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Quality assurance of commercial beeswax Part I. Gas chromatography–electron impact ionization mass spectrometry of hydrocarbons and monoesters☆

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Abstract

The use of low-temperature capillary gas chromatography coupled to electron impact mass spectrometry for the characterization of crude beeswaxes yielded by *Apis mellifera* is described. The system allows the identification of a great number of compounds, some of them not reported till now in beeswax, such as a family of ethyl esters, tetracosyl oleate, and several saturated and unsaturated hydrocarbons. The information acquired makes possible the differentiation between pure beeswax and some foundation beeswax samples where mixture of pure beeswax with another substances is suspected.

Keywords: Waxes; Hydrocarbons; Monoesters

1. Introduction

Some typical physico-chemical parameters used to typify the fatty substances can be measured to establish the quality of beeswax. The values of these parameters must be within a defined value range to consider beeswax as pure. However, the capacity of these procedures to detect adulterations is limited [1–3].

To characterize the composition of the beeswax whose nature is basically lipoid (mainly composed of 14% hydrocarbons, 35% monoesters, 3% diesters and 12% free acids) is preferable to resort to low- or high-temperature gas chromatography. The knowledge of beeswax composition has always been bounded to the enhancement in the performance of the chromatographic columns and most of the published papers deal with packed columns for gas chromatography in combination with thin-layer chromatography techniques. The analysis of beeswax fractions obtained by column chromatography on alumina and silica is also usual [1,4–7]. In the last years, beeswaxes from different bee species have been studied with high-temperature gas chromatography coupled to positive chemical ionization mass spectrometry by using a capillary chromatographic column developed specifically to this purpose [8,9]. Moreover, beeswax composition after pyrolysis and thermal hydrolysis/methylation has also been discussed [10–12].

Recycled beeswaxes, called foundation beeswaxes, are commercialized to be placed in the combs that are introduced in the beehives. In view of the bee-rejection to some of the batches of foundation beeswax sheets, we are looking for ways of quick characterization of the main components of pure beeswax to discriminate it from the adulterated foundation beeswax, on the basis of the presence of strange compounds in this latter.

In this work, low-temperature gas chromatography coupled to electron impact ionization mass spectrometry is used to identify the compounds found and chromatographically discern the pure beeswax of *Apis mellifera* from the beeswax mixed with lower cost strange products. The presence of many compounds in pure beeswax is described for the first time. The ion-fragments found in the spectra are interpreted.

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2. Experimental

2.1. Sample materials and preparation

Pure beeswax (10 samples) and foundation beeswax sheets rejected by the bees (20 samples) were supplied by beekeepers from different provinces of Spain: mainly Guadalajara, Soria, Valencia and Badajoz. Foundation beeswax was directly analyzed, whereas pure beeswax, mixed with rests of the beehive, required a cleanup step previous to the analysis. To this end, beeswaxes were purified by melting. Wax was added to a beaker with boiling water, in proportion 100 g wax/l, and heated for 20 min. Then, the mixture was cooled at room temperature, the wax (less dense) solidified over the water, and the impurities placed at the bottom of the solidified wax removed with a scraper. The treatment was repeated once more.

Samples were kept at room temperature and darkness until their analysis. Then, 10 mg of wax were dissolved in 5 ml of chloroform (Labscan, Dublin, Ireland) to achieve a concentration of 2000 mg/l.

2.2. GC–MS analysis

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 was kept at 50 °C for 1 min and programmed at 5 °C/min to 325 °C, then held there for 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 an 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 at 200 V above autotune. The scan range was 50–650 U (2.48 scans/s).

3. Results and discussion

3.1. Composition of pure beeswax

Fig. 1 shows a total ion chromatogram representative of beeswax composition. The compounds found are grouped and next discussed by families. Table 1 presents the retention time, molecular ion and name of the linear aliphatic hydrocarbons. Also, their relative amounts (%) calculated from the peak areas, taking as a reference the peak area of the most abundant hydrocarbon, 27:0, and the R.S.D.s of the amounts present, are shown. All quantitative data given in this work are the average of the 10 pure beeswaxes analyzed. Table 1 also shows the abbreviations used to designate the long-chain hydrocarbons, which coincide with the corresponding carbon atom number. The presence of 34:0, one of the longer chain and less abundant hydrocarbons, had not been detected till now in the analysis of natural beeswax. The occurrence of hydrocarbons with chain lengths lower than 21:0 had not been mentioned either; only in a work, a chromatographic peak attributed to the coelution of 19:0 and 20:0 had been stated [13]. Hydrocarbons with an even number of carbon atoms are less minor components.

Table 2 shows the retention times, relative mean amounts (referred to 27:0 and expressed in percentage), R.S.D.s, and abbreviations used for the unsaturated and unbranched hydrocarbons found in pure beeswax. They are mainly monounsaturated compounds with an odd number of carbon

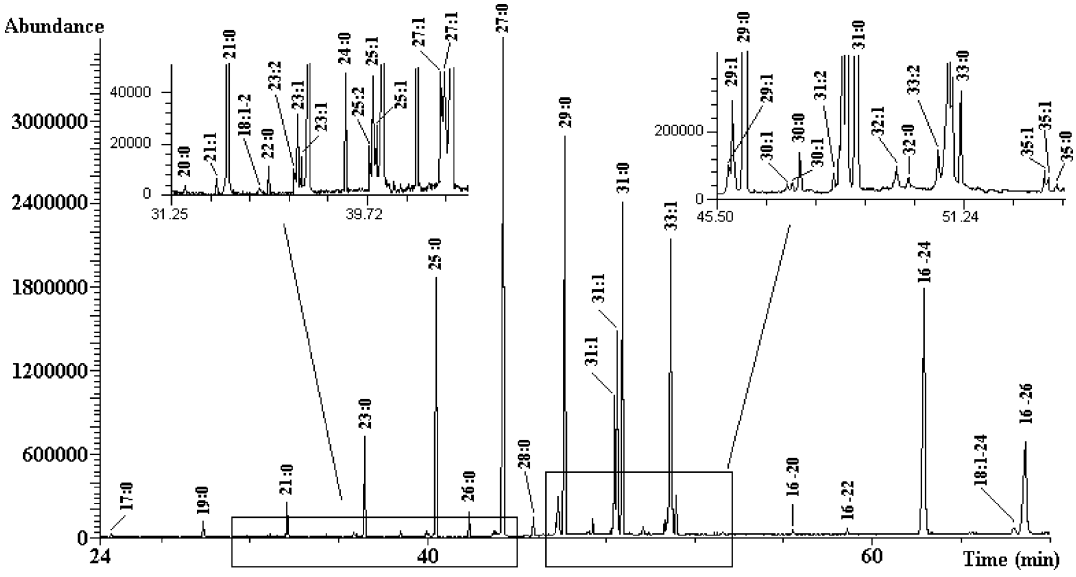

Fig. 1. Total ion chromatogram of the beeswax. See tables for peak identification.

Table 1
Aliphatic hydrocarbons of beeswax

Abbreviation	Retention time (min)	Compound	Molecular ion	Amount (%)	R.S.D.
17	25.76	Heptadecane	240	0.67	0.40
19	29.90	Nonadecane	268	2.37	0.28
20	31.83	Eicosane	282[a]	0.07	0.53
21	33.67	Heneicosane	296	4.66	0.23
22	35.44	Docosane	310[a]	0.20	0.45
23	37.15	Tricosane	324	12.51	0.24
24	38.78	Tetracosane	338	0.81	0.51
25	40.38	Pentacosane	352	35.39	0.21
26	41.87	Hexacosane	366	3.07	0.80
27	43.40	Heptacosane	380	100.0	0.20
28	44.74	Octacosane	394	2.57	0.61
29	46.16	Nonocosane	408	65.25	0.28
30	47.43	Triacontane	422	2.37	0.93
31	48.76	Hentriacontane	436	50.50	0.25
32	49.95	Dotriacontane	450	0.61	0.65
33	51.16	Tritriacontane	464	5.49	0.33
34	52.37	Tetratriacontane	478	0.05	0.57
35	53.46	Pentatriacontane	492[a]	0.19	0.73

[a] Molecular ion not observed.

atoms, being distinguished two compound series. One of them comprised the compounds ranging from 21:1 to 35:1, besides the olefins 30:1 and 32:1; the other isomer series, less abundant, aroused from the olefins 23:1, 25:1, 27:1, 29:1, 30:1, 31:1 and 33:1. The chromatographic peaks for the alkenes were partially overlapped as can be seen in Fig. 1, and the coelution of other isomers cannot be ruled out. The presence of homologue series of isomers for the monounsaturated hydrocarbons of 31, 33 and 35 carbon atoms had already been reported [8]; the odd-chain alkenes, with double bond at 10 position and *cis* configuration, predominate [14]. The alkenes with chain lengths lower than 27:1 had been described in bumble beeswax by Tulloch and Craig [15], who detected only up to the hydrocarbon 23:1 after obtaining wax fractions and oxidizing them for their determination.

Moreover, four dienes (23:2, 25:2, 31:2 and 33:2), in which the double bond position has not been established, have been observed. It had not been detected the presence of higher-molecular mass dienes whose existence was expected

Table 2
Unsaturated hydrocarbons of beeswax

Abbreviation	Retention time (min)	Compound	Molecular ion	Amount (%)	R.S.D.
21:1	33.19	Heneicosene	294[a]	0.12	0.83
23:2	36.63	Tricosadiene	320[a]	0.11	0.75
23:1	36.70	Tricosene	322	0.66	0.60
23:1	36.82	Tricosene isomer	322	0.15	0.81
25:2	39.90	Pentacosadiene	348	0.20	0.52
25:1	39.96	Pentacosene	350	0.86	0.77
25:1	40.07	Pentacosene isomer	350	0.32	1.01
27:1	42.98	Heptacosene	378	1.16	0.41
27:1	43.05	Heptacosene isomer	378	1.73	0.62
29:1	45.77	Nonacosene	406	2.77	0.31
29:1	45.86	Nonacosene isomer	406	7.09	0.22
30:1	47.14	Triacontene	420	0.58	0.94
30:1	47.25	Triacontene isomer	420	0.47	1.05
31:2	48.22	Hentriacontadiene	432	0.84	0.75
31:1	48.42	Hentriacontene	434	21.17	0.31
31:1	48.52	Hentriacontene isomer	434	33.27	0.28
32:1	49.70	Dotriacontene	448	3.29	0.21
33:2	50.65	Tritriacontadiene	460	3.04	0.32
33:1	50.93	Tritriacontene	462	57.58	0.23
33:1	50.99	Tritriacontene isomer	462	5.80	0.25
35:1	53.16	Pentatriacontene	490	1.56	0.44
35:1	53.22	Pentatriacontene isomer	490	0.84	0.43

[a] Molecular ion not observed.

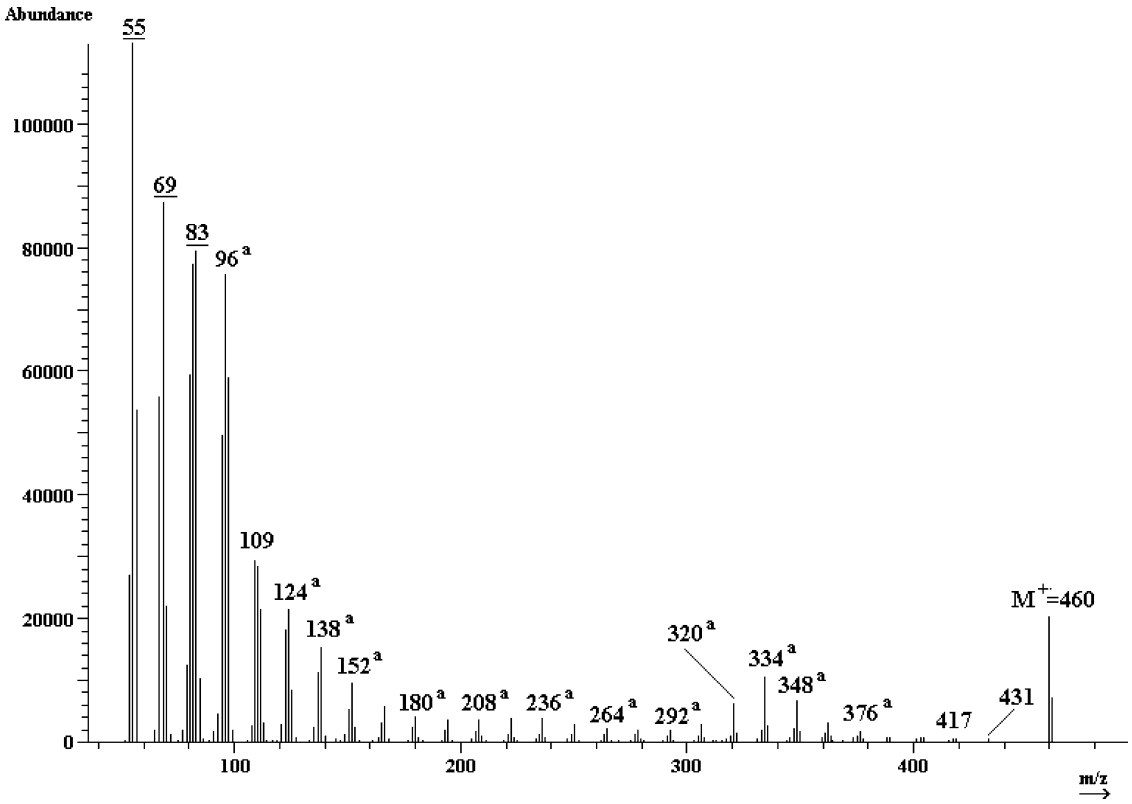

Fig. 2. Electron impact spectrum of tritriacontadiene. Ion series: $C_nH_{2n-3}^+$; $C_nH_{2n-1}^+$ (underlined); $^aC_nH_{2n-2}^+$.

[8,16]. The 23:2 and 25:2 dienes had not been observed before. The alkenes constituted of 33 carbon atoms, either monounsaturated or diunsaturated, were the most abundant. The amount of 31:1 was also notable.

As happened for the saturated hydrocarbons, several alkenes were identified by the use of spectrum libraries, but it was necessary to consider their characteristic fragmentations in electron impact to identify those with higher molecular mass. So, for instance, Fig. 2 shows a spectrum where the C_nH_{2n-1} and C_nH_{2n} ion series are highlighted, besides observing a relatively high abundance of the molecular ion. Obviously, the position of the unsaturations could not be deduced from the mass spectra.

Four monoester compounds—the hexadecanoates of the long-chain alcohols with 20, 22, 24 and 26 carbon atoms—were also detected (Table 3) the presence of a homologue series up to the 16–38 compound has been stated by high-temperature gas-chromatography [6,8]. The 16–20 and 16–22 structures, detected in low amounts and always referred to the 27:0 area, had not been reported previously in beeswax from *A. mellifera*. The 16–22 monoester had already been detected in wax from *Apis dorsata* and *Apis laboriosa* [8]. In some beeswaxes, free hexadecanoic acid was found at a retention time of 31.23 min in amounts ranging from 1 to 4%.

The identification of the palmitates was carried out from the electron impact spectra, which have a well-defined molecular ion. Fig. 3 shows a spectrum and the interpretation of the fragmentations observed.

Table 4 lists the retention times of a compound family whose occurrence in beeswax was unknown. They are 10 ethyl esters of even-chain fatty acids, from 16-2 to 34-2, excepting the 20-2 ester which has not been found in beeswax. The ethyl ester of the oleic acid has also been detected. With the exception of this oleate, 32-2 and 34-2, the ethyl esters coelute with the even-chain aliphatic hydrocarbons as it can be seen in the extracted ion chromatogram of Fig. 4.

Table 3
Palmitates (or hexadecanoates) of beeswax

Abbreviation	Retention time (min)	Compound	Molecular ion	Amount (%)	R.S.D.
16–20	56.38	Eicosyl hexadecanoate	536	0.36	0.42
16–22	58.90	Docosyl hexadecanoate	564	1.29	0.29
16–24	62.34	Tetracosyl hexadecanoate	592	89.82	0.15
16–26	66.87	Hexacosyl hexadecanoate	620	42.98	0.21

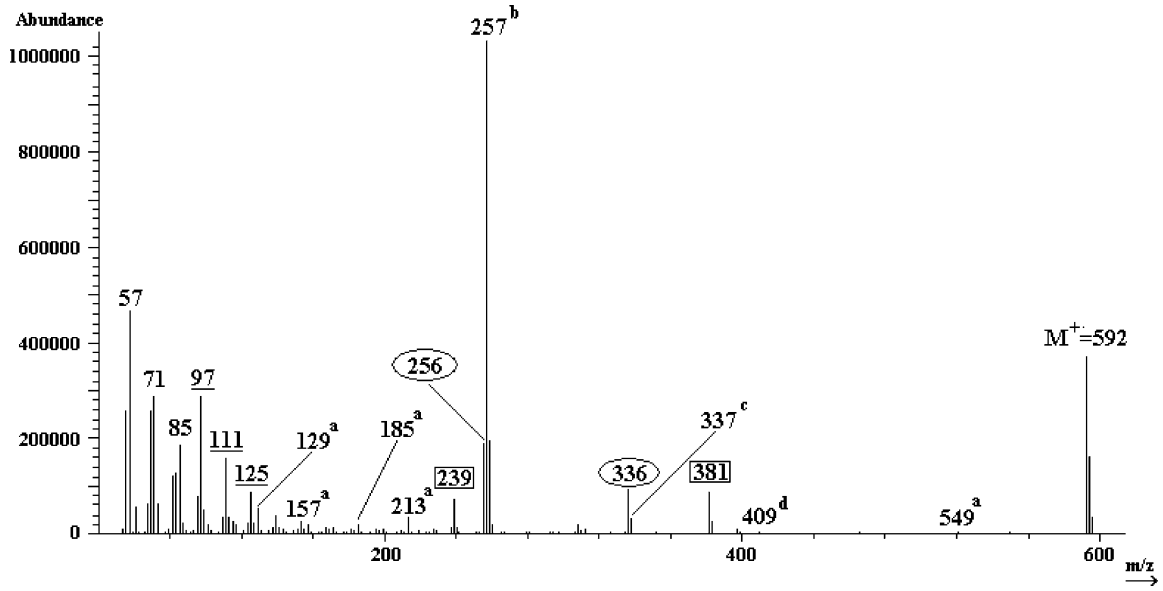

Fig. 3. Electron impact spectrum of tetracosyl palmitate. Ion series: $C_nH_{2n+1}^+$; $C_nH_{2n-1}^+$ (underlined); [a]$C_nH_{2n+1}O^+$; $C_nH_{2n+1}CO_2^+$; Fragmentations: McLafferty rearrangement ($C_{16}H_{32}O_2^+$ and $C_{24}H_{48}^+$ ions) (in circle); α-cleavage at the adjacent carbonyl group ($C_{16}H_{31}O^+$ and $C_{25}H_{49}O_2^+$ ions) (in box). [b]Rearrangement of two hydrogen atoms ($C_{16}H_{33}O_2^+$ ion). [c]Inductive cleavage in C–O bond ($C_{24}H_{49}^+$ ion). [d]γ-H rearrangement to carbonyl group with β-cleavage ($C_{27}H_{53}O_2^+$ ion).

Table 4
Ethyl esters of beeswax

Abbreviation	Retention time (min)	Compound	Molecular ion	Amount (%)	R.S.D.
16-2	31.76	Ethyl hexadecanoate (palmitate)	284	0.66	0.41
18:1-2	34.92	Ethyl 9-octadecenoate (oleate)	310	0.14	0.66
18-2	35.40	Ethyl octadecanoate	312	0.033	0.82
22-2	41.83	Ethyl docosanoate	368	0.039	0.79
24-2	44.72	Ethyl tetracosanoate	396	0.35	0.33
26-2	47.41	Ethyl hexacosanoate	424	0.075	0.60
28-2	49.95	Ethyl octacosanoate	452	0.050	0.93
30-2	52.37	Ethyl triacontanoate	480	0.028	0.88
32-2	54.66	Ethyl dotriacontanoate	508	0.011	1.23
34-2	56.88	Ethyl tetratriacontanoate	536	0.011	1.10

The ethyl oleate whose peak was perfectly resolved (see Fig. 1) was found in a proportion of 1.21% with respect to the 27:0 hydrocarbon. As regards the other ethyl esters, their peak areas recorded in the chromatogram extracted at ion *m/z* 88, selective for this compound family, in relation to 27:0 area are shown in Table 4. In any case, the amounts present of ethyl esters are much lower than those from the even-chain hydrocarbons. As it can be seen in this table, the

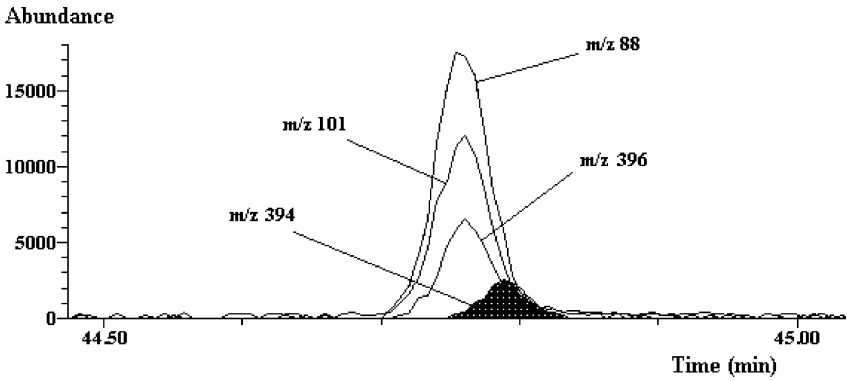

Fig. 4. Extracted ion chromatogram: ethyl tetracosanoate ions: 88, 101 and 396 (molecular ion). Octacosane molecular ion: 394 (shaded).

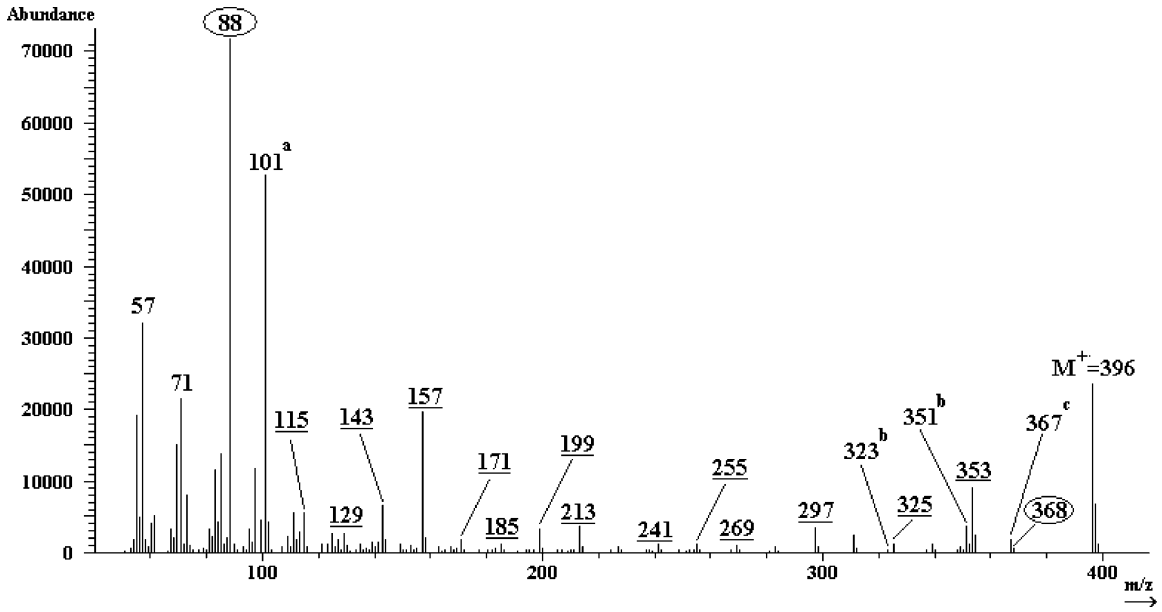

Fig. 5. Electron impact spectrum of ethyl tetracosanoate. Ion series: $C_nH_{2n+1}^+$; $C_nH_{2n+1}CO_2^+$ (underlined). Fragmentations: McLafferty rearrangement ($C_4H_8O_2^+$ and $C_{24}H_{48}O_2^+$ ions) (in circle). [a]γ-H rearrangement to carbonyl group with β-cleavage ($C_5H_9O_2^+$ ion). [b]α-Cleavage at the adjacent carbonyl group ($C_{23}H_{47}^+$ and $C_{24}H_{47}O^+$ ions). [c]Inductive cleavage in C–O bond ($C_{24}H_{47}O_2^+$ ion).

16-2 and 24-2 esters are the prominent ones, this finding is in agreement with the fact that the hexadecanoic and tetracosanoic acids, either free or esterified with long-chain alcohols, are abundant in beeswax; it has been verified by hydrolysis that the hexadecanoic acid is the main one, the oleic acid amount is 10% of the total acids and tetracosanoic acid is one of the most abundant free acids, constituting about a 6% of the total acids in beeswax [2].

Ethyl esters were observed in the spectra and chromatograms by studying the abnormal isotopic distribution of the molecular ion of the even-chain saturated hydrocarbons. The electron impact spectrum of ethyl tetracosanote,

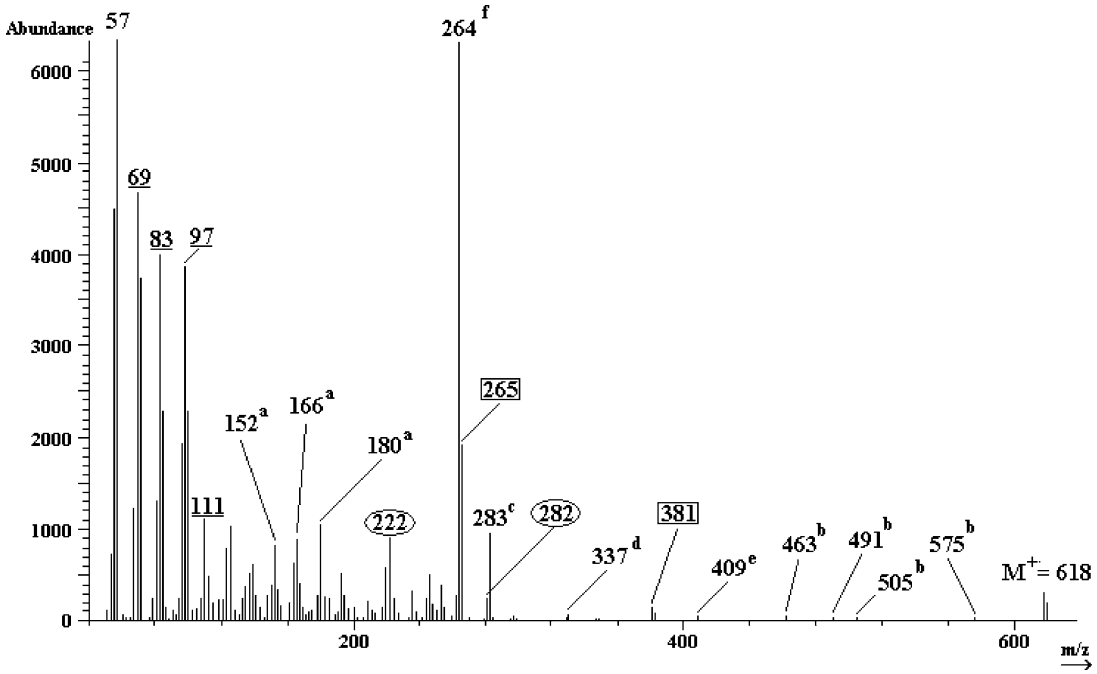

Fig. 6. Electron impact spectrum of tetracosyl oleate. Ion series: $C_nH_{2n+1}^+$; $C_nH_{2n-1}^+$ (underlined); [a]$C_nH_{2n-2}^+$; [b]$C_nH_{2n-1}CO_2^+$. Fragmentations: McLafferty rearrangement ($C_{16}H_{30}^+$ and $C_{18}H_{34}O_2^+$ ions) (in circle); α-cleavage at the adjacent carbonyl group ($C_{18}H_{33}O_2^+$ and $C_{25}H_{49}O_2^+$ ions) (in box). [c]Rearrangement of two hydrogen atoms ($C_{18}H_{35}O_2^+$ ion). [d]Inductive cleavage in C–O bond ($C_{24}H_{49}^+$ ion). [e]γ-H rearrangement to carbonyl group with β-cleavage ($C_{27}H_{53}O_2^+$ ion). [f]Base peak at *m/z* 264: loss of H_2O from $C_{18}H_{34}O_2^+$ or loss of H from; $C_{18}H_{33}O_2^+$.

obtained after subtracting the 28:0 hydrocarbon spectrum, and the interpretation of the fragmentations: ion series, rearrangements and cleavages, is presented in Fig. 5.

Curiously, 18:1-2, 30-2, 32-2 and 34-2 were only observed in the oldest pure beeswaxes (with yellow-brown color). These compounds were not detected in three samples of pure beeswax recently yielded by the honey-bees (yet with white color) in the wires of the comb.

The ethyl esters of the fatty acids are known artifacts formed during the methylation process or in the injection port of a gas chromatograph when ethanol-containing chloroform is used as a solvent. To confirm effectively the occurrence of this ethyl ester family in beeswax, some experiences were carried out with chloroform washed several times with water to remove the ethanol, a stabilizent, as proposed to avoid the formation of artifacts [17]. The experiences confirmed the occurrence of the new family.

Finally, a new component of beeswax, tetracosyl oleate which eluted at 66.51 min and with a relative amount of 2.05%, was detected. Its spectrum and the explanation of some characteristic fragment ions are shown in Fig. 6. Obviously, and the same as for ethyl oleate, the position of the double bond could not be established by the electron impact spectra, but considering that oleic acid is the most abundant monounsaturated acid in beeswax (as mentioned above), it is presumable that this compound could be an oleate. Furthermore, oleates of alcohols with chains longer than 28 carbon atoms have been described in beeswax elsewhere [2,18].

3.2. Strange compounds found in foundation beeswax sheets

The chromatograms obtained for the foundation beeswax samples showed a greater number of chromatographic peaks in comparison with the elution pattern of the pure beeswax, probably as a consequence of the presence of strange substances. Many of these rare compounds were the same in 15 of the foundation beeswax samples analyzed, which suggested that these samples had a similar elaboration process. Table 5 shows the strange compounds found in these samples and their mean abundances also expressed as

Table 5
Strange compounds of commercial foundation beeswax

Retention time (min)	Compound	Molecular ion	Amount (%)	Frequency (%)
29.41	Nonadecene	266	0.09	65
31.23	Hexadecanoic (palmitic) acid	256	5.43	60
33.21	Heneicosene	294	0.22	75
34.34	9*,12*-Octadecadienenoic (linoleic) acid	280	0.21	80
34.47	9*-Octadecenoic (oleic) acid	282	2.71	75
34.59	9*,12*-Octadecadienenoic acid, positional isomer	280	0.57	80
34.83	Ethyl linoleate	308	0.37	60
38.21	Tetracosene	336	0.08	70
38.38	Tetracosene isomer	336	0.27	80
39.81	Propylpentacosane	352	0.38	80
41.35	Propyltricosane	366	0.24	75
41.51	Ethyltetracosane	366	0.41	80
42.84	Methylpropyltricosane	380	0.52	75
43.86	–	–	2.66	30
44.27	Methylpropyltetracosane	394	0.74	80
44.42	Ethylhexacosane	394	0.46	70
44.56	Tetracosanoic acid	368	0.63	40
45.24	Esqualene	410	0.89	80
45.67	Methylpropylpentacosane	408	1.01	75
46.60	–	–	2.70	65
47.00	Methylpropylhexacosane	422	0.95	75
47.89	–	–	0.48	75
49.16	–	–	1.50	40
49.77	Dotriacontene isomer	448	3.15	55
51.42	Tetratriacontene	476	0.37	85
51.56	Tetratriacontene isomer	476	0.57	90
52.39	Tetratriacontane	478	3.75	90
53.01	Pentatriacontadiene	488	0.24	85
54.27	Octadecyl palmitate	508	0.75	45
54.62	Hexatriacontane	506	2.35	80
55.70	Heptatriacontane	520	1.27	70
56.24	Octadecyl oleate	534	0.68	75
56.78	Octatriacontane	534	0.93	65
58.00	Nonatriacontane	548	0.62	75

(–) Compound and molecular ion not known; (*) supposed.

percentage with respect to the hydrocarbon 27:0; from the 33 compounds found, six were not identified. Methylpropylalkanes, olefins and very long-chain linear aliphatic hydrocarbons were often found and relatively abundant, which pointed out the addition of petroleum waxes to beeswaxes. On the other hand, the peak area for the even-chain saturated linear hydrocarbons was considerably higher, which corroborated this supposition because petroleum waxes contain all chain lengths. The occurrence of free long chain fatty acids indicated the possible addition of fatty substances to beeswax.

In consequence, pure beeswax can be distinguished from rejected foundation beeswax by the detection of odd chromatographic peaks in the chromatograms of beeswax solution, which allows having a simple procedure at disposal to test the beeswax quality.

4. Conclusions

Fifty-six components have been identified, some of them tentatively, in pure beeswax from the species *A. mellifera* by low-temperature gas chromatography coupled to electron impact ionization mass spectrometry. Twenty-four compounds had not been reported before, for instance, a new family of ethyl esters from 16-2 to 34-2, excepting the 20-2 ester and including the 18:1-2.

Non-natural pure beeswax compounds, such as branched aliphatic hydrocarbons, olefins and free fatty acids were found in rejected foundation beeswaxes. The observation of these strange compounds in them could be used as an indicator of the adulteration of beeswaxes.

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