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Nonsaponifiable lipid components of the pollen of elder (*Sambucus nigra* L.)

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Abstract

Pollen of the elder (*Sambucus nigra* L.) was extracted with chloroform–methanol. The extract was separated by column chromatography into the following groups of compounds: hydrocarbons (8.7%), polycyclic aromatic hydrocarbons (0.2%), complex esters (5.2%), triglycerides (18.7%), hydroxy esters (27.9%), free fatty acids and alcohols (16.8%), free sterols (6.8%), and triterpenic alcohols (4.0%). The nonsaponifiable components (hydrocarbons, fatty acids, alcohols, and sterols) were examined in detail using spectroscopic and chromatographic methods (IR spectroscopy, GC, and GC–MS). The identified compounds were characterized by their mass spectra and Kováts retention indices. The double bond positions and their configurations in unsaturated compounds are also reported.

Keywords: *Sambucus nigra*; Pollen; Hydrocarbons; Fatty acids; Fatty alcohols; Sterols; Alcohols; Triterpenes; Terpenes; Polynuclear aromatic hydrocarbons

1. Introduction

Elder (*Sambucus nigra* L.) is a bush (up to 5 m high), rarely a tree (height up to 10 m), distributed in the temperate zone of the Northern hemisphere [1]. Elder grows in the flooded forest areas, at the forest edges, and in the bushes rows along river banks. In the last decades, it spread to a great extent onto localities influenced or damaged by human activities [2,3]. The flowering period changes from late April to early August depending on the locality or weather conditions. The inflorescence consists of individual flowers bearing five petals and five stamens each with one anther. The number of flowers in the inflorescence is between 500 and 1700 (1000 in average). Yellow pollen grains have an ellipsoidal shape, size 23.5–25.3×14.5 μm [4]. Pollen of elder is considered allergologically important and its importance (in a negative sense) has a growing trend [3]. It influences negatively about 8% patients suffering allergy [4]. Most of the pollen mass is transferred by wind. The mechanisms of the penetration of allergens through mucous membranes has not been fully explained neither is known the role of particular pollen components in this process.

The composition of the pollen lipids of elder has not been reported before. The purpose of the present study was to analyze its nonsaponifiable fraction in detail as the lipids may play a role in the penetration

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of pollen through membranes and thus, they may contribute to eliciting an allergic reaction.

2. Experimental

2.1. Materials

Light petroleum (fraction b.p. 40–60°C), hexane, diethyl ether and methanol were freshly distilled. Light petroleum for chromatography on silver nitrate impregnated silica gel was treated with conc. sulfuric acid, neutralized and distilled. Chloroform was treated with a solution of $CaCl_2$, dried with $CaCl_2$, distilled and stabilized with ethanol (0.5%, v/v). Standards of *n*-alkanes, methyl esters of fatty acids, 1-alkanols and sterols were from Applied Science Labs., USA, Alltech Associates, USA, and from Sigma, Czech Republic. Standards of α- and β-amyrines originated from Koch-Light Labs., UK, fucosterol was from Steraloids, USA. Dimethyldisulfide (DMDS) was a product of Aldrich, Czech Republic.

2.2. Collection of pollen

The locality of collection was in Prague (elevation 220 m, map field code 5852) approximately 500–1000 m far from streets with residential buildings. Inflorescences were cut-off the bush just before the full opening at the end of May. The flowers (2200 inflorescences, 20.4 kg wet mass) were dried in the dark at room temperature for 14 days (dry mass 3.25 kg, i.e., 15.9% of fresh mass before drying). The pollen was separated from other flower parts using a set of sieves (DIN 4188 mesh sizes 1.0, 0.71, 0.125, and 0.063 mm; Tyler mesh sizes 16, 24, 115, 250). The collected pollen was stored at −18°C until chemical analyses were done.

2.3. Extraction

The pollen (50 g, 120 ml) was extracted continuously at room temperature in a chromatographic column (2.8 cm I.D.) with a mixture chloroform–methanol (1:1, v/v; total volume 4000 ml). After evaporation of solvents in vacuo, 16.79 g of the crude extract was obtained (i.e., 33.65% of the pollen mass). The extract was kept at −18°C until further work-up.

2.4. Column chromatography

The pollen extract (4.0 g) was adsorbed on silica gel (12 g, 60–120 μm) and chromatographed on a column (3.6 cm I.D.) containing silica gel Pitra (200 g, 50–100 μm, water content 10%) [5]. The extract components were eluted with a mixture of light petroleum–diethyl ether (0 to 100% of diethyl ether, v/v; total solvent volume 3000 ml). The mixture was separated into 26 fractions (50 to 250 ml). Eluted compounds were monitored using thin-layer chromatography (TLC), fractions containing compounds with the same R_F were combined. The following fractions were obtained: aliphatic hydrocarbons, polycyclic aromatic hydrocabons (PAHs), complex esters, triglycerides, hydroxyesters, free fatty acids, free alcohols, free sterols and triterpenoids.

2.5. Thin-layer chromatography

Analytical TLC was carried out on glass plates (36×76 mm) coated with Adsorbosil-Plus (Applied Science Labs.; layer thickness 0.2 mm with gypsum (12%). Visualization of the spots were achieved by heating the plates sprayed with conc. sulfuric acid.

Preparative TLC was carried out on glass plates (20×20 cm or smaller) coated with Adsorbosil-Plus (Applied Science Labs.; layer thickness 0.2 mm) with gypsum (12%). Aliphatic hydrocarbons from column chromatography (fractions 1–3, 65 mg) were separated using TLC plates impregnated with $AgNO_3$ (20%). Fractions of alkanes (R_F 0.94, 48 mg, i.e., 77.8% of hydrocarbon mixture) and alkenes (R_F 0.29, 13.7 mg, i.e., 22.2% of hydrocarbon mixture) (purified light petroleum as a solvent). The dry plates were sprayed with Rhodamine 6G (0.05% in ethanol). The chromatographic zones were scraped off and transferred into small columns (0.8 cm I.D.) filled with silica gel (0.5 g, 25–50 μm) and eluted with dry freshly distilled diethyl ether (20 ml). Fractions containing PAHs and those with free acids and free alcohols were rechromatographed in the same way on a non-impregnated silica gel. The R_F values are measured in the middle of the corresponding spot or band.

2.6. Infrared (IR) spectroscopy

Infrared spectra were measured on a Fourier transform (FT) IR spectrometer Model IFS 88 (Bruker, Germany), tetrachloromethane solution, cell 0.1 mm, range 3800–400 cm^{-1}.

2.7. Gas chromatography (GC)

GC was performed with a HP 5890A gas chromatograph (Hewlett-Packard, USA) equipped with a flame ionization detector and a split/splitless injector. Chromatographic conditions (column, injector, and detector temperatures) are given in Tables 2–6. Nitrogen was used as make-up gas (30 ml/min). Data were collected with a HP 3393A integrator (Hewlett-Packard).

2.8. Gas chromatography–mass spectrometry (GC–MS)

The fractions were analyzed using a gas chromatograph with a splitless injector (200°C) and a mass detector (Fisons MD 800), working in the electron impact ionization mode. A DB-5ms column (30 m×0.25 mm, 0.25 μm; J&W Scientific) and helium (flow 0.55 ml/min, measured at 50°C) were used for the separations. The temperature programs were similar to those for GC (see Tables 2, 3, 5, and 6). The identification of compounds was based mostly on their mass spectra compared to those in the National Institute of Standards and Technology (NIST) Library (USA) and on the co-chromatography with commercially available standards.

2.9. Esterification

A method earlier described [6,7] for transesterification of triglycerides was used for the esterification of free fatty acids. The free fatty acids fraction (1.5 mg) was heated with a mixture of methanol–chloroform (3:2, 250 μl) and acetylchloride (29.4 μl) in a sealed ampoule (70°C, 60 min). Neutralization was made with Ag_2CO_3 (57.1 mg) and after centrifugation, the supernatant was analyzed directly using GC or GC–MS.

2.10. Dimethyldisulfide derivatives [8]

Alkenes (1.0 mg) or fatty acid methyl esters (0.7 mg), hexane (350 μl), DMDS (350 μl) and I_2 solution in diethyl ether (5%, w/w, 17.5 μl) were kept in the dark at room temperature for 24 h. The solutions were decolorized with sodium thiosulfate (5% in water), water (300 μl) was added and the organic layer was separated. The extraction with hexane was repeated (2×300 μl). Combined organic layers were washed with water (2×200 μl) and concentrated to a volume of 300 μl. In the case of alkenes, the DMDS adducts were purified by preparative TLC on silica gel (4×10 cm, hexane) prior to the GC–MS analysis.

2.11. Calculations of Kováts retention indices (I) and equivalent chain length (ECL) values

The calculations of *I* were carried out using the method for calculation of ECL values described earlier [9,10]. Mixtures of selected odd- and even-membered *n*-alkanes were used as standards for the co-elution with the samples of alkenes and free sterols. In case of the determination of ECL values, a corresponding mixture of selected methyl esters of saturated fatty acids was used.

3. Results and discussion

A method of continuous extraction of pollen was chosen to obtain maximum extractable compounds. After elution of 4000 ml of the mixture chloroform–methanol no more compounds were extracted.

From the collected elder inflorescences (2200 inflorescences, 3250 g dry mass), 100 g of pure pollen for analyses was obtained. The mass balance in flowers was as follows: inflorescence and petals 3110 g, anthers 8 g, pure pollen 100 g and slightly impure pollen 27 g. The estimated loss of pollen during the work-up procedure was about 5% (6 g). Thus, the total content of dry pure pollen in flowers is estimated to 130–135 g, i.e., 4% of the dry flowers mass. One inflorescence representing 1080 flowers in average produces 60 mg pollen, i.e., 60 μg pollen per flower or 12 μg per anther on average.

By the means of gradient column chromatography

Table 1
Composition of the *Sambucus nigra* L. pollen extract

Fraction	Yield		Group of compounds
	mg	% (w/w)	
1–3	67.5	8.7	Aliphatic hydrocarbons
4–12	1.2	0.2	PAHs+squalane
13	40.2	5.2	Complex esters
14	3.8	0.5	Mixed fraction
15–17	145.4	18.7	Triglycerides
18	8.3	1.1	Mixed fraction
19	217.3	27.9	Hydroxy esters
20	79.3	10.2	Mixed fraction
21, 22	130.4	16.8	Free fatty acids and free alcohols
23	52.7	6.8	Free sterols
24–26	31.4	4.0	Triterpenes
Total	777.5	100	

the extract was separated into the following well defined group of compounds: aliphatic hydrocarbons, PAHs, complex esters, triglycerides, hydroxy esters, free fatty acids with part of free alcohols and free sterols. The composition of these fractions is described below and the quantitative data are summarized in Tables 1–6.

3.1. Aliphatic hydrocarbons

Fractions 1–3 from column chromatography made one spot at TLC (light petroleum) on the non-impregnated silica gel (R_F 0.98). However, the gas chromatogram on a DB-1 column showed a complicated mixture of hydrocarbons, forming several

Table 2
Composition of *n*-alkanes and alkenes in the fraction of hydrocarbons

Number of C atoms	*n*-Alkanes (%)[a]	9-Alkenes			7-Alkenes		
		%[b]	I±SD	n[c]	%[b]	I±SD	n[c]
15	+						
16	0.04						
17	0.13	+					
18	0.14						
19	7.61	0.10			+	1879.09±0.23	4
20	1.38	+					
21	19.46	0.28			1.83	2080.25±0.08	5
22	2.09				+	2179.45	1
23	13.90	2.01	2272.92±0.05	5	1.39	2279.23±0.19	5
24	1.91	0.08	2371.99±0.01	2			
25	23.75	16.56	2471.92±0.24	5	1.07	2478.78±0.19	5
26	2.22	0.12	2570.77±0.59	2	+		
27	23.80	15.31	2671.31±0.33	5	5.15	2678.86±0.09	5
28	0.79	0.31	2770.56±0.42	2	+		
29	2.67	39.28	2872.05±0.69	4	7.45	2879.52±0.28	5
30	+	0.58	2970.48±0.32	3	+		
31	0.11	7.75	3071.23±0.19	4	0.51	3079.18	1
32	+	0.05					
33	+	0.17					
Total	100	82.60			17.40		

GC conditions: fused-silica column DB-1 (30 m×0.25 mm, 0.25 μm), oven 140°C, then 5°C/min to 320°C (20 min), injector 250°C, detector 250°C, average flow velocity of carrier (H_2) 50 cm/s (at 140°C), split ratio 33:1.
GC conditions for measurements of Kováts retention indices I: DB-1 (30 m×0.25 mm, 0.25 μm), oven 230°C, injector 250°C, detector 250°C, average flow velocity of carrier (H_2) 40 cm/s, split ratio 30:1; coinjected mixture of *n*-alkanes: C_{19}–C_{29}.

[a] Area % of *n*-alkanes fraction.
[b] Area % of alkene fraction.
[c] Number of measurements; + traces.

Table 3
Composition of acids in the fraction of free fatty acids (preparative TLC, R_F 0.60)

Number of C atoms	Saturated (%)[a]	Unsaturated *n*-9 (%)[a]	ECL±SD	*n*[b]
11	0.09	0.22		
12	+	0.12		
13	0.16	0.36		
14	+	0.25		
15	0.32	0.80		
16	0.65	+	16.2433±0.0088	3
18	0.40	0.38	18.2742±0.0048	5
20	1.06	0.32	20.2772±0.0019	5
21	+			
22	1.88	0.15	22.2677±0.0019	4
		0.57*	22.6513±0.0062	4
23	0.69			
24	3.37	0.78	24.2631±0.0031	5
25	0.62	+		
26	1.65	3.02	26.2635±0.0039	5
27	0.63	0.33	27.2499	1
28	1.20	10.04	28.2644±0.0055	5
29	0.29	3.55	29.2591±0.0074	5
30	0.39	38.87	30.2945±0.0060	5
		1.18*	30.3597±0.0034	4
31		6.09	31.2654±0.0073	5
32		17.87	32.2798±0.0068	5
33		0.81		
34		0.89		
Total	13.40	86.60		

GC conditions: fused-silica column DB-1 (30 m×0.25 mm, 0.25 μm), oven 140°C, then 5°C/min to 320°C (20 min), injector 250°C, detector 250°C, average flow velocity of carrier (H_2) 50 cm/s (at 140°C), split ratio 33:1.
GC conditions for measurements of ECL values: DB-WAX (30 m×0.25 mm, 0.25 μm): oven 240°C, injector 250°C, detector 250°C, average flow velocity of carrier (H_2) 40 cm/s, split ratio 30:1; coinjected mixture of fatty acid methyl esters: 20:0–31:0 (excluding 27:0).
[a] Area % of fatty acids methyl esters fraction.
[b] Number of measurements; + traces; * double bond position unknown.

homologous series. Therefore, part of the hydrocarbons mixture was subjected to preparative a TLC run on silica gel impregnated with silver nitrate [11] and thus separated into two parts: alkanes (R_F 0.98) and alkenes (R_F 0.57) (R_F values measured at analytical TLC on SiO_2 impregnated with 20% $AgNO_3$).

Alkanes represent a homologous series from *n*-C_{15} to *n*-C_{33} with odd-numbered members predominating and with maximum at C_{25} and C_{27} (Table 1). This pattern is common in cuticular lipids of higher plants [12].

The relatively high proportion of unsaturated hydrocarbons is noteworthy (28.9% of the hydrocarbons fraction, value based on the GC integration of fractions 1–3 before separation, or 22.2%, value based on weighing the separated fractions). Absorption maxims at 3005 and 1653 cm^{-1}, present in IR spectra (band in range 960–970 cm^{-1} was not present), confirm the presence of (*Z*)-double bonds. GC on a DB-1 column showed two well separated homologous series of olefins. The double bonds positions as determined by DMDS derivatization [8] were 9 and 7. 9-Alkenes (82.6%) have shorter retention times than 7-alkenes (17.4%). Kováts retention indices *I* were measured and calculated for most of them (Table 1). It was shown earlier [13,14] that a shift of double bond on the hydrocarbon chain

Table 4
Composition of acids in the fraction of free fatty acids (preparative TLC, R_F 0.49)

Compound	%[a]	ECL±SD	n[b]
14:0	0.53	13.9994±0.0024	5
15:0	0.34		
16:0	35.69	16.0074±0.0039	5
16:1*n*-9	0.39	16.2494±0.0017	5
16:1*n*-7	0.38	16.3084±0.0006	4
17:0	0.22		
18:0	3.15	17.9980±0.0012	5
18:1*n*-9	14.26	18.2332±0.0014	5
18:1*n*-7	2.86	18.2994±0.0015	5
18:2*n*-6	12.79	18.7067±0.0012	5
19:0	0.10		
18:3*n*-3	12.48	19.3574±0.0015	5
20:0	2.32		
20:1*n*-9	2.48	20.2100±0.0019	5
21:0	+		
22:0	0.96		
22:1*n*-9	0.30		
23:0	0.22		
24:0	0.59		
24:1*n*-9	0.37		
25:0	+		
26:0	+		
26:1*n*-9	0.38		
27:0	+		
28:0	+		
28:1*n*-9	0.67		
30:0	0.77		
30:1*n*-9	1.18		
31:0	+		
Unidentified	6.57		
Total	100		

GC conditions: fused-silica column DB-WAX (30 m×0.25 mm, 0.25 μm), oven 200°C (20 min), then 5°C/min to 240°C (20 min), injector 240°C, detector 250°C, average flow velocity of carrier (H_2) 50 cm/s (at 200°C), split ratio 30:1.
GC conditions for measurements of ECL values: DB-WAX (30 m×0.25 mm, 0.25 μm), oven 200°C, injector 240°C, detector 250°C, average flow velocity of carrier (H_2) 40 cm/s, split ratio 30:1; coinjected mixture of fatty acid methyl esters: 13:0, 15:0, 17:0, 19:0, 20:0, 21:0, 22:0.
[a] Area % of fatty acids methyl esters fraction.
[b] Number of measurements; + traces.

Table 5
Composition of fraction containing 1-alkanols and amyrines

Number of C atoms	Area %
20	+
21	0.13
22	1.08
23	0.22
24	0.65
25	0.23
26	7.57
27	2.15
28	54.03
29	0.66
30	7.63
β-Amyrin	2.91
α-Amyrin	11.00
31	0.40
32	3.19
33	0.40
34	0.40
1-Alkanols (sum)	78.74
Amyrines (sum)	13.91
Unidentified	7.35
Total	100

+ Traces.
GC conditions: fused-silica column DB-1 (30 m×0.25 mm, 0.25 μm), oven 140°C, then 5°C/min to 320°C (20 min), injector 250°C, detector 250°C, average flow velocity of carrier (H_2) 50 cm/s (at 140°C), split ratio 33:1.

toward its center causes shortening of retention time. Alkenes with the double bond in the middle of the chain have the shortest retention times [15]. 9-Alkenes have *I* values on average 28.5 units lower than the corresponding *n*-alkanes, while in 7-alkenes this difference is only 20.7 units.

3.2. PAHs and squalene

Fractions 4–12 from column chromatography were rechromatographed by preparative TLC on non-impregnated silica gel (hexane). Two fractions were obtained (R_F 0.73 and 0.66), the mass of each was lower than 0.1 mg. Based on the mass spectra and co-elution with standards, phenathrene (59%) and pyrene (9%) were identified in the first fraction (R_F 0.73). In the second fraction (R_F 0.66), fluoranthene (9%) and squalene (76%) formed the main components. The minor components were not fully identified, but their mass spectra show the pattern of PAHs. Concerning their very low content in the

Table 6
Composition of fraction containing free sterols

Compound	Area %	$I \pm$SD	n[a]	Note
Campesterol	16.42	3193.06±0.23	4	
Stigmasterol	1.65	3221.93±0.41	4	
Unidentified	0.84	3258.09±0.24	3	M_r 412, mass spectrom similar to that of chondrilasterol or stigma-7.25-dien-3-ol
β-Sitosterol	66.49	3275.00±0.10	4	
Unidentified	13.90	3286.57±0.07	3	M_r 412, mass spectrom identical with that of fucosterol
Unidentified	0.70	3309.74±0.15	3	M_r 412, mass spectrom nearly identical with that of fucosterol, only ion at m/z 97 is higher

GC conditions: fused-silica column DB-1 (30 m×0.25 mm, 0.25 μm), oven 260°C, then 2°C/min to 310°C (5 min), injector 260°C, detector 270°C, average flow velocity of carrier (H_2) 50 cm/s at 260°C.
GC conditions for measurements of Kováts retention indices I: DB-1 (30 m×0.25 mm, 0.25 μm), oven 270°C, injector 270°C, detector 270°C, average flow velocity of carrier (H_2) 40 cm/s), split ratio 30:1; coinjected mixture of n-alkanes C_{26}, C_{28}, C_{30}, C_{34} and C_{36}.
Kováts retention indices I of standard sterols: 3193.53±0.40 (campesterol); 3222.15±0.22 (stigmasterol); 3275.18±0.01 (β-sitosterol), 3273.74±0.12 (fucosterol).
[a] Number of measurements.

whole pollen extract we assume that PAHs were contaminants from the environment.

3.3. Free fatty acids

Fractions 21 and 22 from column chromatography made one oval and tailing spot when checked by analytical TLC. The spot shape was characteristic for free acids. A careful preparative TLC separation (hexane–diethyl ether, 8:2) gave three fractions: R_F 0.60 (26.0%), 0.49 (61.5%), and 0.34 (12.5%). IR spectra of first two fractions indicated aliphatic carboxylic acids having (*Z*)-double bonds (bands 3534, 3011, 1760, 1711, 1658 cm^{-1}, the band in the 960–970 cm^{-1} range was not present). The third fraction (R_F 0.34) containing aliphatic alcohols is described below (Section 3.4).

The two free fatty acids fractions were esterified separately and analyzed both on a non-polar column (DB-1) and on a polar one (DB-WAX). The first fraction consisted of saturated and unsaturated acids mostly from C_{24} to C_{34} with a maximum at $C_{30:1}$ (Table 3). Such long-chain fatty acids are not commercially available for comparison. Therefore, we have prepared DMDS adducts of the methylated fraction and analyzed mass spectra of the obtained products (Table 3). All homologues had double bonds in the positions *n*-9 (characteristic mass spectral fragment at m/z 173).

The second fraction was analyzed by GC only and the components were characterized by comparison of their retention times with those of standards and by calculation of the ECL values [9,10]. Besides the small amounts of saturated acids (from C_{14} to C_{30} with maximum at C_{18}) mostly unsaturated fatty acids with (*Z*)-double bonds were present in this fraction (18:1*n*-9, 18:1*n*-7, 18:2*n*-6, 18:3*n*-3 and 20:1*n*-9; Table 4). These acids are rather common in natural materials.

3.4. Free alcohols

From the free fatty acid fraction, another part was separated by preparative TLC (a band with R_F 0.34). The R_F value corresponded to that of 1-octadecanol (hexane–diethyl ether, 7:3). The GC and GC–MS analyses showed a homologous series of 1-alkanols of chain lengths C_{20} to C_{34} with a maximum at C_{28} and with even number of carbons in the chains mainly. This fraction also contained substantial amounts of α- and β-amyrines the structure of which was confirmed by mass spectra and by co-injection with authentic standards. The data are summarized in Table 5.

3.5. Free sterols and triterpenes

Fraction 23 from column chromatography when checked by TLC had R_F 0.22 (hexane–diethyl ether, 8:2) which corresponded to that of the β-sitosterol

standard. The infrared spectrum showed absorptions at 3622, 3032, 3013 and 1052 cm^{-1}. The fraction components were characterized by calculated *I* values and the values were compared with those of commercial standards (Table 6). Furthermore, the structures were confirmed by comparison of mass spectra. Campesterol, stigmasterol and β-sitosterol were identified in the sterols fraction. The two components eluting after β-sitosterol had a mass spectra identical with fucosterol, but its retention times and the calculated *I* values were not identical. Thus, the structure of this component could not be determined.

Fractions 24–26 from column chromatography with R_F 0.15 contained three peaks in the gas chromatogram with the following retention indices *I*: 3454.72±0.01; 3458.67±0.2 and 3473.15±0.20. The mass spectrum of the last compound (main peak) showed the following fragments: *m/z* 43, 69, 95, 109 (base peak), 135, 207, 357 and 426 (molecular ion). We have not succeeded in finding an identical spectrum in the library. The molecular mass corresponds to a triterpenic alcohol, however, the structure could not be fully determined. This unidentified compound made only minor component (under 1%) of the pollen extract.

4. Conclusions

It can be seen from the detailed analysis of the nonsaponifiable components that the composition of extractable compounds from the elder pollen is very complex. Besides compounds common in natural materials we have found substantial amounts of less common substances such as alkenes. We have analyzed earlier green parts of many plants species, but alkenes were rather rare in those materials [11]. On the other hand, pollen and some flower parts were shown to contain substantial amounts of alkenes [16].

An interesting result is the content of both saturated and unsaturated free fatty acids of very long chains. More literature reports of these compounds occur due to the development of modern fused-silica columns that enable analyzing methyl esters of fatty acids up to C_{34} on polar phases or up to C_{40} or longer on non-polar phases [17].

Determination of the *I* and ECL values using older methods of two standards (compounds having shorter and longer retention times compared to the measured compound) gave an indication of the identity of the analyzed compound, but they were not very accurate. On the other hand, methods used in our study are based on several standards [9,10]. Therefore, these methods are more accurate, the obtained values are more reliable and they can be used for identifications of analyzed compounds.

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