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IDENTIFICATION OF WAX ESTERS OF THE FRESH-WATER GREEN ALGA *CHLORELLA KESSLERI* BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Wax esters were detected in the fresh-water green alga *Chlorella kessleri* cultivated heterotrophically and their individual molecular classes of up to 50 carbon atoms were identified by means of capillary gas chromatography–mass spectrometry. The wax esters are composed from very long-chain fatty acids up to dotriacontanoic acid and alcohols up to 13-eicosenol with hexadecanol as a major representative. Monoenic acids have an unusual position of the double bond, namely at the *n*-18 position.

INTRODUCTION

Wax esters are distributed in both the animal and plant kingdoms[1], primarily in higher forms, whereas in lower animals and plants they have been detected only rarely, *e.g.*, in photosynthesizing fresh-water (*Euglena*[2]) and/or marine (dinoflagellates[3] and cryptomonads[4]) protozoans.

Fatty acids with a chain longer than 24 carbon atoms are common constituents of these wax esters and have been detected in the fresh-water algae *Chlorella*[5–7] and *Euglena*[2] and in the marine algae *Stauroneis*[8] and *Emiliana*[9]. The position of the double bond in the above unsaturated acids has not yet been reported and has been determined only in unsaturated acids of mycobacteria[10], phylogenetically highly unrelated organisms.

Gas chromatography (GC) on packed columns with a thermostable non-polar stationary phase[11] separates wax esters according to the number of carbon atoms. Separation according to the number of carbon atoms and simultaneously according to the degree of unsaturation can be achieved on a capillary column of the SE-52 type[12]. However, satisfactory discrimination of saturated esters differing only in the combination of acyl and alkyl chains, *i.e.* compounds with an identical number of carbon atoms and the same unsaturation, on either packed or capillary columns has not yet been reported. Thus, for instance, on a Silar 10C wall-coated open tubular column it was possible to separate wax esters with 28–44 carbon atoms and with 0–6 double bonds[13]. On this column the combinations 14:0–14:0 (acid–alcohol) and

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8:0–20:0 could be separated, but the combinations 14:0–14:0 and 12:0–16:0 could not. The separation improves with increasing number of double bonds but is insufficient for two double bonds.

It was the aim of this work to detect the possible occurrence of wax esters in the fresh-water green alga *Chlorella kessleri,* to separate their mixture according to the number of carbon atoms and unsaturation and to identify individual molecular classes including fatty acids and alcohols, *i.e.,* the basic constituents of wax esters, using methods that avoid the above-mentioned shortcomings of the methods reported previously.

EXPERIMENTAL

Isolation and derivatization

A 100-g amount of lyophilized *C. kessleri* alga cultivated heterotrophically and obtained from the Department of Autotrophic Microorganisms of the Institute of Microbiology in Třeboň, Czechoslovakia, was extracted three times with 2 l of *n*-hexane–isopropanol (2:1). The combined extracts were evaporated to dryness and chromatographed on a silica gel column with chloroform. The fraction of non-polar lipids was further purified by means of preparative thin-layer chromatography (TLC) on silica gel H plates (Merck, Darmstadt, F.R.G.) with chloroform and the band of wax esters of R_F 0.6–0.7 (12.3 mg) (for palmityl palmitate $R_F = 0.6$) was then analysed by gas chromatography–mass spectrometry (GC–MS).

A 10-mg amount of wax esters was transesterified with sodium methoxide[14] and the methyl esters and alcohols obtained were separated by TLC and alcohols were converted into trimethyl silyl (TMS) ethers using trimethylchlorosilane plus hexamethyldisilazane. Methyl esters were separated by high-performance liquid chromatography (Spectra-Physics 8000 with an RP-1 column) [15] and degraded oxidatively[14], yielding monocarboxylic and dicarboxylic acids. After reaction with diazomethane[14] they were identified by GC–MS.

Standards of wax esters were prepared[13] from even-carbon acids 24:0–30:0 (Fluka, Buchs, Switzerland) and odd-carbon 25:0–29:0 (Carl Roth, Karlsruhe, F.R.G.), 24:1 (Sigma, St. Louis, MO, U.S.A.) and even carbon 10:0–18:0 and 15:0 (Fluka) and 16:1 and 20:1 alcohols (Sigma).

GC–MS

GC–MS of wax esters was performed with a Finnigan-MAT 1020 B instrument and GC–MS of methyl esters of very long-chain fatty acids, methyl esters of mono- and dicarboxylic acids and TMS derivatives of alcohols with a Hewlett-Packard HP 5995 instrument.

Wax esters. An Ultra 1 (0.11 μm) (cross-linked methylsilicone, Hewlett-Packard, Avondale, PA, U.S.A.) fused-silica capillary column (25 m × 0.2 mm I.D.) with oncolumn injection at 100°C was used. The temperature was programmed from 250°C to 350°C at 2°C/min. The linear velocity of the carrier gas (helium) was 70 cm/s. The ionization energy was 70 eV and scans over the range 120–800 a.m.u. were made in 0.5 s. Isothermal separation was carried out at 245°C under the conditions described above.

Methyl esters of very long-chain fatty acids. An HP-1 (0.11 μm) (cross-linked methylsilicone, Hewlett-Packard) fused-silica capillary column (25 m × 0.2 mm I.D.) was used. The injection temperature was 250°C, the splitting ratio was 1:50 and the temperature was programmed from 250°C to 350°C at 5°C/min. The linear velocity of the carrier gas (helium) was 40 cm/s, the ionization energy was 70 eV, the scan speed was 390 a.m.u. and the mass range was 40–600 a.m.u.

Methyl esters of mono- and dicarboxylic acids. An SP-2330 (0.20 μm) (Supelco, Bellefonte, PA, U.S.A.) (60 m × 0.25 mm I.D.) was used. The temperature was programmed from 100°C to 230°C at 5°C/min and maintained isothermal for 10 min. The linear velocity of the carrier gas (helium) was 20 cm/s. For other conditions see *Methyl esters of very long-chain fatty acids.*

TMS derivatives of alcohols. A fused-silica (0.25 μm) capillary column DC-510 (Macherey, Nagel & Co., Düren, F.R.G.) (40 m × 0.25 mm I.D.) was used. The temperature was programmed from 100°C to 280°C at 1°C/min. For other conditions see *Methyl esters of very long-chain fatty acids.*

RESULTS AND DISCUSSION

Wax esters from C_{34} to C_{50} were detected in wax isolated from the *C. kessleri* cultivated heterotrophically (Table I). With the aid of the capillary column with a non-polar stationary phase it was possible to separate esters having not only different numbers of carbon atoms but also different numbers of double bonds (Fig. 1). Under optimal conditions of isothermal analysis esters having an identical number of double bonds, *e.g.*, for 46:1 (combinations 26:1–20:0, 28:1–18:0, 30:1–16:0), could be discriminated (Fig. 2). The number of theoretical plates of the column for eicosanyl nervonate (standard) was 122 000, *i.e.*, 4880 theoretical plates per metre, which is ten times greater than that reported by Itabashi and Takagi[13] for their column (11 000 theoretical plates for 18:0–18:0). Owing to this advantageous feature it was possible to separate even wax esters in which the position of the ester group differs by two carbon atoms. In agreement with literature data, although on a non-polar column, the retention time of wax isomers increases with shift of the ester group to one end, *i.e.*, with loss of symmetry. Inadequate separation was observed with wax esters differing in the position of the ester group by only a single carbon atom, *e.g.*, 30:0–15:0 and 29:0–16:0 (mixture of standards).

Proportions of individual isomers with identical chain length were quantified by means of the method of Aasen *et al.*[16], who used intensities of the ions RCO_2H^+, $RCO_2H_2^+$ and $(R'-1)^+$ formed from esters of the general formula RCO_2R', where R and R′ are alkyls of an acid or alcohol, to express their percentage occurrence. The relative percentage representation of any sum of ions mentioned above corresponds to the relative occurrence of their combinations. This method can be used with unsaturated esters, as verified by Spencer[17]. Only with monoenic acids it is necessary to use the ions $(RCO-1)^+$ and RCO^+ which are much more intense in monoenic acids than the ions RCO_2H^+ and $RCO_2H_2^+$ found in saturated acids (Fig. 3). With diene wax esters the situation is much simpler as both the alcohol and acid are monoenic and, hence, the possibility of mutual combinations is much lower.

When using the scan from *m/z* 120 it was possible to decrease the threshold to 0.01% of the base peak and thus increase the voltage on the photomultiplier as about 90% of the total ion current from *m/z* 40 to 800 is caused by hydrocarbon ions up

TABLE I

ISOMER COMPOSITION OF THE WAX ESTERS OF *C. KESSLERI*

Chain length and percent[*]	*Saturated acid–alc. (%)*[**]		*Monoenoic acid–alc. (%)*[***]		*Dienoic acid–alc. (%)*[§]	
C$_{34}$	24–10	100	24–10	100		
saturated = 1.0						
monoenoic = 0.4						
dienoic = 0.0						
C$_{35}$	25–10	100				
saturated = 0.1						
monoenoic = 0.0						
dienoic = 0.0						
C$_{36}$	24–12	80	24–12	60		
saturated = 3.5	26–10	20	26–10	40		
monoenoic = 1.8						
dienoic = 0.0						
C$_{37}$	25–12	90				
saturated = 0.3	27–10	10				
monoenoic = 0.0						
dienoic = 0.0						
C$_{38}$	24–14	67	24–14	44		
saturated = 6.4	26–12	29	26–12	50		
monoenoic = 3.9	28–10	4	28–10	6		
dienoic = 0.0						
C$_{39}$	24–15	60				
saturated = 1.7	25–14	30				
monoenoic = 0.4	29–10	10				
dienoic = 0.0						
C$_{40}$	24–16	79	24–16	55	24–16	100
saturated = 17.6	26–14	17	24–16[§§]	8		
monoenoic = 10.3	28–12	4	26–14	29		
			28–12	6		
dienoic = 0.3			30–10	2		
C$_{41}$	25–16	60	25–16[§§]	10		
saturated = 2.6	26–15	30	26–15	90		
monoenoic = 0.8	27–14	7				
dienoic = 0.0	29–12	3				
C$_{42}$	24–18	8	24–18	20	26–16	100
saturated = 11.5	26–16	82	26–16	69		
monoenoic = 13.9	28–14	8	26–16[§§]	4		
dienoic = 0.6	30–12	2	28–14	7		
			30–12	3		
C$_{43}$	25–18	20	27–16[§§]	20		
saturated = 1.3	27–16	50	28–15	80		
monoenoic = 0.3	28–15	20				
dienoic = 0.0	29–14	10				
C$_{44}$	26–18	30	24–20	17	24–20	70
saturated = 5.0	28–16	60	26–18	24	28–16	30
monoenoic = 7.0	30–14	10	28–16	47		
dienoic = 0.8			28–16[§§]	2		
			30–14	10		
C$_{45}$	30–15	15	30–15	50		
saturated = 0.8	27–18	15	25–20	40		
monoenoic = 0.3	29–16	70	29–16[§§]	10		
dienoic = 0.0						

TABLE I *(continued)*

*Chain length and percent**	*Saturated acid–alc. (%)***		*Monoenoic acid–alc. (%)****		*Dienoic acid–alc. (%)*§	
C_{46}	28–18	30	26–20	25	26–20	85
saturated = 1.8	30–16	70	28–18	15	30–16	15
monoenoic = 3.8			30–16	60		
dienoic = 0.9						
C_{47}	29–18	100				
saturated = 0.1						
monoenoic = 0.0						
dienoic = 0.0						
C_{48}	30–18	100	28–20	100		
saturated = 0.1						
monoenoic = 0.2						
dienoic = 0.0						
C_{49}						
saturated = 0.0						
monoenoic = tr						
dienoic = 0.0						
C_{50}	30–20	100	30–20	100		
saturated = 0.0						
monoenoic = 0.1						
dienoic = 0.0						

* Percent of total wax esters.

** Relative percent of saturated wax esters.

*** Relative percent of monoenoic wax esters.

§ Relative percent of dienoic wax esters.

§§ 7-Hexadecenol.

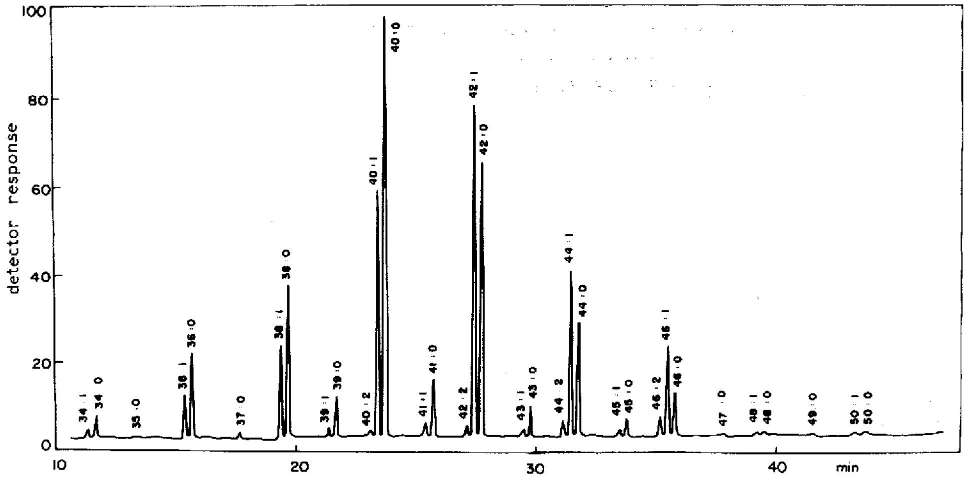

Fig. 1. GC-MS analysis of wax esters from *C. kessleri.*

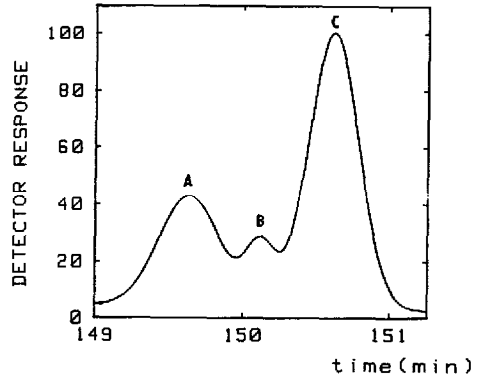

Fig. 2. Separation of peak 46:1 from Fig. 1 during isothermal analysis. (A) 26:1–20:0 (acid–alcohol); (B) 28:1–18:0 (acid–alcohol); (C) 30:1–16:0 (acid–alcohol).

to *m/z* 120, as detected in the standard (palmityl nervonate). The base peak in the whole spectrum, the ion *m/z* 55, would have a value of about 1020% relative to the ion of *m/z* 125. By using the technique described above we also detected molecular ions whose intensity commonly varies within tenths of a percent from that of the base peak[16,18]. Therefore, it is possible to use a quadrupole mass spectrometer in which spectra with low intensity of diagnostic ions were detected as described by

TABLE II

CONTENT OF VERY LONG-CHAIN FATTY ACIDS IN *C. KESSLERI*

Acid	*Content (%)*	*Acid*	*Content (%)*
6–24:1* + 15–24:1	10.10	10–28:1*	5.92
24:0	28.01	28:0	6.54
25:0	2.78	29:0	0.96
8–26:1*	20.99	12–30:1*	4.14
26:0	16.37	30:0	2.32
27:0	1.23	32:0	0.12

* *n*-18 monoenoic acids.

TABLE III

CONTENT OF ALCOHOLS OBTAINED BY HYDROLYSIS OF WAX ESTERS IN *C. KESSLERI*

Alcohol	*Content (%)*	*Alcohol*	*Content (%)*
Decanol	3.6	9-Hexadecenol	3.0
Dodecanol	10.4	Hexadecanol	50.3
Tetradecanol	15.7	Octadecanol	8.8
Pentadecanol	3.9	13-Eicosenol	4.3

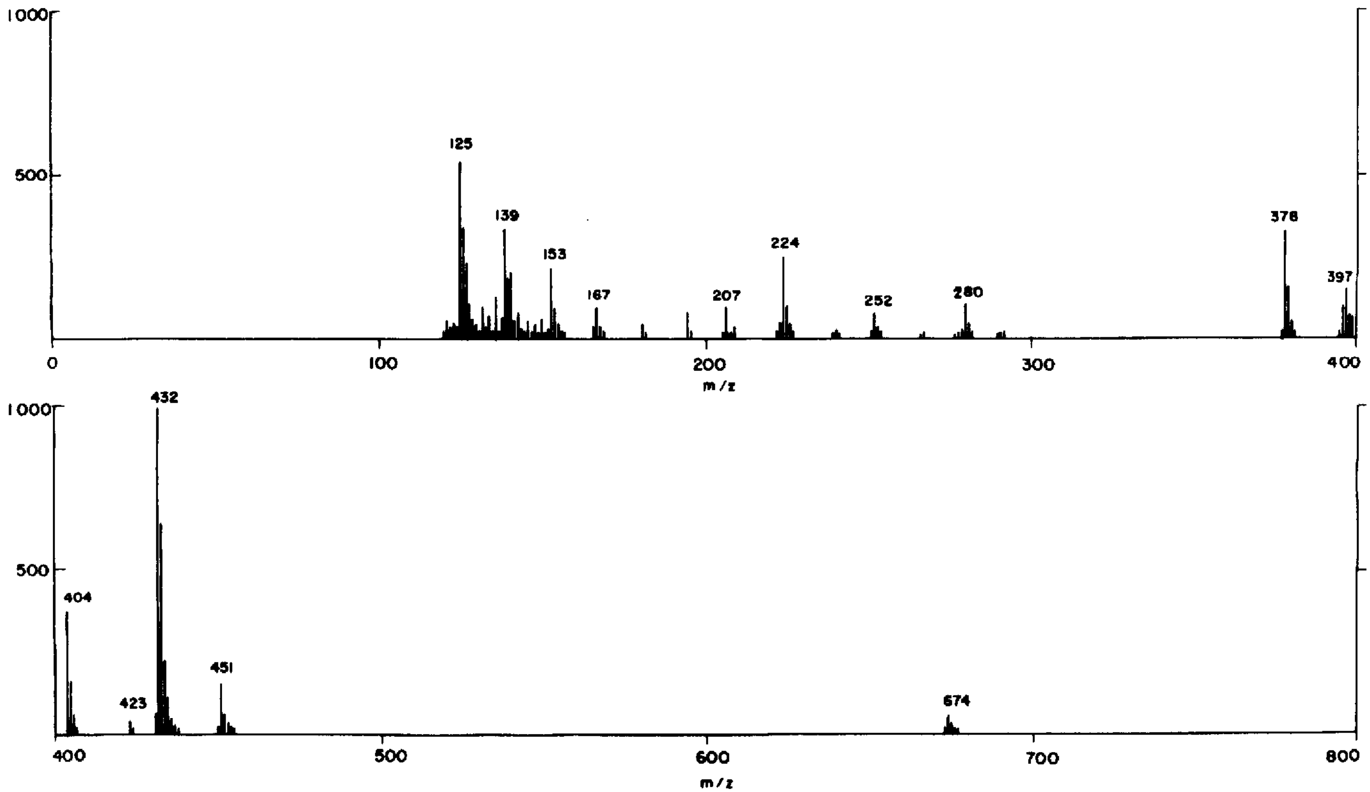

Fig. 3. Mass spectrum of peak 46:1 from Fig. 1, with interpretation of some important fragments: *m/z* 674, molecular ion; series *m/z* 451, 423, 397 and 432, 404, 378, ions $RCO_2H_2^+$ and $(RCO-1)^+$, respectively, obtained by fragmentation of wax esters with C_{30}, C_{28} and C_{26} fatty acids; series *m/z* 280, 252, 224, ions $(R'-1)^+$, obtained by fragmentation of wax esters with C_{20}, C_{18} and C_{16} alcohols.

Wakeham and Frew[12]. Hence it is not necessary to utilize chemical ionization yielding a number of pseudomolecular ions[12,19]. Unfortunately, as is common in quadrupole mass spectrometers, the intensity of ions above m/z 200 is discriminated compared with a sector mass spectrometer[16,18].

Common wax esters mostly contain an acid and alcohol of identical length, *e.g.*, sperm whale oil[17] and wax esters from bacteria of the genus Neisseriaceae[20] and the genus Acinetobacter[21]. Both in *C. kessleri* (Tables II and III) and in other photosynthesizing microorganisms (*Euglena gracilis*[22]) acids that on the average have higher carbon numbers than alcohols have always been detected. This phenomenon is most pronounced in *Emiliana huxleyi*[9], *i.e.*, acids 36:2 and 36:3, whereas even alcohols 14:0–20:0 are present. A gradual transition from saturated acids with a shorter chain to monoenic acids with a longer chain, which is conditioned by the melting point, is the second phenomenon.

Volkman *et al.*[9] and Withers and Nevenzel[3] assumed that waxes may serve as an energy reserve of the cell. It follows from literature data and the results referred to here that photosynthesizing microorganisms cultivated heterotrophically produce several times more waxes than under autotrophic conditions. For instance, Antia *et al.*[4] identified a high content of waxes (70% of all lipids) after heterotrophic cultivation on glycerol. After heterotrophic cultivation *C. kessleri* contained 0.02% of waxes with respect to the dry mass. However, this content is still very low and hence wax esters can hardly be reserve compounds. They rather form a protective surface of the cell, as suggested by Bryn *et al.*[20]. The phylogenetic development of higher organisms forming the series bacteria[20]–green algae–higher plants[1] would thus be confirmed.

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