17. G. Galliard, Phytochemistry, 7, 1915 (1968).
18. É. V. Dyatlovitskaya, T. I. Torkhovskaya, and L. D. Bergel'son, Biokhimiya, 34, No. 1, 177 (1969).
19. E. Stahl, Thin Layer Chromatography, 2nd English Edition: Allen and Unwin, London/Springer, New York (1969).
20. Kh. S. Mukhamedova and S. T. Akramov, Khim. Prir. Soedin., 12 (1979).

LIPIDS FROM EXTRACTS OF *Chlorella vulgaris*

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The composition of ethanolic (I) and gasoline (II) extracts of the cultivated microalga *Chlorella vulgaris* has been studied. With the aid of CC, TLC, qualitative reactions, GLC, and UVS the following classes of compounds have been detected in them. From the neutral lipids: hydrocarbons, carotenoids, traces of sterols and their esters, fatty acid esters, tri- and diacylglycerols, free fatty acids, and chlorophylls; and from the polar lipids: di- and monogalactosylglycerols, phosphatidylethanolamine, lecithin, phosphatidylinositol, phosphatidylserine, and three sphingosine bases. The polar lipids I and II made up 52.4 and 50.2% of the total, respectively. As compared with extract I, extract II was somewhat enriched with neutral lipids, including provitamins of the A group and vitamins of the F group. In the fatty acids of chlorella, 19 components were detected, the main ones being the 16:0 acid and 18:2 and 18:3 acids.

Green and blue-green algae are rich sources of proteins, carbohydrates, vitamins, and lipids [1]. A technology has been developed for the industrial cultivation of some species of these algae, including chlorella, the biomass of which is used as a protein-vitamin additive in animal husbandry, sericulture, and plant growing [2].

An intensive study of the lipids of green and blue-green algae is being performed mainly for scientific purposes: The role of the composition and structure of the lipids in the process of photosynthesis, in the adaptation of the algea to varying conditions of growth, and the value of this class of compounds in chemotaxomony are being investigated [3].

At the same time, the production by some species of algae of a considerable amount of lipids and the pronounced physiological activity of a number of lipid extracts [4] shows the value of the lipids as independent components and the desirability of the complex processing of the algal biomass.

The composition of algal lipids is very labile and is determined largely by the conditions of growth [3, 5].

In the present paper we give information on the composition of the lipids of two samples of extracts of the cultivated alga *Chlorella vulgaris*: an ethanol extract (sample 1) and a gasoline extract (sample 2).

The extracts were separated by column chromatography on silica gel. This led to the isolation of a number of individual classes of lipids and also of some mixed fractions which were then separated by preparative TLC.

The assignment of the lipids to definite classes was made from their chromatographic mobility on TLC in systems 1-5 in comparison with model samples, and also by comparison with literature information [6].

The compositions of the lipids of the two chlorella extracts are given below (% on the weight of the extracts):

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Fraction No.	Class of Lipids	1(C_2H_5OH)	2 (gas.)
	Neutral lipids		
1	Carbohydrates + carotenoids + an unidentified compound (X_1)	0.4	2.2
2	Sterol esters + an unidentified compound (X_2)	1.2	1.8
3	Fatty acid esters and low-molecular-weight alcohols	1.7	2.2
4	Diacylglycerols (TAGs)	2.4	6.4
5	Free fatty acids (FFAs)	2.1	5.8
6	Free sterols	Tr.	Tr.
7	Chlorophylls + diacylglycerols (DAGs)	39.8	31.4
	Polar lipids		
8	Glycolipids		
9	Phospholipids	52.4	50.2
10	Amino alcohols of the sphingosine type		

The extracts were characterized by a complex set of components of the neutral and polar lipids that were qualitatively identical. In the quantitative respect there were certain differences: In the gasoline extract the amount of fractions 1-5 of the neutral lipids was 18.4%, while in the ethanolic extract it was 7.8%, i.e., ethanol extracted from the algae a larger amount of polar lipids, chlorophylls, and diacylglycerols and a smaller amount of the other classes of neutral lipids.

The main component of fraction 1 consisted of carotenoids (R_f 0.96, system 1), which were identified from the yellow coloration of the spot and from its change to blue-green after treatment with 50% H_2SO_4 followed by heating. According to the visible spectrum, the carotenoids consisted of a mixture (λ_{max}^{hexane} 358, 379, 402, 420, 425, 427, 448, 453, 457, 472, 475 nm), its main component being β-carotene (425, 448, 475 nm) [7-9].

The unidentified compound (X_1) was colored black when a chromatogram was treated with 50% H_2SO_4 followed by heating. It, like the other compound (X_2), because of their inertness to alkaline hydrolysis, was assigned to the unsaponifiables.

Fatty acid esters and low-molecular-weight alcohols were identifed from their chromatographic mobilities and their GLC behavior on a polar phase, and also from their capacity for readily undergoing saponification. In their polarity they corresponded to model methyl esters of the fatty acids of cottonseed oil obtained by methylation with diazomethane. No accurate identification of the alcoholic moieties of these esters was performed.

Natural methyl esters have been detected previously in *Chlorella vulgaris* [10], and their presence in the extract that we investigated may be assumed. The possibility of the formation of ethyl esters on prolonged contact with the lipids of ethanol is also known from the literature. Consequently, an ethanolic extract of *Chlorella vulgaris* may also contain secondary ethyl esters.

Free sterols, and also bound sterols, were detected in the extracts in trace amounts.

The bulk of fraction 7 consisted of chlorophylls. By analytical TLC in system 6, seven green spots were detected in fraction 7, with R_f 0.05, 0.08, 0.21, 0.34, 0.39, 0.50, and 0.64. The main spot was that with R_f 0.64, which corresponded to literature information on the mobility of chlorophyll *a* [9]. The assignment to chlorophyll *a* was confirmed by the UV spectrum of fraction 7, where absorption was observed at $\lambda_{max}^{diethyl\ ether}$ 407, 505, 536, 565, 613, and 672 nm [10, 11].

On the basis of the chromatographic mobility of the glycolipids in system 5 and also from the violet coloration of their spots when the plate was treated with α-naphthol, the presence of monogalactosyl- and digalactosyldiacylglycerols (MGDGs and DGDGs) was established. From the ratio of the areas of the spots and the intensities of the colorations of the two components the MGDGs predominated to some extent.

Together with the two components of glycolipid nature, five classes of phospholipids were detected in the extracts. Phospholipids were revealed with the Vaskovsky reagent and corresponded to the chromatographic mobilities of model samples. On the basis of a comparison of the

TABLE 1. Composition of the Fatty Acids of the Total Lipids and of the Separate Acyl-Containing Fractions of Extracts of *Chlorella vulgaris* (samples 1 and 2) (%, GLC)

Acid	Extract		Fatty acid esters		TAGs		FFAs		DAGs		Polar lipids	
	1	2	1	2	1	2	1	2	1	2	1	2
X_1	—	—	—	2.1	—	—	1.7	Tr.	—	—	Tr.	Tr.
10:0	—	—	Tr.	Tr.	—	—	0.7	1.0	—	—	Tr.	Tr.
12:0	0.2	0.7	Tr.	—	—	—	0,6	0,7	1,2	1,2	0,4	Tr.
13:0	—	—	—	—	—	—	0,6	Tr.	Tr.	1,5	—	—
14:0	0,7	0,9	0.7	0,2	1,8	2,2	2,6	0.3	2,0	1,9	0,5	Tr.
X_2	—	—	—	—	—	—	1.4	Tr.	—	Tr.	—	—
14:1	0.4	0,6	—	—	—	—	1.2	Tr.	—	Tr.	—	—
14:2	Tr.	—	—	1,0	—	—	5,5	0,8	1.8	1.2	0,5	—
16:0	37,4	30,9	26.4	13,0	26,3	21,5	17,0	15,7	27.4	15,6	45,3	51,2
16:1	5,1	7,1	7,3	3,3	3,7	2.5	4,5	2,3	4,0	3,4	3,7	3.3
16:2	5,6	7,6	7,2	4.1	1,8	1,1	4,4	2.9	5,2	1,3	2,6	1,9
16:3+18:0	3,4	9.7	12,7	10,4	3,8	9,8	8.2	6.2	12,1	9,3	5.6	2.0
18:1	11,3	4.6	5.5	3,7	9,5	11,0	4.8	Tr.	5.6	3.7	4,9	Tr.
18:2	14,8	18,2	17,1	19,2	11,0	11,9	11.7	14,2	13,8	4,8	20.5	26,1
18:3	7,1	17,2	15,4	41.2	21,3	24.4	13.4	55.9	26,9	56,1	13.7	15,5
20:1	14,0	2,5	7.7	1,8	6,6	5.4	—	—	—	—	—	—
X_3	—	—	—	—	—	2,1	—		—	—	—	—
X_4	Tr.	Tr.	—	—	3,3	0,9	15.2	—	—	—	0,7	—
X_5	Tr.	Tr.	—	—	10.9	7.2	6.5	—	—	—	1.6	—
Vitamins F (18:2+18:3)	21,8	35,4	32,5	60,4	32,3	36,3	25.9	70.1	42,0	70,9	34,2	41.6
Σsat	43,9	40.1	34,3	19.4	40,8	32,0	35,5	20 6	35 8	21,5	50.4	53,1
Σunsat	56,1	59,9	65,7	80.6	59,2	68,0	64,5	79,4	64,2	78,5	49.6	46,9

areas of the spots, the main component among them was a phosphatidylglycerol. Phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol were present in appreciable amounts, and phosphatidylserine in trace amounts.

In addition, in the fractions of polar lipids of the two extracts three higher amino alcohols of the sphingosine type were detected. The spots of these compounds were colored pink-crimson on treatment with ninhydrin — a specific agent for revealing compounds with a primary amino group. We did not identify two compounds of the polar lipids. These compounds contained choline (revelation with the Dragendorff reagent). The qualitative characteristics of the polar lipids are given below:

R_f of the spot (in system 5)	P-containing lipids (Vaskovsky reagent)	Prim. $-NH_2$ groups (ninhydrin)	Choline-containing compounds (Dragendorff reagent)	Sugars (α-Naphthol)	Identification
0.77	—	—	—	+	MGDGs
0.62	—	—	—	+	DGDGs
0.62	+	+	—	—	Phosphatidylethanolamine
0.60	—	—	+	—	Unidentified
0.48	++		—	—	Phosphatidylglycerol
0.40	—	—	+	—	Unidentified
0.33	+	—	+	—	Phosphatidylcholine
0.23	+	—	—	—	Phosphatidylinositol
0.15	+	+	—	—	Phosphatidylserine
0.11	—	+	—	—	Higher amino alcohols
0.08	—	+	—	—	of the sphingosine
0.04	—	+	—	—	type

To determine the total fatty acid composition, part of the initial extracts and the acyl-containing fractions were saponified, the acids obtained were methylated with diazomethane, and the fatty acid methyl esters were analyzed by GLC. The FFAs were methylated without preliminary treatment with alkali.

The fatty acid compositions of the extracts are given in Table 1. The main acids of the *Chlorella vulgaris* extracts were palmitic, linoleic, and linolenic. The latter, as is well known is a vitamin of the F group. The amounts of vitamins F (18:2 + 18:3) in the gasoline

extract were greater than that in the ethanol extract. However, for the same reason the ethanol extract was more resistant to oxidation on storage.

The polar lipids were enriched with palmitic acid but the total amounts of the 18:2 and 18:3 acids in these fractions were also appreciable.

Thus, as compared with the lipids of extracts of higher plants [12], the lipids of extracts of the microalga *Chlorella vulgaris* are enriched with glyco- and phospholipids and contain provitamins of the A group, vitamins of the F group, and free fatty acids which, as has been observed, may exhibit antimicrobial activity [4]. Such biologically active lipids may be of interest for a number of branches of industry.

EXPERIMENTAL

UV-Visible spectra were taken on a Hitachi spectrophotometer in hexane (carotenoids) and diethyl ether (chlorophylls).

Gas-liquid chromatography was performed on a Chrom-4 instrument with a flame-ionization detector using a 0.4 × 250 cm steel column filled with 15% of Reoplex-400 on Chromaton N-AW in the isothermal regime at 202°C with a rate of flow of carrier gas (helium) of 100 ml/min.

The acids were identified as described in [10].

The cultivation of the alga was autotrophic under conditions of solar irradiation in a mineral medium containing chemical fertilizers (urea, ammofos [ammonium phosphate], kalimagnesiya [potassium-magnesium sulfate] and ferric chloride) as sources of biogens. The temperature of the suspension was from 35 to 39°C, and the apparatus was of the closed type.

The biomass of a culture of *Chlorella vulgaris* (strain LARG-3M) was obtained in the Andizhan hydrolysis factory and was dried in a spray-dryer.

The lipids were extracted with ethanol and with type A gasoline at their boiling points.

Column chromatography was performed on a 3 × 30 cm column containing silica gel L 100/250 (Czechoslovakia), the fractions being eluted first with benzene and then with hexane–benzene (1:1), hexane–diethyl ether (1:1), diethyl ether, chloroform, and methanol (300 ml each).

Preparative thin-layer chromatography was performed on silica gel of type L 5/40 (Czechoslovakia) with the addition of 10% of $CaSO_4$, and the analytical variant on the same sorbent and also on Silufol in the following systems: 1) hexane–diethyl ether–acetic acid (80:20:1); 2) heptane–methyl ethyl ketone–acetic acid (43:7:0.5) with two runs of the solvent [13]; hexane–diethyl ether: 3) (6.5:3.5) and 4) (8:2); 5) chloroform–methanol–water (65:25:4); and 6) hexane–acetone–benzene–isopropanol (69.5:25:4:1.5).

To purify the less polar compounds (from the hydrocarbon to the triacylglycerols) eluted from the column we used system 4, and for the free fatty acids system 3.

Analytical GLC of the chlorophylls was performed on silica gel L 5/40, with the addition of $CaCO_3$ in a ratio of 1:1.

Chromatograms were visualized with I_2 vapor and with 50% H_2SO_4 followed by heating to 120°C for 3-4 min; phospholipids were detected with the Vaskovsky reagent, compounds containing a primary amino group with ninhydrin, choline-containing compounds with the Dragendorff reagent, and glycolipids with α-naphthol [6].

As markers for TLC we used carbohydrates, sterol esters, fatty acid methyl esters, triacyl-, diazyl-, and monoacylglycerols, free fatty acids, and β-sitosterol [14] and phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol [15] isolated from higher plants.

The lipids were saponified with a 10% aqueous ethanolic (H_2O–CH_3OH (1:9, v/v)) solution of KOH in a proportion of 5 ml of solution per 20 mg of lipids.

The separation of the unsaponifiable compounds from the soap solution by extraction with hexane was difficult because of the persistent dark green coloration which masked the clarify of the phase-separation boundary between the aqueous and the organic layers. At this stage, to achieve sharpness of the boundary a small volume of 10% NaCl solution was added to the soap solution.

SUMMARY

1. Ethanol and gasoline extracts of the cultivated alga *Chlorella vulgaris* have been found to contain 11 classes of neutral compounds (48-50% of the weight of the extracts), two classes of glycolipids, five classes of phospholipids, and three sphingosine bases (52-50%).

2. As compared with the ethanolic extract, the gasoline extract of the chlorella was enriched with provitamins of the A group and vitamins of the F group.

LITERATURE CITED

1. A. M. Muzafarov and T. T. Taubaev, Prikl. Biokhim. i Mikrobiol., No. 19, 3 (1983).
2. T. T. Taubaev, in: Proceedings of the VIth Conference on Sporophytic Plants of Central Asia and Kazakhstan [in Russian], Dushanbe (1978), p. 4.
3. E. G. Sud'ina and G. I. Lozovaya, Principles of Evolutionary Plant Biochemistry [in Russian], Kiev (1982), p. 196; G. S. Kalacheva, I. N. Trubachev, Fiziol. Rast., No. 21, 56 (1974).
4. A. A. Akhunov, S. D. Gusakova, T. T. Taubaev, and A. U. Umarov, Khim. Prir. Soedin., 379 (1978).
5. I. N. Trubachev, I. I. Gitel'zon, G. S. Kalacheva, V. A. Barashkov, V. N. Belyanin, and R. I. Andreeva, Prikl. Biokhim. Mikrobiol., No. 12, 196 (1976).
6. M. Kates, Techniques of Lipidology, North Holland, Amsterdam/American Elsevier, New York (1972).
7. Handbook on Methods of Investigation, Technical Chemistry, and the Control and Accounting of Production in the Oils and Fats Industry [in Russian], Leningrad, Vol. 1 (1967), p. 470.
8. F. N. Foppen, Chromatographic Reviews, 14, 133 (1971).
9. F. Bjornlanol, Phytochemistry, 21, 1715 (1982).
10. V. M. Khasanova, S. D. Gusakova, T. T. Taubaev, Khim. Prir. Soedin., 49 (1978).
11. F. L. Kalinin, V. P. Lobov, and V. A. Zhidkov, Handbook of Biochemistry [in Russian], Kiev (1971), p. 747.
12. T. V. Khomova and S. D. Gusakova, Maslo-zhir. Prom-st', 30 (1981).
13. L. B. Sugak, V. M. Merezhinskii, Biokhim., Mezhved. Sb., 2, 57 (1974).
14. T. V. Khomova, S. D. Gusakova, Yu. M. Murdakhaev, and A. U. Umarov, Khim. Prir. Soedin., 30 (1984).
15. F. Yu. Gazizov, A. Sh. Isamukhamedov, and S. T. Akramov, Khim. Prir. Soedin., 485 (1980).