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Journal of Chromatography A, 1007 (2003) 101–116
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Quality assurance of commercial beeswax II. Gas chromatography–electron impact ionization mass spectrometry of alcohols and acids☆

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Received 7 March 2003; received in revised form 5 May 2003; accepted 23 May 2003

Abstract

Gas chromatography with mass spectrometric detection was used to find the fraction of alcohols and acids present in pure beeswax from *Apis mellifera*. Some new compounds not described till now were found, such as a family of unsaturated linear fatty acids, several hydroxyacids and 1,2,3-propanetriol monoesters. The chromatographic profiles obtained from pure beeswax and bee-rejected foundation beeswax can be used to discriminate them; they mainly differ in the amount of some acids and alcohols.

Keywords: *Apis mellifera*; Beeswax; Waxes; Alcohols; Organic acids

1. Introduction

Commercial foundation beeswax is not often accepted by honey-bees. In view of the necessity of differentiating *Apis mellifera* pure beeswax and the rejected beeswax, the possibility of discrimination on the basis of their acid and alcohol content is investigated in this work, continuing a previous study [1]. In this way, the nature and amount of acids and alcohols that compose the beeswax esters are determined after hydrolyzing them. Analysis methods for free acids and alcohols in beeswax are also proposed and applied.

Beeswax is a natural product composed of over 60% of mono-, di- and polyester compounds with high molecular mass. In minor proportion, it also contains about 13% free acids and alcohols. Prior to gas chromatography analysis, a derivatization step is required to analyze compounds such as the above mentioned on account of their low-volatility, great chemical reactivity and easy thermal decomposition.

In GC analysis of the crude beeswax composition, the acidic and alcoholic groups have been sequentially derivatized to form the corresponding *tert.*-butyldimethylsilyl esters and trimethylsilyl ethers [2]. However, it is more common to turn to an esterifica-

☆Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November, 2002.

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00962-2

tion of the acids with diazomethane and an acetylation of the alcohols with acetic anhydride [3–7]. For the acid analysis, a treatment with trifluoro-*N*-methyl-*N*-trimethylsilylacetamide has also been used [8].

Another option for beeswax analysis dealt with its acidic or alkaline hydrolysis (in methanol or ethanol) and posterior derivatization of the released compounds to determine the simple components (total acids and alcohols) [3,9,10]; these two steps can be carried out together with a thermally assisted hydrolysis and methylation in the presence of tetramethylammonium hydroxide [11].

Some major compounds of beeswax have also been detected by supercritical fluid chromatography [12,13]. Furthermore, it is possible to analyze either the crude beeswax or the fractions obtained by thin-layer chromatography (TLC) and packed-column chromatography. TLC has been for many years a complementary technique to GC to gather information about the beeswax composition. It was also considered necessary owing to the low resolution of the packed-columns used in GC in the past [3,14].

In most of these manuscripts, the identification of the target analytes was performed by comparing retention times. Moreover, beeswax fractions have been analyzed by spectroscopic techniques, mainly nuclear magnetic resonance [10,15,16] and infrared spectroscopy [17].

Few studies have used mass spectrometry to characterize beeswaxes till now. One of them works with positive chemical ionization to confirm the molecular ions of the expected compounds in beeswax [18], another one works with electron impact to characterize the compounds of low molecular mass in crude beeswax [1]. Mass spectrometry has also been applied to determine the pyrolysis products of beeswax [19].

In this work, the acids and alcohols are determined by gas chromatography with mass spectrometry detection in the electron impact mode. To this end, three sample treatments are suitably combined: a soft hydrolysis with boron trifluoride–methanol (reagent not previously assayed for this aim) that allows the simultaneous esterification of the released acids, an alcohol acetylation with acetic anhydride, and an extraction procedure to isolate the free acids.

2. Experimental

2.1. Materials and reagents

Residue analysis grade hexane, acetonitrile, chloroform and methanol were supplied by Labscan (Dublin, Ireland). Ultrapure water was obtained from a Milli-Ro 6 plus apparatus (Millipore, Milford, MA, USA). Potassium carbonate was purchased from Panreac (Barcelona, Spain). Acetic anhydride, pyridine, and boron trifluoride (BF_3) 20% in methanol from Merck (Darmstadt, Germany) were used for derivatization. A model 5810R refrigerated centrifuge was supplied by Eppendorf (Hamburg, Germany) and a rotary evaporator by Buchi (Flawil, Switzerland).

2.2. Sampling

Pure beeswaxes (10 samples) and commercial foundation beeswax sheets (20 samples, most of them rejected by the bees) used for the conditioning of beehives were supplied by apiarists from different provinces of Spain. The foundation beeswaxes were directly analyzed whereas the pure ones, mixed with rests from the beehive, required a previous clean-up [1]. Samples were kept at room temperature and darkness until their analysis.

2.3. Analysis of beeswax acids

2.3.1. Total acids

Beeswax (5 mg) was dissolved in 4 ml of chloroform and mixed with 2 ml of methanol and 2 ml of BF_3 in methanol at 20%. Then, simultaneous hydrolysis and methylation was carried out in 10-ml vials at 90 °C for 1 h. After that, 2 ml of water were added to the mixture, the vial was manually shaken for 30 s and the organic phase separated by centrifugation at 2500×*g* for 5 min. The aqueous phase was removed and the organic layer was then washed with water twice to remove the excess of reagents, making them ready for injecting.

2.3.2. Free acids

Beeswax (5 mg) and 4 ml of hexane were added to 4 ml of acetonitrile placed in a 10-ml vial. The

mixture was mechanically shaken for 10 min and the lower layer (acetonitrile) containing the free acids was collected; the extraction was repeated two more times. Then, the three acetonitrile portions were combined and evaporated to dryness in a rotary evaporator at 35 °C under a gentle vacuum. Finally, the residue was dissolved in 4 ml of chloroform and derivatized with BF_3–methanol according to the above mentioned procedure.

2.4. Analysis of beeswax alcohols

2.4.1. Total alcohols

Once hydrolysis and simultaneous esterification of the acids were made (see Section 2.3.1), a portion of 2 ml was taken and mixed with 0.5 ml of acetic anhydride and 50 μl of pyridine. Alcohols were acetylated in 10-ml vials at 90 °C for 2 h. The mixture was allowed to cool at room temperature and, then, 2 ml of 1 *M* potassium carbonate were added to remove the excess of anhydride. The vial was shaken for 1 min and the phases were separated by centrifugation at 2500×*g* for 10 min. The upper aqueous phase was removed and the treatment with carbonate was repeated twice. The organic phase containing the alcohol acetates was ready for injection.

2.4.2. Free alcohols

Beeswax (5 mg) was dissolved in 4 ml of chloroform and a portion of 2 ml was subjected to the procedure in Section 2.4.1.

2.5. Chromatographic equipment

A Hewlett-Packard 6890 gas chromatograph (Little Falls Site, Wilmington, DE, USA) was directly coupled to a Hewlett-Packard 5973 mass spectrometer. The chromatograph was fitted with a 30 m×0.25 mm, 0.25 μm HP-5 column from Hewlett-Packard. The oven temperature program was 50 °C (1 min), then 5 °C/min to 325 °C (15 min). The carrier gas (helium) flow was kept constant at 1 ml/min (equivalent to a pressure of 52.8 kPa at 50 °C). Pulsed splitless injection (1 μl) was performed with a HP7673A automatic sampler at an injection port temperature of 280 °C; the pressure pulse was of 172 kPa for 1 min and the purge valve was on at 1 min; the transfer line temperature was 328 °C. The MS temperatures were as follows: ion source, 230 °C, quadrupole, 150 °C. Electron multiplier voltage was maintained 200 V above autotune. The scan range was 50–650 u (2.48 scans/s).

3. Results and discussion

3.1. Acids

Fig. 1 shows the total ion chromatogram obtained after hydrolysis, esterification and acetylation of a pure beeswax sample. The total saturated fatty acids detected in the pure beeswax, the abbreviations used and their mean amount (in %), calculated from the peak areas and in relation to the most abundant compound in the chromatogram, are shown in Table 1. As it can be seen, the presence of acids with an even number of carbon atoms, besides two odd-chain acids (21:0 and 23:0) in low amount, was observed in pure beeswax. In previous papers, most of them published about 25 years ago, only the occurrence of even chain fatty acids in beeswax was reported [2,6,15]. However, an old study [20] already foresaw the likely presence of odd-chain acids, from which the 23:0 would be the predominant one. As it was expected, 16:0 acid was the most abundant compound in the beeswax, 24:0 acid was the second most abundant acid, in a proportion close to 2:1, somewhat different from the data published in other works [4,21]. It is necessary to point out that in most manuscripts, there is not a differentiation between natural and commercial beeswaxes.

Table 1 shows the odd- and even-chain free saturated acids detected in the experimentation. According to the scientific literature, the presence of some free acids whose chain length was lower than 20:0 had been occasionally observed [2,6,22]. 24:0 acid is the predominant, followed by 26:0, 28:0 and 16:0, in decreasing order. The amount of 16:0 acid is higher than those estimated by other authors [4,7].

The electron impact spectrum of a saturated fatty acid methyl ester, and its characteristic fragmentations, is shown in Fig. 2. The extraction of free acids with acetonitrile was practically complete. In the

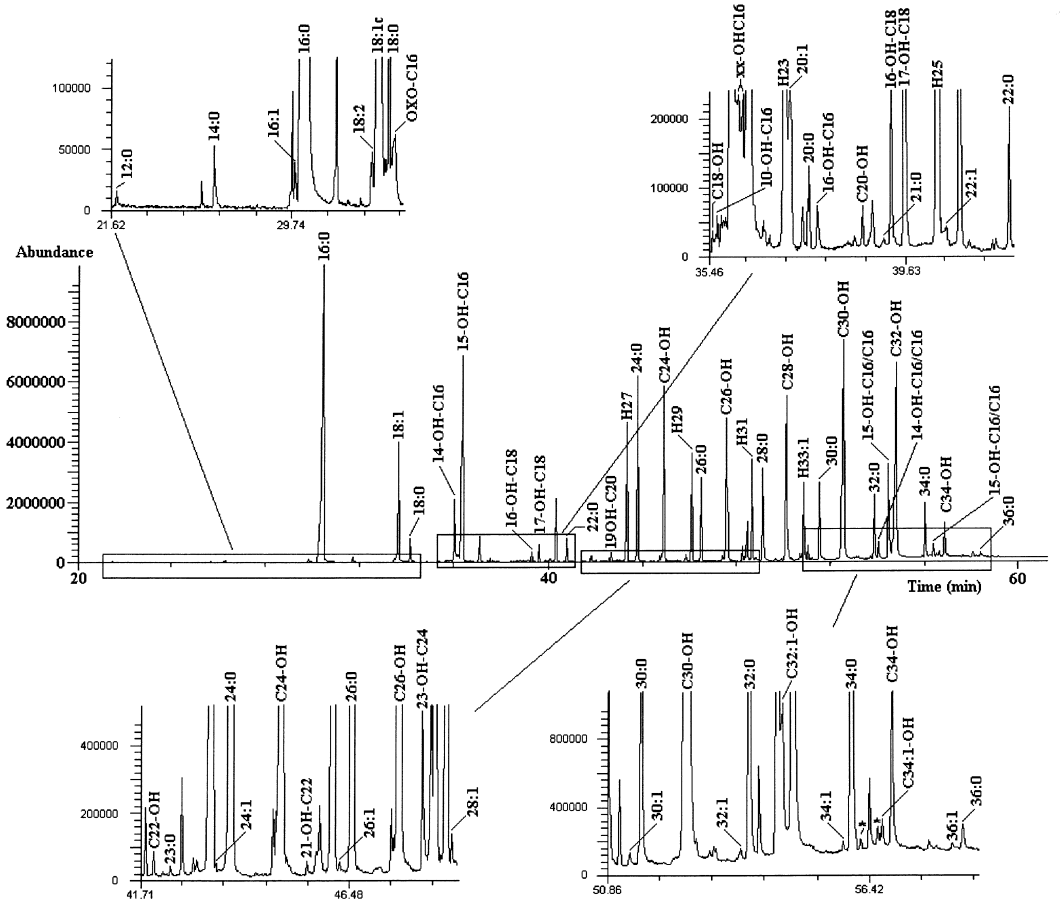

Fig. 1. Total ion chromatogram of a beeswax sample treated with BF_3–MeOH and acetic anhydride. Saturated aliphatic hydrocarbons identified by the letter H and the carbon atom number. See tables to identify other peaks. *Unknown monoesters.

posterior re-extraction of some samples dissolved in hexane with acetonitrile, the free acid peak heights were always lower than 4% in relation to the peak heights achieved in the first extraction. The hexane–0.5 *M* NaOH and chloroform–acetonitrile partitions were also assayed, they provided bad results. The extraction with acetonitrile also allowed the detection of the fatty acid ethyl esters from the beeswax [1].

Table 2 shows the free and total unsaturated fatty acids found in pure beeswax, it also shows their abbreviations and relative mean amounts (in %). An even-chain monounsaturated acid homologous series, from 16:1 to 36:1 and esterified with alcohols in the unhydrolyzed beeswax, was also detected. The existence of monoenoic acid esters containing from 38 to 52 carbon atoms [20] and from 40 to 54 carbon atoms [4] had been reported elsewhere; in any case, the specific structure of the unsaturated fatty acids had not been described, with the exception of oleic acid whose presence had been ascertained, either free or esterified [2,9].

In this work, the existence of free 18:2 acid in beeswax, in lower proportion than free 18:1 acid, has also been established. With reference to the latter, small amounts of *trans* isomer have been detected, this finding is in agreement with the animal origin of this type of wax. Fig. 3 shows the ion chromatogram of a free acid extract after derivatization.

The spectrum and the explanation of the fragmentations of a methyl ester of an unsaturated fatty acid can be seen in Fig. 4. The double bond position cannot be assigned from the electron impact spectra.

Table 1
Saturated fatty acids of beeswax

Abbreviation	Retention time of the derivative (min)	Acid	Molecular ion of the derivative	Total (%)	Free (%)
12:0	21.82	Dodecanoic	214	0.10	–
14:0	26.30	Tetradecanoic	242	0.23	0.52
15:0	28.42	Pentadecanoic	256	–	0.21
16:0	30.49	Hexadecanoic	270	100.0	14.36
18:0	34.14	Octadecanoic	298	5.87	2.75
20:0	37.60	Eicosanoic	326	0.90	0.60
21:0	39.23	Heneicosanoic	340	0.03	–
22:0	40.80	Docosanoic	354	6.89	13.23
23:0	42.30	Tricosanoic	368	0.19	0.30
24:0	43.82	Tetracosanoic	382	45.26	100.00
26:0	46.55	Hexacosanoic	410	15.74	23.30
28:0	49.15	Octacosanoic	438	15.35	15.96
30:0	51.59	Triacontanoic	466	11.73	8.78
32:0	53.87	Dotriacontanoic	494	9.71	4.50
34:0	56.04	Tetratriacontanoic	522	9.31	3.41
36:0	58.42	Hexatriacontanoic	550	0.88	0.06

–, Not detected. The relative amount of the total acids is referred to 16:0 acid and expressed in %; the free acids are referred to 24:0 acid.

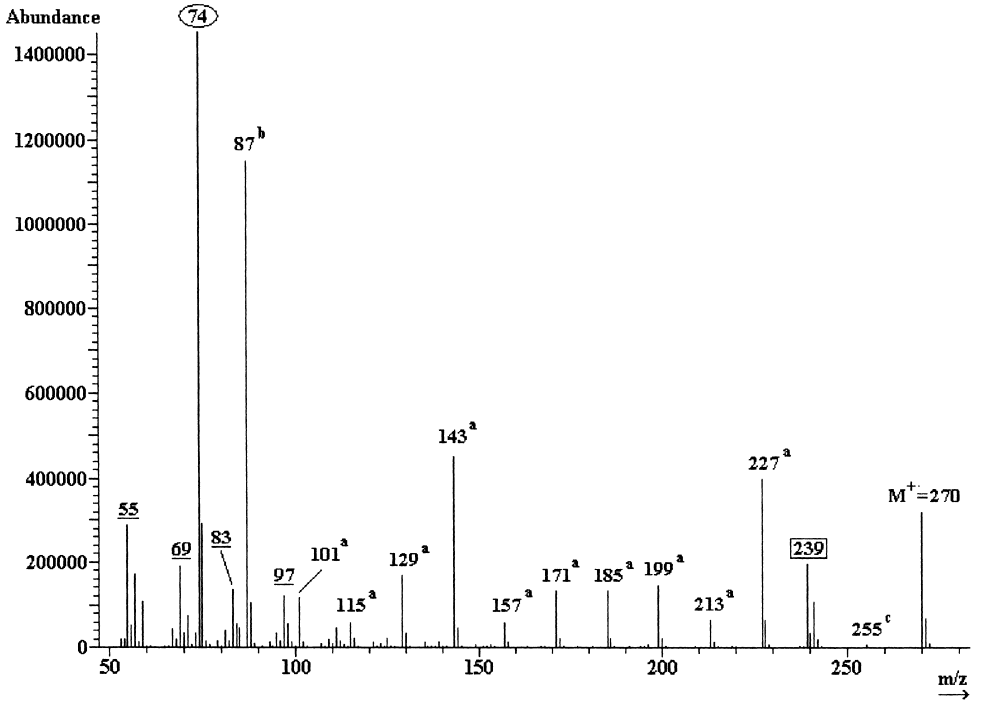

Fig. 2. Electron impact spectrum of methyl hexadecanoate. Ion series: $C_nH^+_{2n-1}$, underlined; $^aC_nH_{2n+1}CO^+_2$. Fragmentations: McLafferty rearrangement ($C_3H_6O^+_2$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{16}H_{31}O^+$ ion), in the box; ^{b}γ-H rearrangement to carbonyl group with β-cleavage ($C_4H_7O^+_2$ ion); ^{c}inductive cleavage in C–O bond ($C_{16}H_{31}O^+_2$ ion).

Table 2
Unsaturated fatty acids of beeswax

Abbreviation	Retention time of the derivative (min)	Acid	Molecular ion of the derivative	Total (%)	Free (%)
16:1	29.98	7?-Hexadecenoic	268	0.10	–
18:2	33.57	9,12-Octadecadienoic	294	0.17	0.75
18:1c	33.69	*Cis* 9-Octadecenoic	296	23.17	4.48
18:1t	33.77	*Trans* 9-Octadecenoic	296	0.12	–
20:1	37.16	11?-Eicosenoic	324	0.47	–
22:1	40.41	13?-Docosenoic	352	0.08	–
24:1	43.43	15?-Tetracosenoic	380	0.05	–
26:1	46.23	17?-Hexacosenoic	408	0.06	–
28:1	48.86	19?-Octacosenoic	436	0.08	–
30:1	51.32	21?-Triacontenoic	464	0.15	–
32:1	53.64	23?-Dotriacontenoic	492	0.27	–
34:1	55.84	25?-Tetratriacontenoic	520	0.23	–
36:1	58.22	27?-Hexatriacontenoic	548	0.004	–

–, Not detected. The relative amount of the total is referred to 16:0 acid and expressed in %; the free acids are referred to 24:0 acid.

The only certain information deduced is that they are not methyl esters of 2-monoenoic acids because a selective ion for these compounds, at m/z 113, was not observed in the spectra. Concerning this, the most common natural monoenoic fatty acids are those whose unsaturation is in the middle of their structure, as a consequence, it is presumable that the detected acids were the compounds mentioned in Table 2. So, for instance, the 18:1 compound should be oleic acid, whose presence had already been confirmed by other authors, and the 18:2 compound should be linoleic acid.

3.2. Hydroxyacids and related compounds

Table 3 shows the hydroxyacids found in pure

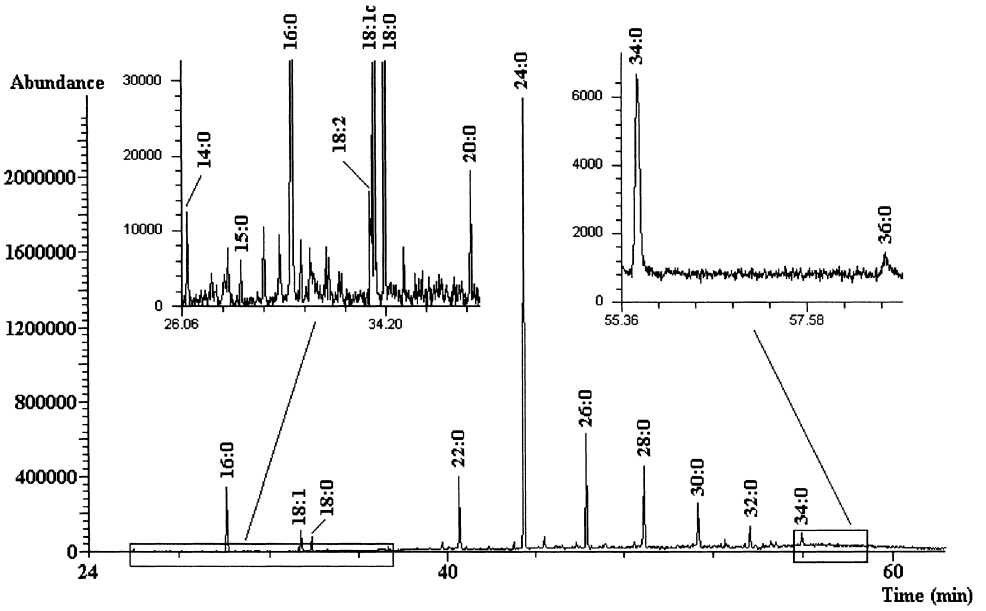

Fig. 3. Chromatogram of esterified free acids of the beeswax. See tables for peak identification.

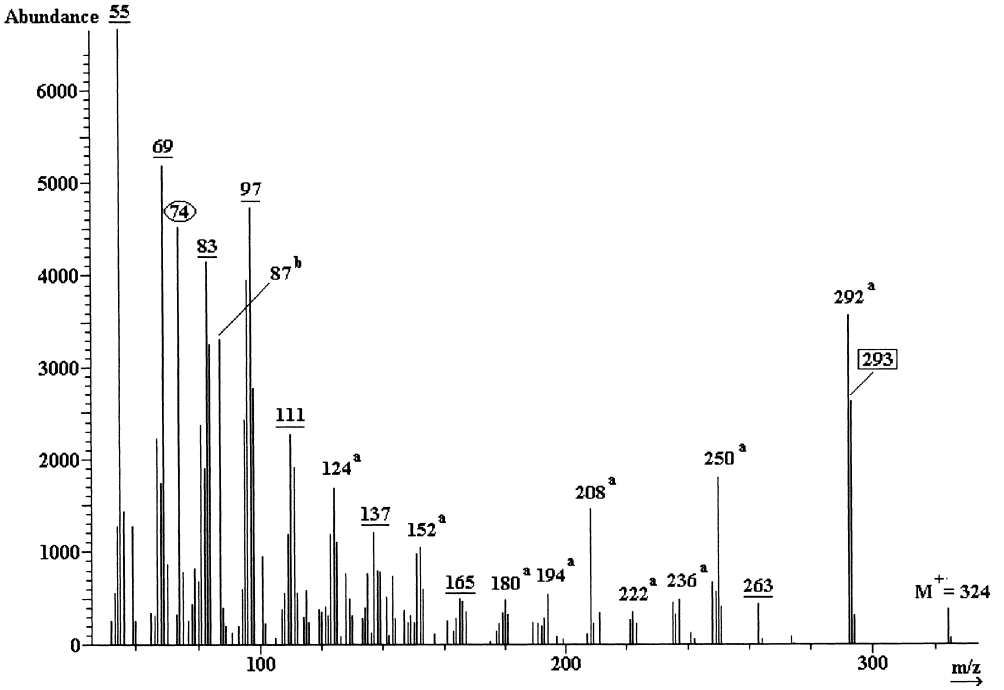

Fig. 4. Electron impact spectrum of methyl eicosenoate. Ion series: $C_nH^+_{2n-1}$, underlined; $^aC_nH^+_{2n-2}$. Fragmentations: McLafferty rearrangement ($C_3H_6O_2^+$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{20}H_{37}O^+$ ion), in the box; ^{b}γ-H rearrangement to carbonyl group with β-cleavage ($C_4H_7O_2^+$ ion).

Table 3
Oxo and hydroxy acids of beeswax

Abbreviation	Retention time of the derivative (min)	Acid	Molecular ion of the derivative	Total (%)
Oxo-C_{16}	34.24	15-Oxohexadecanoic	284	0.56
10-OH-C_{16}	35.66	10-Hydroxyhexadecanoic	328[a]	0.12
11-OH-C_{16}	35.74	11-Hydroxyhexadecanoic	328[a]	0.27
12?-OH-C_{16}	35.82	12?-Hydroxyhexadecanoic	328[a]	0.03
13?-OH-C_{16}	35.87	13?-Hydroxyhexadecanoic	328[a]	0.06
14-OH-C_{16}	36.02	14-Hydroxyhexadecanoic	328[a]	8.84
xx-OH-C_{16}	36.12	Hydroxyhexadecanoic	328[a]	0.10
xx-OH-C_{16}	36.18	Hydroxyhexadecanoic	328[a]	0.22
15-OH-C_{16}	36.37	15-Hydroxyhexadecanoic	328	36.31
16-OH-C_{16}	37.79	16-Hydroxyhexadecanoic	328[a]	0.35
16-OH-C_{18}	39.32	16-Hydroxyoctadecanoic	356[a]	1.21
17-OH-C_{18}	39.61	17-Hydroxyoctadecanoic	356[a]	2.73
19-OH-C_{20}	42.66	19-Hydroxyeicosanoic	384[a]	0.09
21-OH-C_{22}	45.55	21-Hydroxydocosanoic	384[a]	1.40
23-OH-C_{24}	48.16	23-Hydroxytetracosanoic	440[a]	2.09

–, Not detected. The relative amount of the hydroxyacids is referred to 16:0 acid and expressed in %.
[a] Molecular ion not observed.

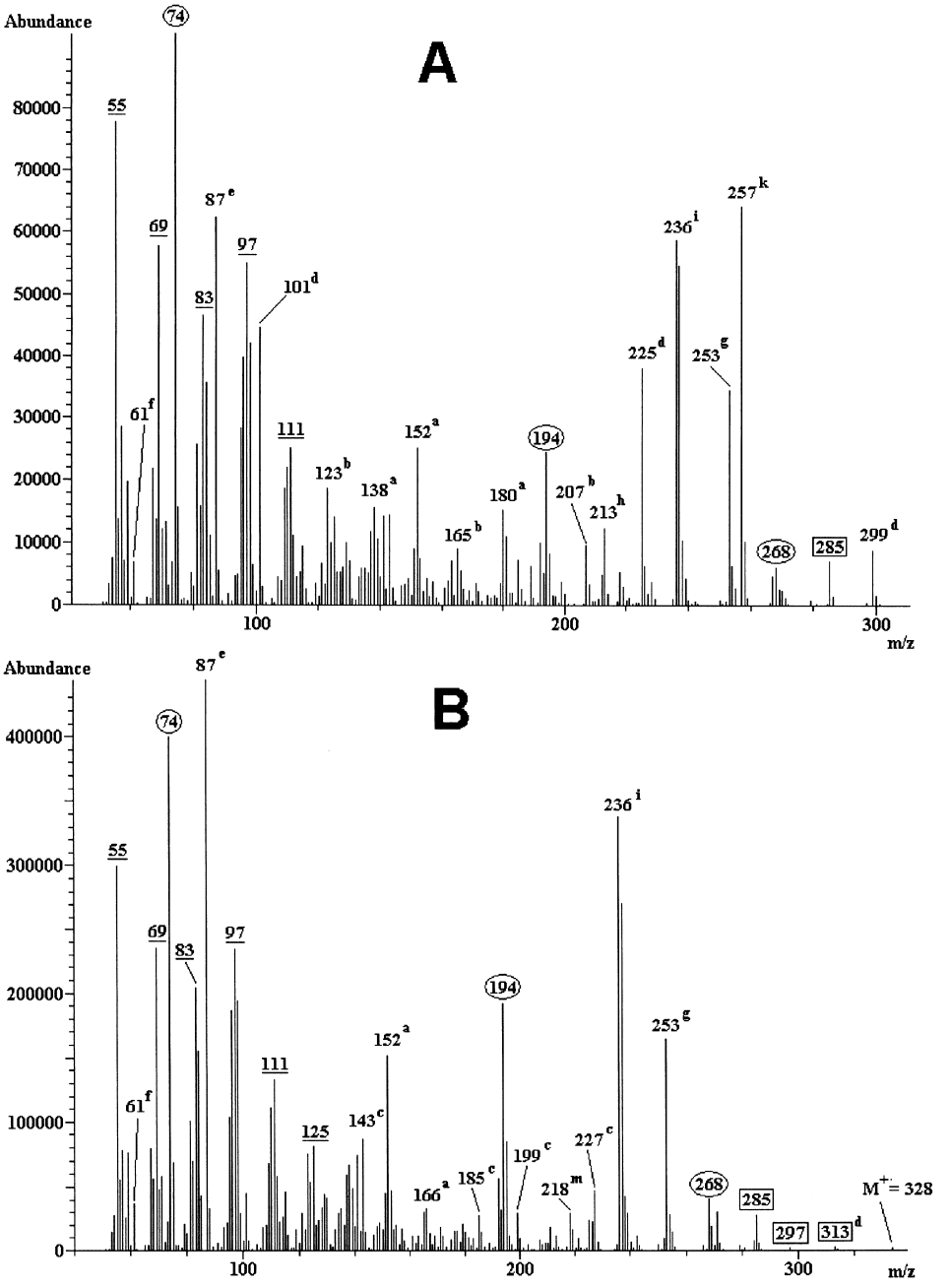

Fig. 5. Electron impact spectra of (a) methyl 14-acetylhexadecanoate and (b) methyl 15-acetylhexadecanoate. Ion series: $C_nH^+_{2n-1}$, underlined; [a]$C_nH^+_{2n-2}$; [b]$C_nH^+_{2n-3}$; [c]$C_nH_{2n+1}CO^+_2$. Fragmentations: McLafferty rearrangement in the molecular ion ($C_{17}H_{32}O^+_2$ ion) and in the $C_{17}H_{32}O^+_2$ ion ($C_{14}H^+_{26}$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{17}H_{33}O^+_3$, $C_{18}H_{33}O^+_3$ and $C_{18}H_{33}O^+_4$ ions), in the box; [d]α-cleavage at the adjacent C–O bond ($C_{18}H_{33}O^+_4$, $C_5H_9O^+_2$ and $C_{17}H_{31}O^+_4$ ions); [e]γ-H rearrangement to carbonyl group with β-cleavage ($C_4H_7O^+_2$ ion); [f]rearrangement of two hydrogen atoms ($C_2H_5O^+_2$ ion); [g]inductive cleavage in C–O bond in the $C_{17}H_{32}O^+_2$ ion ($C_{16}H_{29}O^+_2$ ion); [h]allylic cleavage in the $C_{17}H_{32}O^+_2$ ion (*m*/*z* 254) arisen from a McLafferty rearrangement in the acid group ($C_{13}H_{25}O^+_2$ ion); [i]peak at *m*/*z* 236: loss of CH_3OH from $C_{17}H_{32}O^+_2$; [k]peak at *m*/*z* 257: loss of $CH_2{=}CH_2$ from $C_{17}H_{33}O^+_3$; [m]peak at *m*/*z* 218: loss of H_2O from ion at *m*/*z* 236.

beeswax after hydrolysis and derivatization step. Nine of them are related to the hydroxyhexadecanoic acid. The major compounds are 15-OH-C_{16} and 14-OH-C_{16} hydroxyhexadecanoic acids, which agrees with the reviewed literature [9,22]. The electron impact spectra for these last two components are shown in Fig. 5; many typical ions of the hydroxyhexadecanoic acid family can be observed, such as m/z 87, 97, 152, 194, 236, 253, and 268, all independent of the hydroxyl group position. There are also distinctive fragmentations for each isomer according to the hydroxyl group position, these are due to the α-cleavages adjacent to the C–O bond, either in the molecular ion or in ions arisen from McLafferty rearrangements initiated in the acid moiety. So, it has been possible to distinguish some hydroxyhexadecanoic acid isomers from the obtained spectra, such as 10-OH-C_{16} (ion at m/z 243), 11-OH-C_{16} (ion at m/z 257), 14-OH-C_{16} (ions at m/z 101 and 299) and 16-OH-C_{16} (ion at m/z 255). On the basis of the boiling points, the retention times of the isomers must increase as the hydroxy group gets a more terminal position in the chain. The retention times of the identified compounds agree with this, and for this reason, the compounds eluted at 35.82 and 35.87 min could be the corresponding hydroxyhexadecanoic acids, in which the hydroxy groups are attached to positions 12 and 13.

The elucidation of the structure of another two hydroxyacids, eluted at 36.12 and 36.18 min, has not been possible. All of them present the typical ions of the family. If the acid group position changed, new characteristic ions should be observed in the spectra; they have not been observed. The presence of hydroxyhexadecanoic acids in pure beeswax, excepting the major compounds, 15-OH-C_{16} and 14-OH-C_{16}, had not been known.

Five other hydroxyacids, all previously described in the bibliography, were also detected. The 16-OH-C_{18} (m/z 101, 253, 285 and 327) and 17-OH-C_{18} (m/z 341) hydroxyacids were identified by using their characteristic ions, in parentheses. Three other low abundant compounds, 19-OH-C_{20}, 21-OH-C_{22} and 23-OH-C_{24} were found; in these ones, the position of the hydroxy groups could not be established from their spectra, although they must be the above mentioned ones according to the findings of other authors.

Finally, for the first time, the presence of methyl 15-oxohexadecanoate has been observed in the hydrolyzed beeswax chromatogram (Fig. 1 and Table 3).

Five monoester compounds were also observed in the chromatogram obtained from the hydrolyzed and esterified beeswax (Table 4); these compounds could be in free state in the beeswax or could come from polyester hydrolysis because the hydrolysis capacity of the BF_3–methanol reagent is limited. These monoesters result from palmitic and oleic acids esterified with 14- and 15-hydroxyhexadecanoic acids (see spectrum in Fig. 6). Another two chemical species not identified and related to the oleic and palmitic acids were detected, their spectra were similar and characterized by the ions at m/z 239, 264, 297, 313 and the ion series C_nH_{2n-1}.

3.3. Monoalcohols and diols

Table 5 shows the total fatty monoalcohols (most of them bond to acid compounds) and free fatty

Table 4
Monoesters of beeswax

Abbreviation	Retention time of the derivative (min)	Compound	Molecular ion of the derivative	Total (%)
14-OH-C_{16}/C_{16}	54.12	1-Ethyl-13-methoxycarbonyl tridecanoyl hexadecanoate	524[a]	3.47
15-OH-C_{16}/C_{16}	54.47	1-Methyl-14-methoxycarbonyl tetradecanoyl hexadecanoate	524	18.12
	56.26	Unknown	Unknown	0.28
15-OH-$C_{16}/C_{18:1}$	56.41	1-Methyl-14-methoxycarbonyl tetradecanoyl 9-octadecenoate	550	2.31
	56.59	Unknown	Unknown	0.84

The relative amount is referred to 16:0 acid and expressed in %.
[a] Molecular ion not observed.

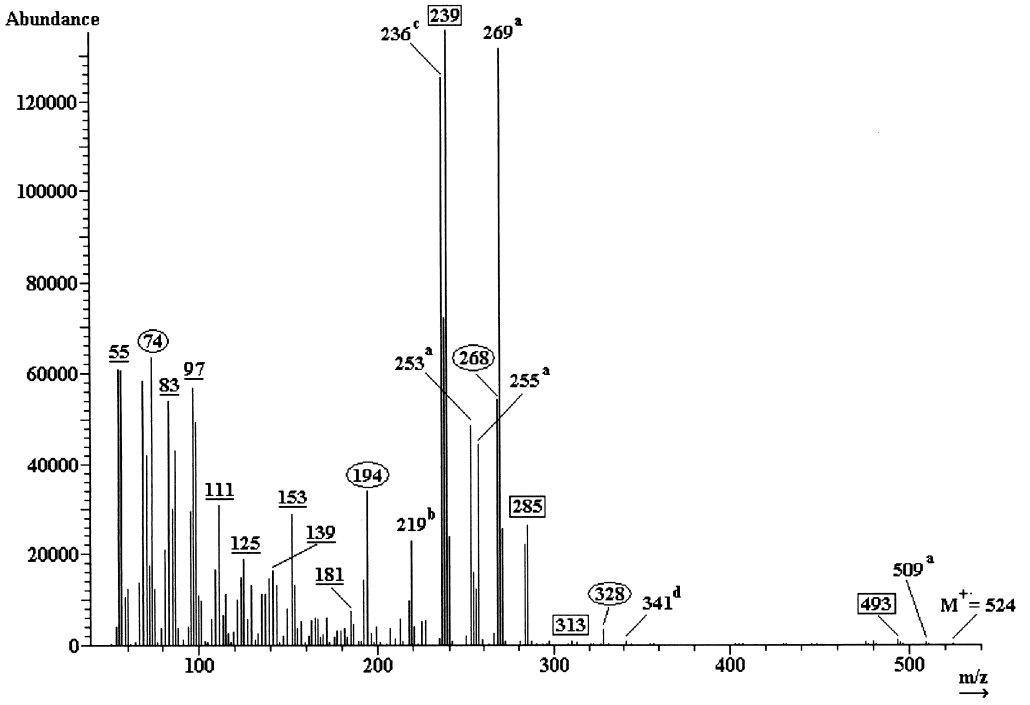

Fig. 6. Electron impact spectrum of 1-methyl 14-methoxycarbonyltetradecanoyl hexadecanoate. Ion series: $C_nH^+_{2n-1}$, underlined. Fragmentations: McLafferty rearrangement in the molecular ion ($C_3H_6O_2^+$, $C_{17}H_{32}O_2^+$ and $C_{19}H_{36}O_4^+$ ions) and in the $C_{17}H_{32}O_2^+$ ion ($C_{14}H_{26}^+$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{16}H_{31}O^+$, $C_{17}H_{33}O_3^+$, $C_{18}H_{33}O_4^+$ and $C_{32}H_{61}O_3^+$ ions), in the box; [a]inductive cleavage in the C–O bond ($C_{16}H_{31}O_2^+$, $C_{17}H_{33}O_2^+$ and $C_{32}H_{61}O_4^+$ ions) and in the $C_{17}H_{32}O_2^+$ ion ($C_{16}H_{29}O_2^+$ ion); [b]peak at m/z 219: loss of CH_3OH and H_2O from $C_{17}H_{33}O_2^+$ or loss of CH_3O and H_2O from $C_{17}H_{32}O_2^+$; [c]peak at m/z 236: loss of CH_3OH from $C_{17}H_{32}O_2^+$; [d]γ-H rearrangement to carbonyl group with β-cleavage ($C_{20}H_{37}O_4^+$ ion).

Table 5
Fatty alcohols of beeswax

Abbreviation	Retention time of the derivative (min)	Compound	Molecular ion of the derivative	Total (%)	Free (%)
C_{18}-OH	35.60	1-Octadecanol	312	0.04	–
C_{20}-OH	38.94	1-Eicosanol	340	0.03	–
C_{22}-OH	42.01	1-Docosanol	368	0.38	–
C_{24}-OH	44.95	1-Tetracosanol	396	38.85	7.29
C_{26}-OH	47.62	1-Hexacosanol	424	32.04	7.67
C_{28}-OH	50.15	1-Octacosanol	452	35.96	20.35
C_{30}-OH	52.57	1-Triacontanol	480	69.14	100.0
$C_{32:1}$-OH	54.53	xx-Dotriaconten-1-ol	506	0.45	–
C_{32}-OH	54.79	1-Dotriacontanol	508	48.49	91.72
$C_{34:1}$-OH	56.69	xx-Tetratriaconten-1-ol	534	0.31	–
C_{34}-OH	56.89	1-Tetratriacontanol	536	4.12	8.65

–, Not detected. The relative amount of the total alcohols is referred to 16:0 acid and expressed in %; the free alcohols are referred to C_{30}-OH alcohol.

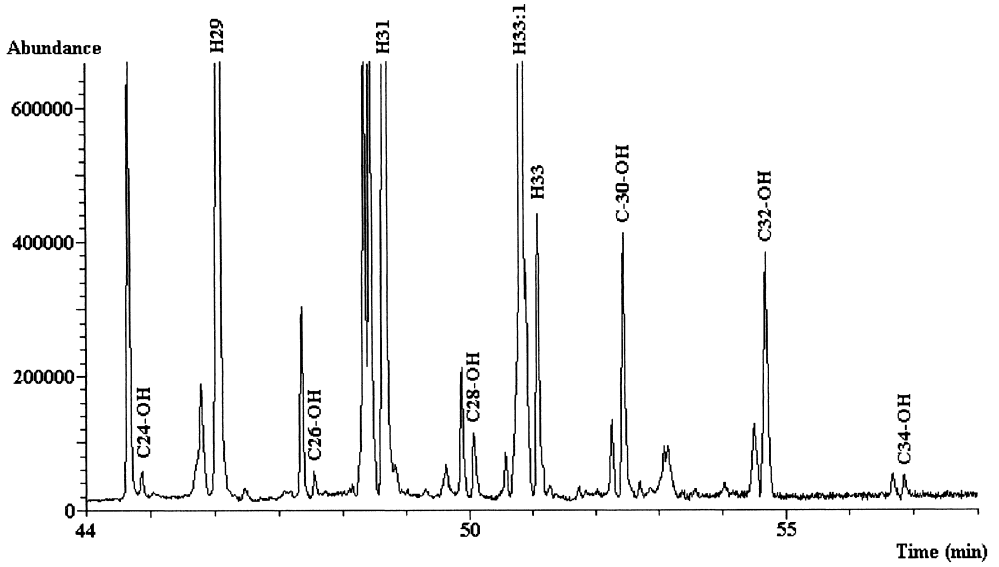

Fig. 7. Chromatogram of acetylated free alcohols of the beeswax. See tables for peak identification.

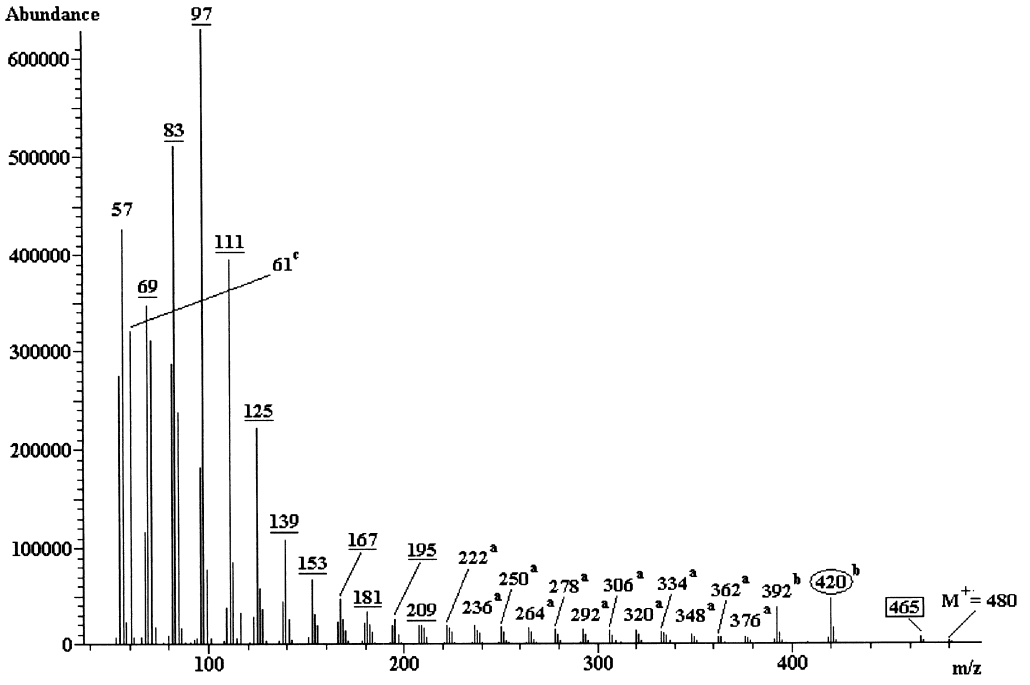

Fig. 8. Electron impact spectrum of triacontyl acetate. Ion series: $C_nH^+_{2n+1}$; $C_nH^+_{2n-1}$, underlined; [a]$C_nH^+_{2n-2}$; [b]$C_nH^+_{2n}$. Fragmentations: McLafferty rearrangement ($C_{30}H^+_{60}$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{31}H_{61}O^+_2$ ion), in the box; [c]rearrangement of two hydrogen atoms ($C_2H_5O^+_2$ ion).

Table 6
Diols of beeswax

Abbreviation	Retention time of the derivative (min)	Compound	Molecular ion of the derivative	Total (%)
1,23-C_{24}-diol	49.16	1,23-Tetracosanediol	454[a]	0.18
1,25-C_{26}-diol	51.59	1,25-Hexacosanediol	482[a]	0.08
1,27-C_{28}-diol	53.89	1,27-Octacosanediol	510[a]	0.11
1,29-C_{30}-diol	56.05	1,29-Triacontanediol	538[a]	0.03

The relative amount is estimated from the peak area for the ion chromatogram extracted at m/z 61, and referred to the most abundant compound in the chromatogram: the methylated 16:0 acid.

[a] Molecular ion not observed.

monoalcohols (in minority) found in beeswax. The total monoalcohols consist of even-chain primary alcohols with a chain-length from 18 to 34 carbon atoms; their relative amounts were lower than those of the total acids (see Fig. 1 and Table 5). The C_{18}-OH, C_{20}-OH and C_{22}-OH contents were remarkably low, perhaps for this reason, they had not been mentioned in previous works [4,22,23]. Two unsaturated alcohols, $C_{32:1}$-OH and $C_{34:1}$-OH, arising probably from their corresponding palmitates [24], were also detected.

Fig. 7 shows the chromatogram recorded in the free alcohol fraction. This revealed the existence of primary alcohols comprised within C_{24}-OH and C_{34}-OH, all with even-chain. As it happened for the total alcohols, the predominant components were C_{30}-OH

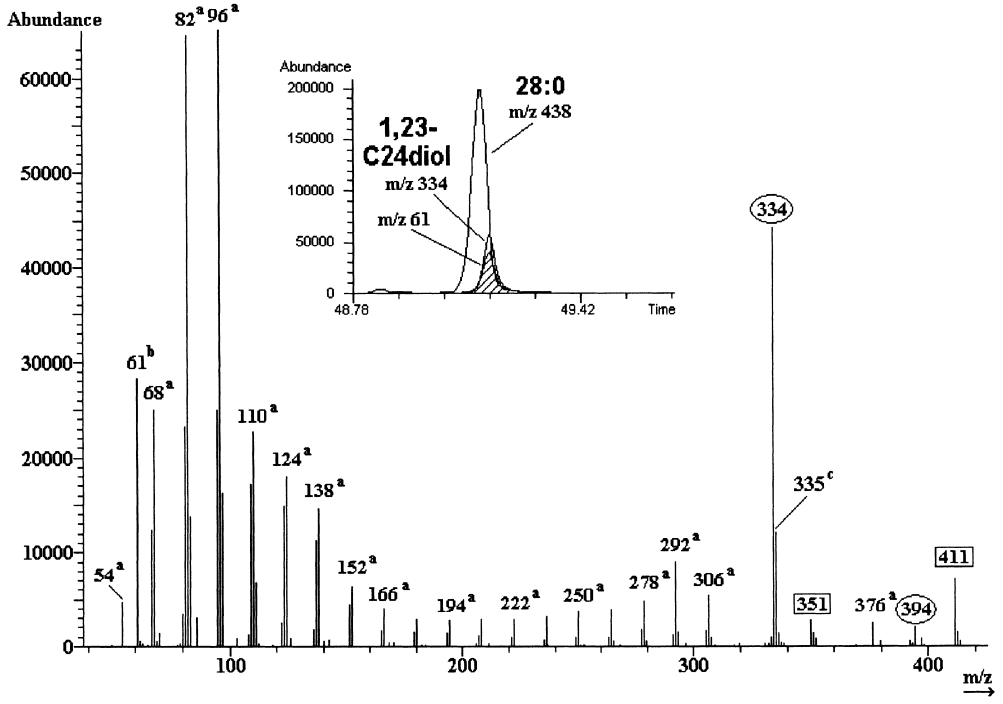

Fig. 9. Electron impact spectrum of 1,23-tetracosanediol diacetate. Ion series: [a]$C_nH^+_{2n-2}$. Fragmentations: McLafferty rearrangement in the molecular ion ($C_{26}H_{50}O_2^+$ ion) and in the $C_{26}H_{50}O_2^+$ ion ($C_{24}H_{46}^+$ ion), in the circle; α-cleavage at the adjacent carbonyl group ($C_{26}H_{51}O_3^+$ ion), in the box; [b]rearrangement of two hydrogen atoms ($C_2H_5O_2^+$ ion); [c]inductive cleavage in the C–O bond of the ion $C_{26}H_{50}O_2^+$ ($C_{24}H_{47}^+$ ion).

Table 7
1,2,3-Propanetriols of beeswax

Abbreviation	Retention time of the derivative (min)	Compound	Molecular ion of the derivative	Free (%)
C_{14}/2-triol	41.38	1,3-Bis(hydroxy)propyl tetradecanoate	386[a]	0.22
C_{14}/1-triol	41.47	2,3-Bis(hydroxy)propyl tetradecanoate	386[a]	1.71
C_{16}/2-triol	44.37	1,3-Bis(hydroxy)propyl hexadecanoate	414[a]	8.50
C_{16}/1-triol	44.48	2,3-Bis(hydroxy)propyl hexadecanoate	414[a]	110.29
C_{18}/2-triol	47.16	1,3-Bis(hydroxy)propyl octadecanoate	442[a]	7.67
C_{18}/1-triol	47.57	2,3-Bis(hydroxy)propyl octadecanoate	442[a]	110.56

The relative amount is referred to 24:0 acid and expressed in %.
[a] Molecular ion not observed.

and C_{32}-OH, followed in decreasing order by C_{28}-OH, C_{26}-OH and C_{24}-OH. The presence of free alcohols with odd number of carbon atoms, C_{33}-OH and C_{35}-OH, recently mentioned [2], has not been ascertained in this work. The electron impact spectrum for triacontyl acetate, the main ion series and typical fragmentations can be seen in Fig. 8.

Four diols whose chromatographic peaks overlapped with the methyl esters from the acids were also found in the hydrolyzed and acetylated beeswax (see Table 6). These compounds are the most abundant from the diol family [9] and they are not free in beeswax, on the contrary they form hydroxy-monoesters and monoesters [2,25]. Fig. 9 shows the spectrum of 1,23-C_{24}-diol and its coelution with an acid methyl ester.

3.4. Compounds related to the 1,2,3-propanetriol

Extracts obtained with acetonitrile were also acetylated after methylating the acids. An unknown compound series derived from 1,2,3-propanetriol was

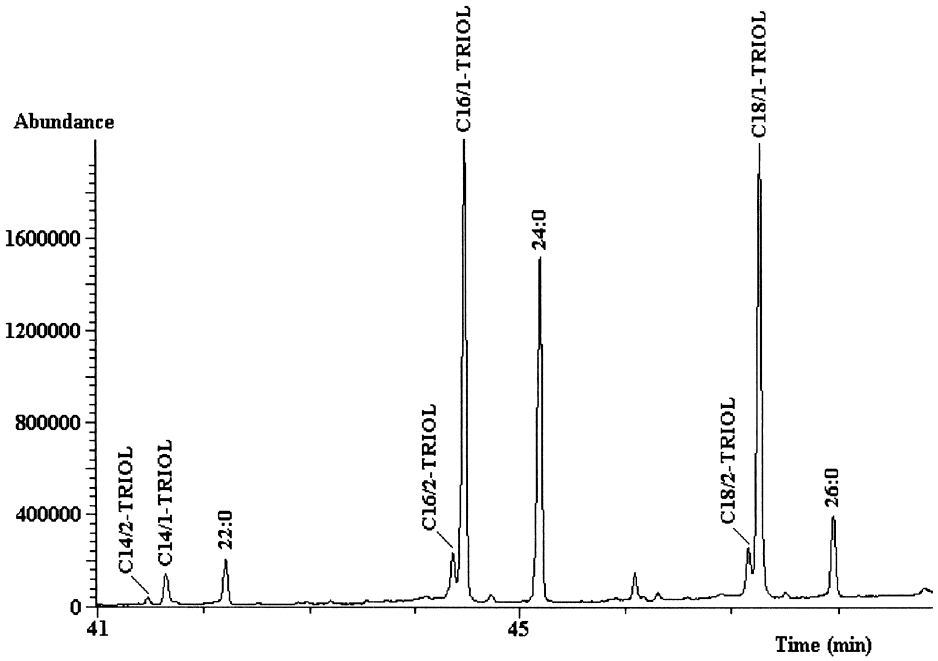

Fig. 10. Chromatogram of acetonitrile extract of the beeswax.

discovered in beeswax (Table 7). This series consisted of six compounds: the dihydroxypropyl monoesters of 14:0, 16:0 and 18:0 acids at positions 1 and 2, the esterification at position 1 of the propanetriol and the esterification of 16:0 and 18:0 acids (C_{16}/1-triol and C_{18}/1-triol compounds) being preferential; the relative amount of the two mentioned compounds is higher than the amount of the predominant free acid (the 24:0 one) as can be seen in the table and chromatogram (Fig. 10). This family of compounds was also observed when the extracts obtained with acetonitrile were directly acetylated.

Fig. 11 shows the spectrum for one of these compounds, and its corresponding fragmentations. The ion at m/z 145 ($C_6H_9O_4^+$) was characteristic of the fatty acid esterifications at position 1 and was attributed to an α-cleavage adjacent to a C–O bond.

3.5. Foundation beeswax sheets

The analysis of foundation beeswax sheets pointed out the presence of non-natural compounds in beeswax, such as branched hydrocarbons and olefins which could be also detected by injecting the beeswax sample dissolved in an appropriate organic solvent [1]. The occurrence of acids and alcohols different from those mentioned in this paper was not observed, although differences in the present amounts were noticed. Table 8 summarizes the mean data obtained in the analysis of foundation beeswaxes. The amount of free acids, 16:0 and 18:0, increased notably, about 2–3 times, whereas the amount of others seemed to decrease slightly, especially that for the higher molecular mass acids. The content in free unsaturated acids, 18:1 and 18:2, also increased sharply, 6 and 3 times, respectively, and the hydroxyacid and monoester contents decreased, these last ones about 40–50%. The C_{18}-OH and C_{20}-OH total alcohols increased their relative amount 5–6 times, always in comparison with pure beeswaxes, while $C_{32:1}$-OH and $C_{34:1}$-OH unsaturated alcohol contents increased 2–3 times. As

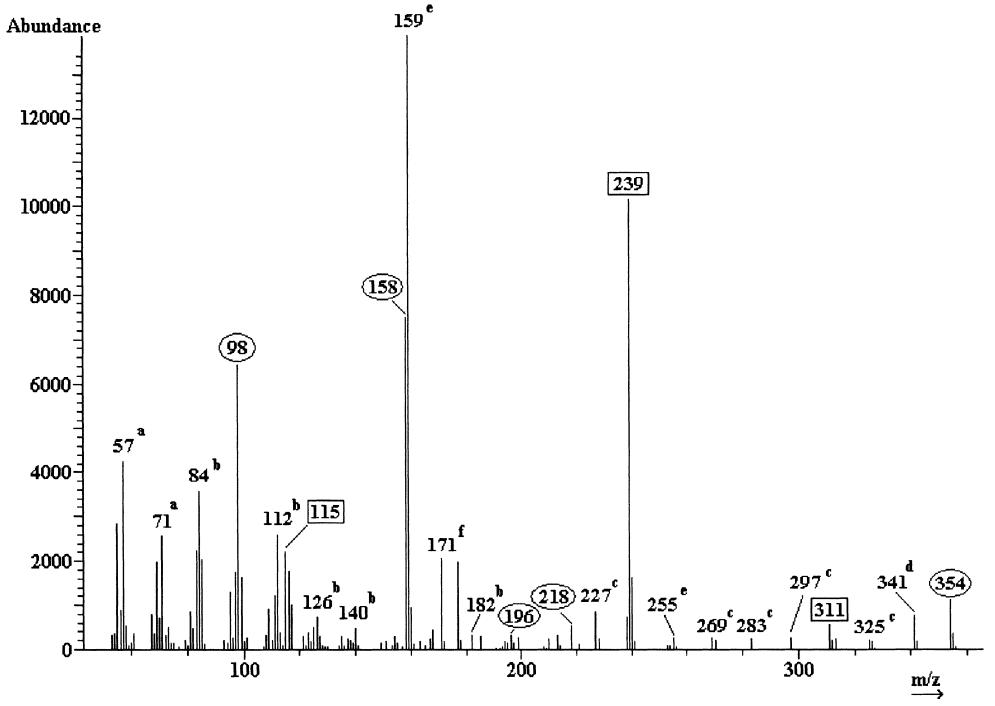

Fig. 11. Electron impact spectrum of 1,3-bis(acetyl) propylhexadecanoate. Ion series: [a]$C_nH_{2n+1}^+$; [b]$C_nH_{2n}^+$; [c]$C_nH_{2n+1}COO^+$. Fragmentations: McLafferty rearrangement in the molecular ion ($C_{14}H_{28}^+$ and $C_9H_{14}O_6^+$ and $C_{21}H_{38}O_4^+$ and $C_7H_{10}O_4^+$ ions) and in the $C_7H_{10}O_4^+$ ion ($C_5H_6O_2^+$ ion), within the circle; α-cleavage at the adjacent carbonyl group in the molecular ion ($C_{16}H_{31}O^+$ ion), the $C_{21}H_{38}O_4^+$ ion ($C_{19}H_{35}O_3^+$ ion) and the $C_7H_{10}O_4^+$ ion ($C_5H_7O_3^+$ ion); [d]α-cleavage in the C–O bond ($C_{20}H_{37}O_4^+$ ion), within the box; [e]inductive cleavage in the C–O bond ($C_{16}H_{31}O_2^+$ and $C_7H_{11}O_4^+$ ions); [f]γ-H rearrangement to carbonyl group with β-cleavage in the $C_{21}H_{38}O_4^+$ ion ($C_8H_{11}O_4^+$ ion).

Table 8
Compounds and mean values found in the analysis of foundation beeswax

Compound	Total (%)	Free (%)	Compound	Total (%)	Free (%)
Saturated acids			Unsaturated acids		
12:0	0.11	–	16:1	0.15	–
14:0	0.14	0.72	18:2	0.82[a]	2.40[a]
15:0	–	0.19	18:1c	29.39	26.82[a]
16:0	100.0	40.05[a]	18:1t	0.17	–
18:0	5.39	5.98[a]	20:1	0.38	–
20:0	0.87	0.84	22:1	0.12	–
21:0	0.03	–	24:1	0.04	–
22:0	4.02	12.02	26:1	0.06	–
23:0	0.15	0.50[a]	28:1	0.10	–
24:0	33.87	100.00	30:1	0.33[a]	–
26:0	12.32	22.94	32:1	0.36	–
28:0	12.32	16.76	34:1	0.26	–
30:0	10.30	8.83	36:1	0.13[a]	–
32:0	7.69	3.98			
34:0	7.51	2.58	Monoesters		
36:0	0.92	–	14OH-C_{16}/C_{16}	2.03	–
			15OH-C_{16}/C_{16}	7.04	–
Hydroxyacids			15OH-C_{16}/$C_{18:1}$	1.24	–
Oxo-C_{16}	0.45	–			
10-OH-C_{16}	0.06	–	Alcohols		
11-OH-C_{16}	0.14	–	C_{18}-OH	0.30[a]	–
12?-OH-C_{16}	–	–	C_{20}-OH	0.17[a]	–
13?-OH-C_{16}	0.05	–	C_{22}-OH	0.43	–
14-OH-C_{16}	5.46	–	C_{24}-OH	26.99	26.14[a]
xx-OH-C_{16}	–	–	C_{26}-OH	21.92	20.34[a]
xx-OH-C_{16}	0.28	–	C_{28}-OH	35.96	32.14
15-OH-C_{16}	33.85	–	C_{30}-OH	57.71	100.0
16-OH-C_{16}	0.56	–	$C_{32:1}$-OH	2.07[a]	–
16-OH-C_{18}	1.01	–	C_{32}-OH	42.69	59.58
17-OH-C_{18}	2.14	–	$C_{34:1}$-OH	1.38	–
19-OH-C_{20}	2.18	–	C_{34}-OH	4.37[a]	5.52
21-OH-C_{22}	0.17	–			
23-OH-C_{24}	1.86	–			
			Esterified propanetriols		
Diols			C_{14}/2-triol	–	0.32
1,23-C_{24}-diol	0.12	–	C_{14}/1-triol	–	2.81
1,25-C_{26}-diol	0.05	–	C_{16}/2-triol	–	14.31
1,27-C_{28}-diol	0.06	–	C_{16}/1-triol	–	214.23
1,29-C_{30}-diol	0.02	–	C_{18}/2-triol	–	14.19
			C_{18}/1-triol	–	217.65

–, Not detected. Relative amounts are calculated as stated in previous tables.
[a] Compounds whose relative amounts change acutely in the commercial beeswax. They could be appropriate to discriminate adulterated beeswaxes.

regards the short-chain free alcohols, their amounts also increased 3 times. The amounts of diols decreased somewhat and, finally, the monoesterified propanetriol content increased twice in relation to pure beeswax.

So, the commercial foundation beeswaxes had chromatographic profiles different from the natural beeswaxes, likely as a consequence of their mixture with other cheaper substances during the manufacture process. These strange substances are responsible for the modification of the naturally-occurring fatty compound contents, increasing their concen-

trations and, even, causing the loss of minor compounds if the dilution is excessive.

The chromatogram from a typical pure beeswax after a concrete sample preparation could be used as a fingerprint for quality assurance of the commercial beeswax. The observation of strange chromatographic peaks or changes in the relative amounts of the own-beeswax compounds could discriminate the adulterated foundation beeswax sheets. The more amenable compounds to detect adulterations on the basis of concentration variations are noted in Table 8.

4. Conclusions

The constituents of high molecular mass esters from pure beeswax, and their electron impact spectra, have been described. The presence of some hydroxyacids, monoesters of the saturated fatty acids with 1,2,3-propanetriol, a homologous series of unsaturated fatty acids and some free acids and alcohols has been detected in pure beeswax from *Apis mellifera* for the first time.

Most of the commercial foundation beeswaxes have chromatographic profiles that differ in the acid and alcohol content, either total or free, which indicates their mixture with other substances. The different chemical composition of commercial beeswaxes in relation to natural beeswaxes could explain the reject cases observed in beehives.

Acknowledgements

The authors thank the Spanish Instituto Nacional de Investigaciones Agrarias for providing funds for this investigation (projects API99-003 and API01-005).

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