CHROM. 5804

# GAS CHROMATOGRAPHIC ANALYSIS OF THE HIGHER FATTY ACIDS OF THE ALGA *CHLORELLA VULGARIS* (*PYRENOIDOSA*)

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(Received September 21st, 1971)

SUMMARY

Fatty acids of the alga *Chlorella vulgaris* (*pyrenoidosa*) were analyzed by the gas–liquid chromatography of their methyl esters. By use of two (polar and non-polar) stationary phases, comparison with standards and bromination $C_{14}$–$C_{20}$ fatty acids were detected and identified. In addition to the major acids (palmitic, oleic, linoleic and linolenic) the alga lipids contain *n*-14:0, 14:1, 14:2, 14:3, *n*-15:0, *ai*-15:0, 16:1, 16:2, 16:3, *n*-17:0, *ai*-17:0, *n*-18:0, *n*-19:0 and *n*-20:0 fatty acids at levels in excess of 0.1 %. The results obtained are of importance in the analytical control of the radiochemical purity of $^{14}$C-labelled higher fatty acids prepared from the alga. The presence of odd (straight-chain and branched) acids is interesting from biochemical viewpoint.

## INTRODUCTION

The lipids of *Chlorella vulgaris* (*pyrenoidosa*) contain four well-known major fatty acids (FA) of the $C_{16}$ and $C_{18}$ series (16:0, 18:1, 18:2, 18:3) and several minor components[1–5]. Not all of these minor components have been identified satisfactorily. A basic study in this field was made by MANGOLD AND SCHLENK[2], who found in *Chlorella pyrenoidosa* the following fatty acids: one 12:0, 14:0, 14:1, 14:2, 14:3, 15:0, 16:0, 16:1, 16:2, 16:3, two 17, one 18:0, 18:1, 18:2, 18:3, 19, three 20, two 22 and one 24. The attention of JAMES[6], HARRIS AND JAMES[7], NICHOLS[8,9], BARRON[10] AND MIYACHI[11] was drawn to the biosynthesis and metabolism of fatty acids. Other publications[12,13] contain few details.

In recent years a new technique has been used in the analysis of lipids: the combination of thin-layer (TLC) and gas–liquid chromatography (GLC). With this combined chromatographic technique whole lipid classes were analyzed (first separated by TLC and then chromatographed by GLC, *e.g.* ref. 14) or a detailed analysis of FA was performed. So KORN[15] analyzed the fatty acids of *Euglena gratilis*. He showed that some overlapping occurs during GLC and after elimination of this by use of TLC he was able to detect over fifty fatty acids.

## EXPERIMENTAL

For the purpose of analysis of the algae and the control of radiochemical purity of $^{14}$C-labelled fatty acids (prepared from radioactive algae[16]) we used a technique

usually applied to the analysis of more complex mixtures of volatile compounds: GLC of methyl esters on two stationary phases. We tried to identify all *Chlorella* fatty acids with a relative abundance higher than 0.5 %. A polar polyester diethylene glycol succinate (DEGS) and a non-polar grease, Apiezon-L, were chosen as stationary phases.

*Microorganisms*

A pure culture of the alga *Chlorella vulgaris* No. 82 from the collection of autotrophic organism, Czechoslovak Academy of Sciences, Prague, precultivated as reported by BASLEROVÁ AND DVOŘÁKOVÁ[17], was grown under conditions similar to those for cultivation in an atmosphere of $^{14}CO_2$ (ref. 16).

*Chemicals*

Myristic, palmitic*, stearic, oleic, linoleic** and linolenic** acids were supplied by Lachema (ČSSR), Reanal (Hungary)* and Fluka (Switzerland)** and 14-methylhexadecanoic acid was obtained thanks to the kindness of Dr. J. HRADEC of the Oncological Institute, Prague.

*Preparation of fatty acid methyl esters*

After centrifugation of *Chlorella* cells and washing free of nutrient medium, lipids were extracted with hot 96 % ethanol and with an ethanol–diethyl ether mixture (3 : 1). The remaining part of the sugars was removed from the lipid extract by water extraction; the dried lipids were then transesterified with methanolic hydrochloric acid. After the extraction of methyl esters by petroleum ether they were purified by vacuum sublimation.

*Gas–liquid chromatography*

This was carried out on a column of length 2 m and I.D. 4 mm filled with 15 % DEGS on Chromaton N-AW-HMDS 60/80 (Lachema), in a Packard Chromatograph 7409 with an argon-ionisation detector (AID) and on a column of length 1.5 m and I.D. 3 mm filled with 15 % Apiezon-L on Chromaton N-AW-NMDS 60/80 (Lachema), in a Becker-Delft Chromatograph C-ASTGV with a flame-ionisation detector (FID).

For the labelled fatty acids the simultaneous determination of radioactivity, after combustion in Tricarb furnace Packard 325, was carried out by a 2-ml gas proportional counter. The counter unit consisted of a Berthold LB 80074 preamplifier with high voltage supply, an amplifier and a Berthold LB 242K dual-channel rate meter.

## RESULTS AND DISCUSSION

We identified in the *Chlorella vulgaris* lipids *n*-fatty acids with even numbers of carbons saturated (14, 16, 18 and 20), mono-, di- and tri-unsaturated acids (14, 16 and 18) and *n*- and *anteiso*-fatty acids with odd numbers of carbons (15, 17, 19). As was expected, we found the overlapping[15] of peaks of *Chlorella* fatty acid methyl esters. This is obvious from the semi-logarithmic plots of retention times of the fatty acid methyl esters determined in *Chlorella versus* number of carbons in chain (Figs. 1 and 2). It is also evident from the GLC analysis of *Chlorella* fatty acids shown in Figs. 3 and 4.

The identification of individual compounds was carried out by comparison with standard substances or by bromination (the unsaturated acids then disappearing on GLC). The minor components, with the exception of 14-methylhexadecanoic acid which was isolated from lanolin, were identified using the regularities of their retention times. The position of the double bond in unsaturated acids was not verified and the position of the methyl group was verified only for 14-methylhexadecanoic acid (a component of carcinolipin) by its biochemical behaviour[18–20]. The other

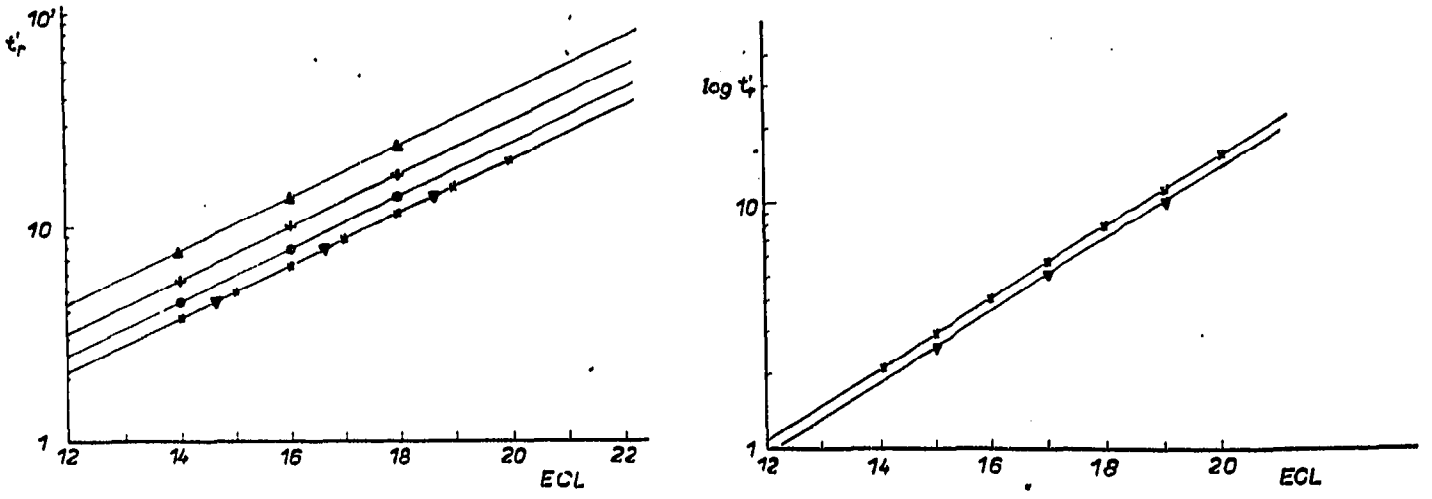


Fig. 1. The retention time of fatty acid methyl esters *versus* the equivalent chain length (polar phase). x, straight-chain saturated; ▼, *anteiso* saturated; ●, mono-unsaturated; +, di-unsaturated; ▲, tri-unsaturated.

Fig. 2. The retention time of fatty acid methyl esters *versus* the equivalent chain length (non-polar phase). ×, straight-chain saturated; ▼, *anteiso* saturated.

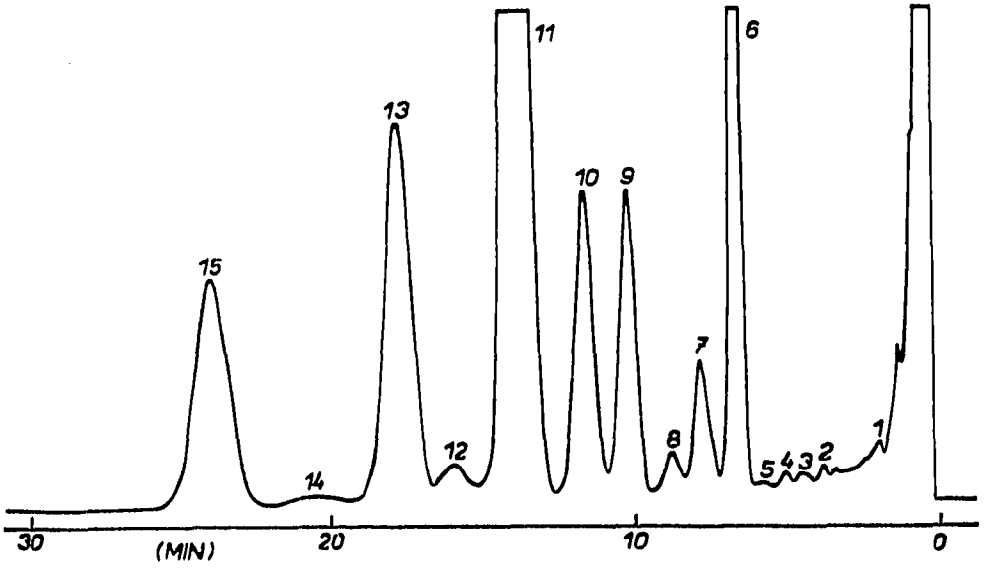


Fig. 3. Gas chromatogram of fatty acid methyl esters, prepared from triglyceride fraction of *Chlorella* lipids (TLC) on diethylene glycol succinate at 190°. Carrier gas: argon (30 ml/min). Argon ionisation detector. Column: length 2 m × 4 mm I.D. Esters: 1 = 12:0; 2 = 14:0; 3 = 14:1 and *ai*-15; 4 = 15:0; 5 = 14:2; 6 = 16:0; 7 = 16:1, *ai*-17 and 14:3; 8 = 17:0; 9 = 16:2; 10 = 18:0; 11 = 18:1, 16:3 and *ai*-19; 12 = 19:0; 13 = 18:2; 14 = 20:0; 15 = 18:3.

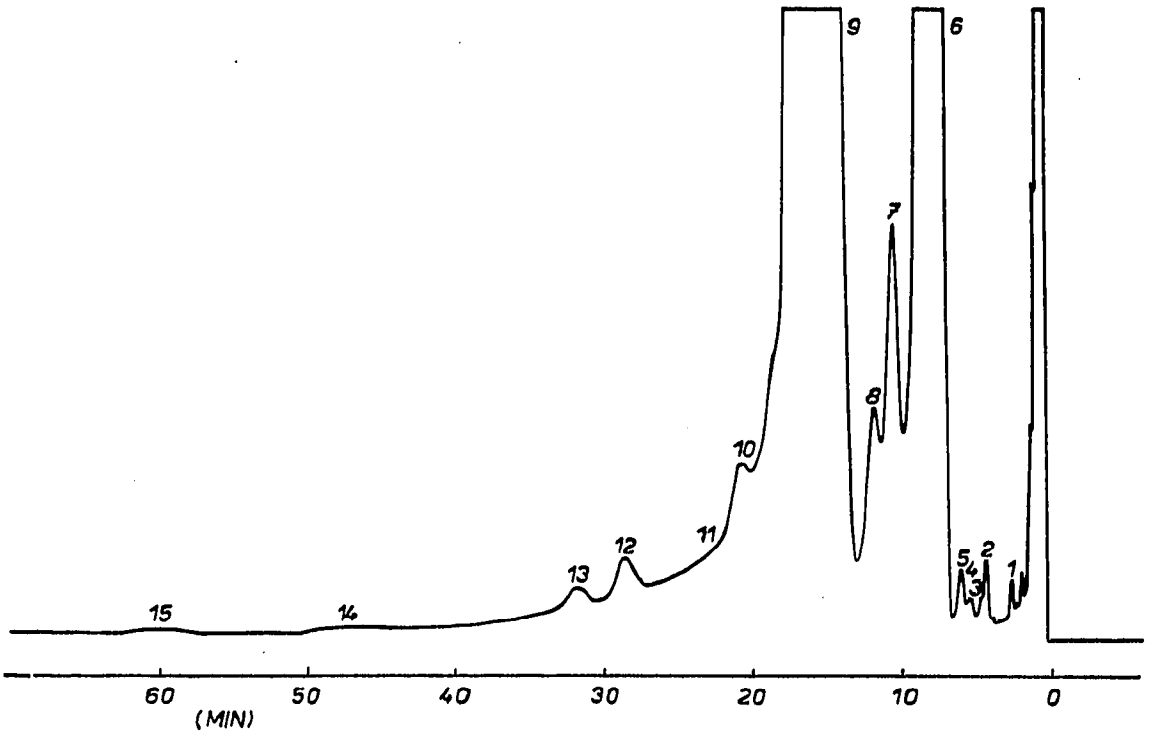


Fig. 4. Gas chromatogram of *Chlorella* fatty acid methyl esters on Apiezon-L at 230°. Carrier gas nitrogen (30 ml/min). Flame ionization detector. Column: length 1.5 m × 3 mm I.D. Esters 1 = 12:0; 2 = 14:1, 14:2 and 14:3; 3 = 14:0; 4 = *ai*-15; 5 = 15:0; 6 = 16:0, 16:1 and 16:2 7 = *ai*-17; 8 = 17:0; 9 = 18:0, 18:1, 18:2 and 18:3; 10 = *ai*-19; 11 = 19:0; 12 = 20:1, 20:2 13 = 20:0; 14 = 22:0; 15 = 23:0.

TABLE I

COMPOSITION OF *Chlorella* FATTY ACID METHYL ESTERS

| *Methyl ester of acid* | *Weight* (%) |
|---|---|
| 12:0 | 0.1 |
| 14:0 | 0.1 |
| 14:1 | 0.5 |
| 14:2 | 0.1 |
| 14:3 | 0.1 |
| *ai*-15 | 0.1 |
| 15:0 | 0.5 |
| 16:0 | 15–18 |
| 16:1 | 0.5–1 |
| 16:2 | 3–5 |
| 16:3 | 10–12 |
| *ai*-17 | 1–1.5 |
| 17:0 | 0.5 |
| 18:0 | 3–5 |
| 18:1 | 20–25 |
| 18:2 | 13–18 |
| 18:3 | 13–18 |
| *ai*-19 | 0.2 |
| 19:0 | 0.1 |
| 20:0 | 0.1–0.2 |
| 20:1 | 0.2 |
| 20:2 | 0.2 |
| higher acids than $C_{20}$ | traces |

*anteiso*-acids (Fig. 2) originate evidently from biosynthesis, as do the straight-chain odd- and even-numbered acids. The results obtained agree generally with the work of SCHLENK AND MANGOLD[2], who discussed the possibility of the presence of odd-numbered acids. We found these acids in the bacteria-free culture of *Chlorella* as well as in the $^{14}C$-labelled fatty acids of *Chlorella vulgaris* and identified them more precisely. The quantitative composition of *Chlorella* fatty acids (Table I) differs insignificantly from published data[2]. The representative separation of *Chlorella* fatty acid methyl esters on DEGS is shown on a typical radio-gas chromatogram in Fig. 5. The composition of *Chlorella* fatty acids is important to the biosynthetic preparation of $^{14}C$-labelled fatty acids and their isolation (combination GLC–TLC or two-phase GLC) and analysis. It is evident (Figs. 1 and 2) that for example in oleic acid separated on DEGS, 16:3 and *ai*-19:0 acids can also be present when TLC or separation on Apiezon-L or a similar phase was not carried out. This fact is naturally important for the choice of an isolation method.

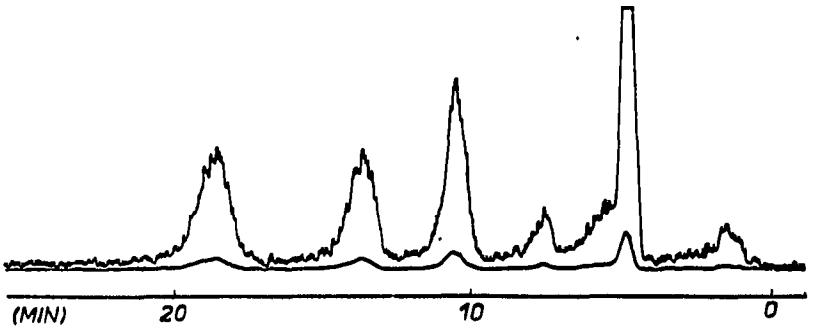


Fig. 5. Radio-gas chromatogram of *Chlorella* fatty acid methyl esters labelled with $^{14}C$ on diethylene glycol succinate, using proportional counter detection (20% $CH_4$, 80% Ar). A composition equivalent to that of inactive algae is found (16:0, 16:2, 18:1, 18:2 and 18:3 are the main components, see Fig. 3).

## CONCLUSIONS

A gas chromatographic separation of the *Chlorella* higher fatty acids as their methyl esters is achieved only by chromatography on polar and non-polar stationary phases. In *Chlorella vulgaris* were found: (1) even-numbered straight-chain fatty acids as follows: saturated ($C_{14}$–$C_{20}$), mono-unsaturated ($C_{14}$–$C_{18}$), di-unsaturated ($C_{14}$–$C_{18}$), tri-unsaturated ($C_{14}$–$C_{18}$); (2) odd-numbered saturated fatty acids ($C_{15}$–$C_{19}$), both straight-chain and their *anteiso* derivatives.

## ACKNOWLEDGEMENTS

This research was supported by the Czechoslovak Atomic Energy Commission, Prague. We thank Dr. J. HRADEC from the Oncological Institute for a kind gift of 14-methylhexadecanoic acid, Dr. J. DVOŘÁKOVÁ, ČSAV, Prague, for discussions of our results and Dr. J. KOLINA, ÚVVVR, Prague, for kind supply of cultivated *Chlorella vulgaris*.

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