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DETERMINATION OF FATTY ACIDS IN ALGAE BY CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Capillary gas chromatography and mass spectrometry were used to determine fatty acids in algae of the genera *Chlorella* and *Scenedesmus*, and in the blue-green alga *Spirulina platensis*. Most algae studied were cultivated autotrophically; *Chlorella kessleri* was also cultivated heterotrophically. In addition to commonly occurring fatty acids in green and blue-green algae, further acids, not previously described in these organisms, were detected. The novel features of these fatty acids were the branching position, cyclopropane ring, number and position of double bonds and chain length.

INTRODUCTION

Fatty acids are the most frequently studied lipid components of freshwater green algae and blue-green algae. The use of various analytical methods, *e.g.*, gas chromatography[1,2] (GC), gas chromatography–mass spectrometry[3] (GC–MS), liquid chromatography[4,5] (LC) and infrared, mass and nuclear magnetic resonance spectroscopy[6], for their separation and determination has been investigated. These methods were employed in the study of the relationship between cultivation conditions and fatty acid content (autotrophic and heterotrophic conditions[7], illumination[8], composition of cultivation media[9]), treatment of the initial algal material and the spectrum of isolated fatty acids[7] and representation of fatty acids in individual genera of freshwater algae[1].

This paper extends further the systematic work on compounds in an industrially significant collection of algae and is linked with our previous studies[10–12]. It is aimed at appraising technical and commercial aspects of fatty acid profiles by capillary GC–MS.

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EXPERIMENTAL

Samples

Algae (*Chlorella kessleri*, *C. vulgaris* and *Scenedesmus acuminatus*) and the blue-green alga (*Spirulina platensis*) were supplied by the Department of Autotrophic Microorganisms, Institute of Microbiology, Czechoslovak Academy of Sciences, Třeboň, in the form of a powder (moisture content about 5%) prepared by spray drying of cells harvested after cultivation.

Methyl esters and pyrrolidines of fatty acids

Fifty grams of dry algae were extracted with chloroform–methanol (1:1) in a Soxhlet extractor. The extract was evaporated to dryness and saponified by boiling for 2 h in 500 ml of 2 *M* sodium hydroxide solution in 50% ethanol. The non-saponified portion was extracted with diethyl ether, the solution was acidified to pH 1 with hydrochloric acid and fatty acids were extracted with chloroform. Methyl esters of fatty acids were prepared by reaction with a 14% boron trifluoride solution in methanol (Supelco, U.S.A.)[10]. Methyl esters of fatty acids were separated by thin-layer chromatography (TLC) (light petroleum–diethyl ether, 9:1) on silica gel G (Merck, F.R.G.). Pyrrolidines of fatty acids were prepared by boiling 10 mg of methyl esters of fatty acids with 1 ml of pyrrolidine and 0.1 ml of acetic acid for 1 h[13]. The pyrrolidines were purified by TLC (light petroleum–diethyl ether, 1:1) on silica gel G (Merck).

Gas chromatography–mass spectrometry

The mixtures of both fatty acid methyl esters and pyrrolidines were analysed using a Hewlett-Packard 5992 B instrument with a SCOT glass capillary column (77 m × 0.5 mm I.D.) (SGE, Australia) with SE-30 as the stationary phase.

The operating conditions of the instrument were as follows: ionization energy, 70 eV; scan speed, 690 a.m.u. sec^{-1}; mass range, 4–600 a.m.u.; data treated with an HP 9825 A computer connected on-line with the GC–MS system. For the separation of methyl esters, the capillary column oven temperature was programmed from 160 to 280°C at 4°C min^{-1}; for the separation of pyrrolidines, the temperature was programmed from 240 to 280°C at 2°C min^{-1}, the separation then proceeding isothermally for 30 min. The carrier gas (helium) flow-rate was 1.5 ml min^{-1} in the capillary column. The injector temperature was 288°C. The quantitative evaluation was based on total ion current. The accuracy of repeated analyses was ±0.01%.

Mass spectrometry

Mass spectra were measured using a Varian-MAT 311 (Varian, U.S.A.) with the following conditions: energy of ionizing electrons, 70 eV; ionizing current, 1 mA; temperature of the direct inlet system, 20–200°C; composition of ions at high resolution was determined with an error lower than 4 ppm. This method was used for methyl esters of linoleic and linolenic acid (standards; Lachema, Brno, Czechoslovakia).

RESULTS AND DISCUSSION

Gas chromatography

When using the capillary SCOT column with SE-30 as the stationary phase, methyl esters of fatty acids could be well separated. This can be demonstrated by the separation of homologues and discrimination of branched methyl esters. In spite of the fact that the SE-30 separates components according to their boiling points, methyl esters of unsaturated fatty acids with different numbers of double bonds can be resolved using this column. A typical chromatogram of methyl esters of fatty acids (*C. kessleri*, heterotrophy) is shown in Fig. 1.

Localization of double bonds and/or cyclopropane ring

The position of double bonds and or cyclopropane rings is usually determined after the synthesis of suitable derivatives. In this work we used the method of Anderson[13], which has only been used rarely, the principle of which is based on the GC–MS of pyrrolidines of fatty acids. Ions of only the polar moiety of the molecule occur in their mass spectra. Next to the double bond or cyclopropane ring, the regular interval of 14 a.m.u. between maxima of groups (corresponding to the CH_2 group) is interrupted by an interval of 12 a.m.u. The method is limited primarily by the chromatographic behaviour of pyrrolidines of fatty acids. Much higher temperatures must be used for elution than with methyl esters of fatty acids (by up to 50°C), which has not been possible with pyrrolidines of fatty acids above C_{24}, and that individual pyrrolidines with identical numbers of carbon atoms and differing only in the double bond position are much more poorly separated. Another method[14] used to determine double bond positions in methyl esters with two or more double bonds is based on representation of ions of m/z 150 (dominant for double bonds in positions $\omega 6$, 9 etc.) and m/z 108 (dominant for double bonds in positions $\omega 3$, 6 etc.). However, it was found according to the mass spectrum of methyl esters of linoleic and linolenic acid determined by the high-resolution method that the ion of m/z 150 is composed of $C_{10}H_{14}O$ and $C_{11}H_{18}$ ion at a ratio of 3:1 in the methyl ester of linoleic acid and at a ratio of 1:1 in the linoleic acid. The ion of m/z 108 has the composition C_8H_{12}. In spite of the above finding, the method may suitably supplement that of Anderson[13].

Fatty acids (saturated, unsaturated and with a cyclopropane ring)

The fatty acids identified are summarized in Table I (previously described in green and blue-green algae) and in Table II (the novel compounds in green and blue-green algae). Saturated and unsaturated fatty acids were identified according to methods already described[19,20]. When the branching is located at the end of the chain, in positions $\omega 3$ (anteisoacids) and $\omega 2$ (isoacids) the characteristic ion M − 57 is detected in their methyl esters. After analysis of these results it was possible to derive a relationship between the frequency of the M − 57 ion and the position of branching in the chain; in methyl esters of isoacids, detector response is reaching values up to one twentieth of the base peak, while in methyl esters of anteisoacids, detector response is reaching values up to one third of the base peak.

Fatty acids with a cyclopropane ring can hardly be determined on the basis of retention times, as these are identical with those of unsaturated fatty acids. Literature data[21] make it possible to discriminate between monounsaturated and cyclopropane

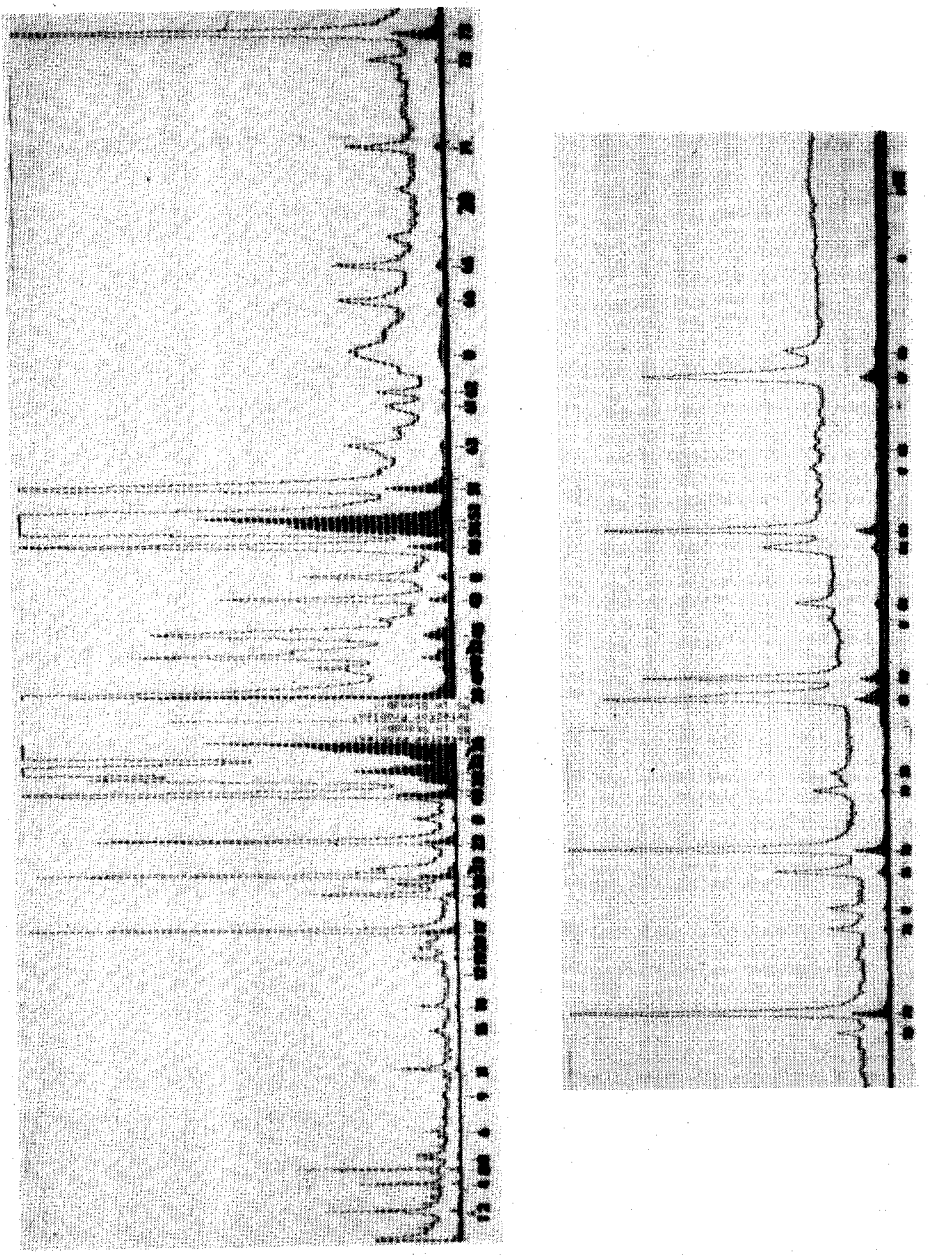

Fig. 1. Gas chromatogram of methyl esters of fatty acids in *C. kessleri* (heterotrophy) obtained on an SE-30 SCOT column. Small numbers = peak numbers (see Tables I and II). Large numbers (20, 40) = minutes. Shaded curves = 10 times lower sensitivity. U = unidentified. Ordinate: total ion current.

SCOT column. Small numbers = peak numbers (see Tables I and II). Large numbers (20, 40) = minutes.

TABLE I

PERCENTAGE CONTENT OF FATTY ACIDS IN GREEN AND BLUE-GREEN ALGAE (PREVIOUSLY DESCRIBED)

Results (%) relative to total fatty acid content.

*Peak No.**	*Fatty acid***	*C. kessleri (heterotrophy)*	*C. kessleri (autotrophy)*	*C. vulgaris*	*S. acuminatus*	*S. platensis*	*Reference*
6	10:0	0.16	0.96	0.98	0	1.59	15
11	12:0	0.39	3.59	2.33	0	2.23	16
16	13:2	0	0	0	0	0.49	10
17	14:0	2.76	9.35	6.61	1.61	0.44	1
20	14:1	0.16	0.40	0.56	0	0.26 + 0.17	6
21	14:3	0	0	0	0	0.15	16
22	15:0	2.35	5.89	4.26	1.30	1.39	6
23	*i*-15:0	2.71	2.30	1.26	0	5.07	6
27	15:1	0	0.26	0	0	0.15	6
28	16:0	27.96	16.30	8.07	43.18	7.35	1
33	7-16:1	5.29	0	9.86	0	11.25	17
35	9-16:1	0	0	9.22	0	0	17
36	16:1	1.77	0	0	1.07	0.47	1
38	7,10-16:2	2.06	0	0	0	0	6
39	16:2	0	0	0.71	0	0	9
40	7,10,13-16:3	0	4.17	0	0	0	18
41	16:3	0	0	0	0.54	0	18
42	17:0	0.50	1.16	1.77	1.39	0.60	8
43	*i*-17:0	0	0	0	0	0.36	6
50	17:1	0	0	1.85	0	0	3
51	18:0	4.09	4.42	5.67	31.95	5.50	1
52	9-18:1	9.30	3.50	8.95	4.47	1.34	6
53	18:1	0	0	0.40 + 0.36	0	0.66	1
55	9,12-18:2	0	7.76	0	0	2.04	6
56	18:2	0	1.07	0	0	0.17 + 0.55	1
57	6,9,12-18:3	0	3.83	0	0	10.52	18
58	9,12,15-18:3	3.67	0	0	1.92	8.11	18
59	18:3	0	2.10	0	0	0	9
60	19:0	0	0	0.29	0	0.44	16
62	19:1	0.23	0	3.33 + 0.91	0	0.48 + 0.13	11
64	19:2	0	0	0	0	0.80	11
65	20:0	0.44	0.19	0.73	1.12	0.56	15
68	20:1	0	0	0.66	0	0.11	16
69	20:2	0	0	0	0	0.81 + 0.65	17
70	20:3	0	0	0	0	0.33	11
72	22:0	2.89	0	0.88	0.85	0.46	11
73	22:1	0.35	0	0	0	0.12	17

* See Fig. 1 for *C. kessleri* (heterotrophy).

** First number, number of carbon atoms in the chain; second number, number of double bonds; number(s) before the hyphen, position(s) of double bond(s); position of double bond not localized, only the degree of unsaturation is given; *i* = isoacid.

methyl esters on the basis of intensity ratios of the ions M^+ and M − 32. The value of the ionization energy only slightly influences the representation of ions.

It follows from the data in Tables I and II that algae can synthesize saturated and unsaturated straight-chain fatty acids and, in addition, iso- and anteisoacids,

TABLE II

PERCENTAGE CONTENT OF FATTY ACIDS IN GREEN AND BLUE-GREEN ALGAE (NOT PREVIOUSLY DESCRIBED IN THESE ORGANISMS)

Results (%) relative to total fatty acid content.

*Peak No.**	*Fatty acid***	*C. kessleri (heterotrophy)*	*C. kessleri (autotrophy)*	*C. vulgaris*	*S. acuminatus*	*S. platensis*
1	7:0	0.21	0	0	0	0
2	8:0	0.94	1.80	3.12	0.40	2.58
3	8:1	0	0	0.19	0	0
4	9:0	0.57	0.57	5.16	1.47	1.39
5	br_2-9:0	0	0	0.14	0	0
7	10:1	0	0	0.19	0	2.68
8	10:2	0.18	0	0	0	0.84
9	11:0	0.70	0.73	0.13	0	0.11
10	*ai*-11:0	0	0.13	0	0	0
13	*i*-13:0	0	0.13	0.11	0	0
14	*ai*-13:0	0.39	0	0	0	0
15	13:1	0.42	0	0	0	0
18	*i*-14:0	0	0.37	0.43	0	1.38
19	*ai*-14:0	0.26	0	0	0	0
24	*ai*-15:0	0	0.33	1.57	0	1.96
25	br_7-15:0	0	0.59	0	0	0
26	10-15:1	0.96	0	0	0	0
29	*i*-16:0	0	13.50	0	0	8.19
30	br_4-16:0	0.29	0	0.54	0	0
31	br_5-16:0	0	0.72	0.45	0	0
32	10,11-c-16:0	1.64	0	0	0	0
34	8-16:1	0	3.25	0	0	0
37	5,8-16:2	0	7.20	0	0	0
44	*ai*-17:0	0	0	6.21	0	0
45	br_2-17:0	1.56	3.16	0	0.80	0
46	br_2-*ai*-17:0	0	0	0	0	4.42
47	9,10-c-17:0	0.21	0	0	0	0
48	8-17:1	0	0	0	0	0.71
49	9-17:1	0.96	0	0	0	0
54	6,9-18:2	4.30	0	2.95	0.86	1.61
61	9,10-c-19:0	0.29	1.70	0	0	0
63	br_2-19:1	0.36	0	0	0	0
66	9-20:1	0.42	0	0	0	0
67	11-20:1	0	0	0.66	0	0.11
71	21:0	0.42	0	0.08	0	0
74	23:0	0.29	0.11	0.36	0	0.13
75	24:0	2.48	0.49	1.76	2.77	0.46
76	15-24:1	0.66	0	0	0	0
77	24:1	0	0.16	0.07	0	0
78	25:0	0.28	0	0.96	0.85	0.12
79	br_2-25:0	0.25	0	0.08	0	0
80	26:0	0.83	0.53	1.38	1.12	0.22
81	26:1	1.15	0	0	0	0
82	27:0	0.18	0	0.23	0	0
83	28:0	0.73	0.23	0.12	0	0.17
84	28:1	0.63	0	0	0	0
85	29:0	0.10	0	0	0	0
86	30:0	0.13	0	0	0	0.11
87	30:1	0.60	0	0	0	0

* See Fig. 1 for *C. kessleri* (heterotrophy).

** First number, number of carbon atoms in the chain; second number, number of double bonds; number(s) before the hyphen, position(s) of double bond(s) or cyclopropane ring; position of double bond not localized, only the degree of unsaturation is given; *i* = isoacid; *ai* = anteisoacid; br_2, br_4, br_5, br_7 = methyl groups on C_2, C_4, C_5, C_7; c = cyclopropane ring.

acids branched on other carbon atoms and cyclopropane acids, the synthesis of which is considered to be typical of bacteria[22]. The data which refer to previously described fatty acids are in accord with the literature (Table I); generally the dominant fatty acids (about 10% and more) in green algae are straight-chain even-numbered $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$ and in the blue-green alga *S. platensis* 6,9,12-$C_{18:3}$ and 9,12,15-$C_{18:3}$. Other fatty acids either previously described or not yet found in green algae represent minor components, with the one exception of *i*-$C_{16:0}$ acid in *C. kessleri* (autotrophy) and *S. platensis* (Table II). The presentation (percentage of total fatty acids) of unidentified components (with their number in parentheses) is as follows: *C. kessleri* (heterotrophy) 5.15 (9), *C. kessleri* (autotrophy) 0.76 (1), *C. vulgaris* 2.31 (12), *S. acuminatus* 2.33 (10) and *S. platensis* 1.89 (12).

Some fatty acid patterns could well be used in the chemotaxonomy of *C. kessleri* (7,10-16:2; 7,10,13-16:3; long-chain fatty acids 15-24:1, 28:1, 29:0, 30:0, 30:1; heterotrophic cultivation), of *C. Kessleri* (8-16:1; 5,8-16:2; autotrophic cultivation) and of the blue-green alga *S. platensis* (6,9,12-18:3; 9,12,15-18:3).

The pronounced variability of the carbon chain length of fatty acids produced by green algae is an interesting result of the work presented here. Usually, the literature data indicate a range between lauric acid (C_{12}) and arachic acid (C_{20}), C_{16} and C_{18} acids being representing about 90%[1,23]. Table II also includes partly C_7, C_8, C_9 acids and partly long-chain fatty acids (with more than 22 carbon atoms). Triacontanoic and triacontenoic acids were detected in *C. kessleri* cultivated under heterotrophic conditions, the proportion of the latter being determined to be 0.6%. Fatty acids with a chain longer than C_{22} have not yet been detected in green algae; even C_{22} acids were found only exceptionally; *e.g.*, Klenk *et al.*[17] described a total of 0.31% for all C_{22} acids. The heterotrophic culture of *C. kessleri* afforded 17.4% of lipid whereas the autotrophic culture of *C. kessleri* gave 10.1% dry weight. Other organisms studied (*C. vulgaris*, *S. acuminatus*, *S. platensis*) yielded less lipid than *C. kessleri* and the total fatty acids contents 6.7 (*C. kessleri*-heterotrophy), 3.1 (*C. kessleri*-autotrophy), 1.5 (*C. vulgaris*), 1.2 (*S. acuminatus*) and 0.1% dry weight (*S. platensis*).

Referring to both fatty acid content and composition, the alga *C. kessleri*, prepared as a mutant strain in the Department of Autotrophic Microorganisms, Institute of Microbiology, Czechoslovak Academy of Sciences, Třeboň[24], cultivated heterotrophically, is considered to be most valuable.

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