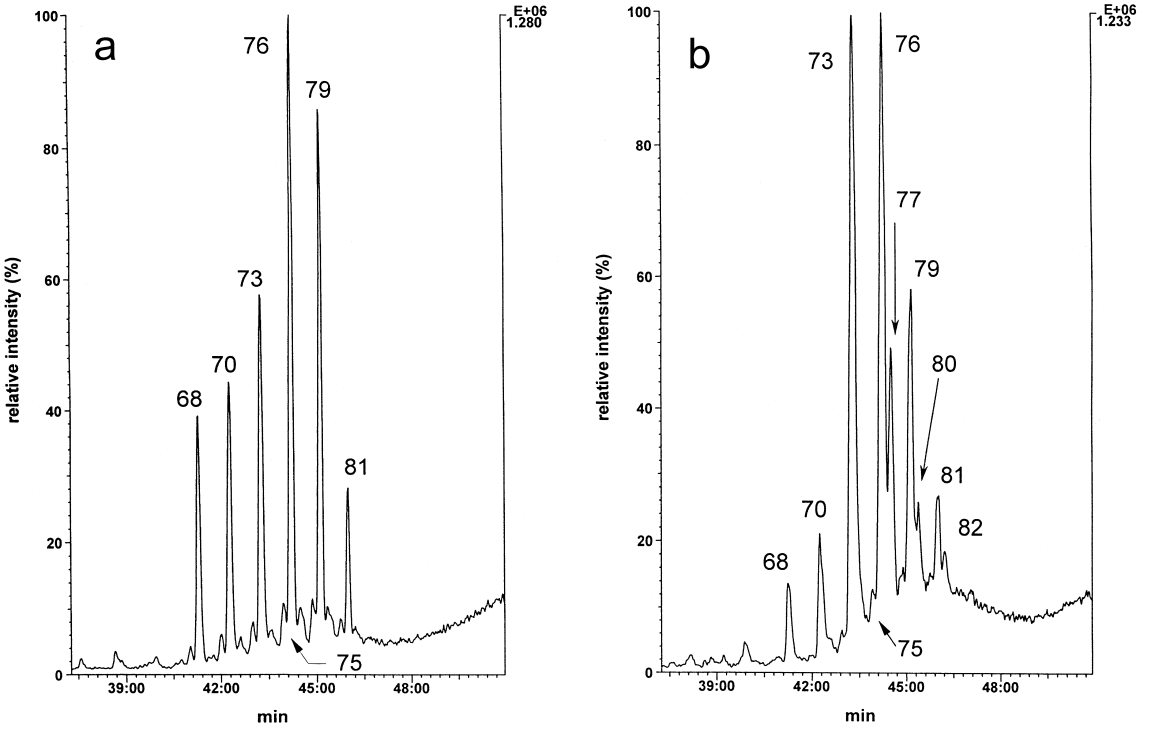

Fig. 11. Ion chromatogram over m/z 820–1100 for the comparison of the peak patterns of diesters and hydroxydiesters in the combwax of (a) *Apis mellifera* and (b) *Apis andreniformis*. The peak numbers correspond to Table 2 in Ref. [11].

enables a rapid and expressive profiling of the individual classes of compounds in crude combwax, even at elution temperatures of 380°C. The amounts of the various compound classes are determined and the proportions of the molecular species give information of the chain-length distribution of the constituents. To our knowledge, methane chemical ionization mass spectra of hydroxy monoesters, diesters and hydroxy diesters were described for the first time.

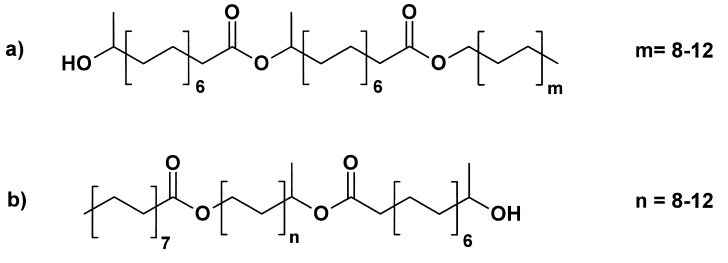

Fig. 12. Proposed structures of hydroxy diesters: (a) hydroxypalmitic acid esters acylated by hydroxypalmitic acid, (b) palmitic acid diol esters acylated by hydroxypalmitic acid.

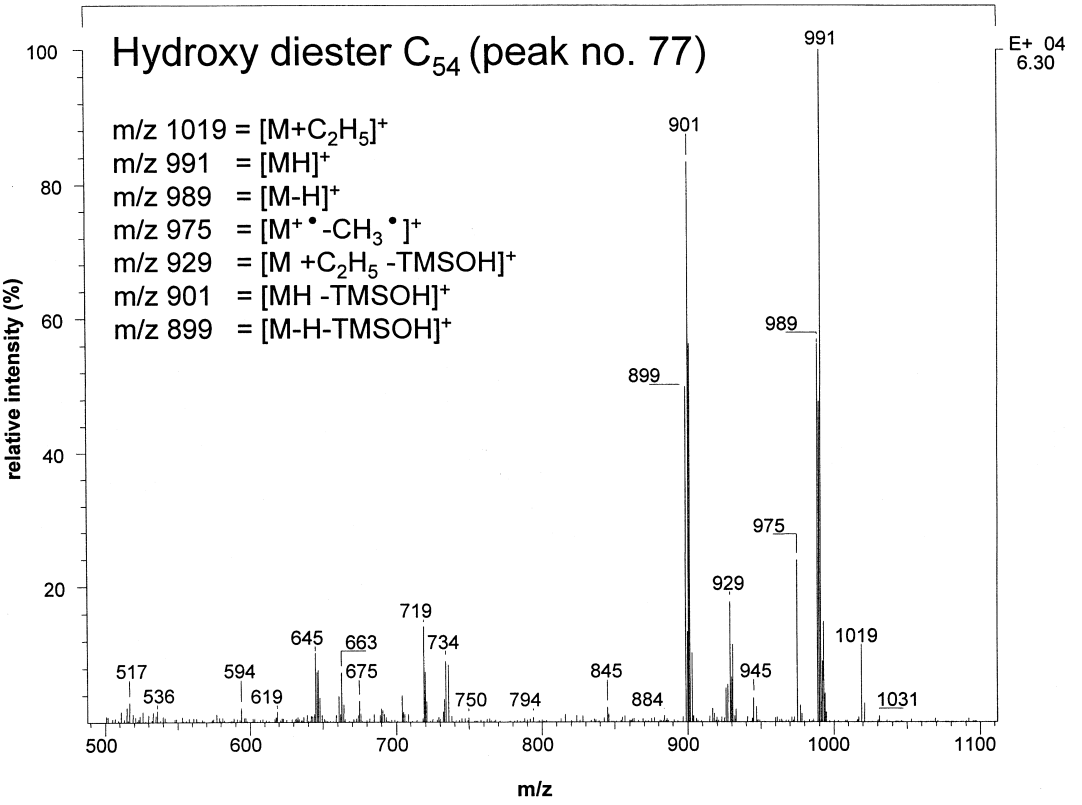

Fig. 13. Positive methane chemical ionization mass spectrum of trimethylsilylated hydroxy diester C_{54} (peak 77). The peak number corresponds to Table 2 in Ref. [11].

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References

[1] W.W. Christie, in: Gas Chromatography and Lipids, 2nd ed., Oily Press, Dundee, 1992, and Refs. cited therein.
[2] W. Blum, R. Aichholz, in: Hochtemperatur Gas Chromatographie, Hüthig Verlag, Heidelberg, 1991.
[3] G.P. Arsenault, J.J. Dolhun, K. Biemann, Chem. Commun. 1 (1970) 1542.
[4] W. Blum, W.J. Richter, Tetrahedron Lett. 11 (1973) 835.
[5] W. Blum, W.J. Richter, Finnigan Spectra 4 (1974) 1.
[6] W. Blum, W.J. Richter, Finnigan Spectra 5 (1975) 3.
[7] W. Blum, W.J. Richter, J. Chromatogr. 132 (1977) 249.
[8] W. Blum, P. Ramstein, G. Eglington, J. High Resolut. Chromatogr. 13 (1990) 85.
[9] R.C. Murphy, in: Mass Spectrometry of Lipids, Plenum Press, New York, 1993, and Refs. cited therein.
[10] Y.Y. Lin, L.L. Smith, Mass Spectrom. Rev. 3 (1984) 319, and Refs. cited therein.
[11] R. Aichholz, E. Lorbeer, J. Chromatogr. 855 (1999) 601.
[12] R. Aichholz, E. Lorbeer, J. Microcol. Sep. 8 (1996) 553.
[13] K. Grob, in: Making and Manipulating Capillary Columns for Gas Chromatography, Hüthig, Heidelberg, 1986, p. 134.
[14] K. Grob, in: Making and Manipulating Capillary Columns for Gas Chromatography, Hüthig, Heidelberg, 1986, p. 101.
[15] W. Blum, R. Aichholz, in: Hochtemperatur Gas Chromatographie, Hüthig, Heidelberg, 1991, p. 79.
[16] A.G. Harrison, in: Chemical Ionization Mass spectrometry, 2nd ed., CRC Press, Boca Raton, FL, 1992, p. 113, and Refs. cited therein.
[17] A.P. Tulloch, Bee World 61 (1980) 47.
[18] A.P. Tulloch, Chem. Phys. Lipids 6 (1971) 235.
[19] R.D. Plattner, G.F. Spencer, Lipids 18 (1983) 68.