•*Technical*

# Comparative Analytical Studies of Fatty Acids of the Alga *Chlorella Pyrenoidosa*[1]

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Gas-liquid chromatography, several versions of paper chromatography, and alkaline isomerization have been applied to one preparation of *Chlorella* fatty acid methyl esters.

GLC outranks other methods in rapidity, reproducibility, and resolving power. The presence of nonvolatile components in the sample can lead to erroneous results. Among PC methods the use of methyl-$C^{14}$ esters is preferable because components which might arise from autoxidation and polymerization are detected. Esters or acids with 14 or fewer C atoms should be analyzed as nonvolatile derivatives. AI, as used here, introduces a systematic error in the determination of linoleic and linolenic acids.

The quantitative results of the methods are in agreement for the major components, which all belong to the $C_{16}$ and $C_{18}$ series.

A number of minor components have been revealed by GLC and/or PC. They are tentatively identified as $<C_{12}$, $C_{12}$, four $C_{14}$, $C_{15}$, two $C_{17}$, $C_{20}$, three $C_{20}$, two $C_{22}$, and $C_{24}$ acids. Several of these compounds have been found independently by different methods in hydrogenated form or as radioactive esters. This enhances the certainty of their identification. Enrichment by fractional distillation was essential for their detection. Possible losses in their distillation and other considerations make uncertain the assay of most of the minor components by GLC or PC.

GAS-LIQUID chromatography (GLC), paper chromatography (PC), and alkaline isomerization (AI) are currently used for analyzing the acidic moiety of fats. Although accuracies of each of the methods quite often have been determined by use of model mixtures of known composition, much less work has been reported on their comparison (1,2,3). The interest in the methods lies in their application to unknown samples. Therefore comparative "field tests" appear even more important than comparison of methods on the basis of results obtained with simple known mixtures.

In the work presented here, GLC, PC, and AI have been applied to one rather complex material in an attempt to arrive at some comparisons which should permit their more discriminate use. In accord with this purpose, instrumentation has been chosen which is commonly available. Similarly the specific techniques used in applying the methods have been described in the literature and are rather widely used. Purposely procedures have not been amplified or improved although the authors felt that this may be desirable after comparison of their results.

Methyl esters of fatty acids obtained from *Chlorella pyrenoidosa* were chosen for the comprehensive analytical study for several reasons. Although traditional methods have been applied to algal fat, particularly to that of *Chlorella pyrenoidosa*, this material is still one of the lesser known fats, and reinvestigation with new techniques is warranted. Because of the rising interest in lipid constituents of microorganisms, a detailed analysis of the fat of an organism which can be greatly influenced by controlling each of several environmental factors was an attractive prospect. The lipids of *Chlorella pyrenoidosa* contain eight major acids of the $C_{16}$ and $C_{18}$ series as well as several minor components. The original material and samples derived from it provide types of mixtures often encountered in analyses connected with the isolation or synthesis of fatty acids. Analyses were carried out with the methyl esters (or acids) and with samples obtained from them by hydrogenation or distillation. These latter procedures also facilitated the search for minor components.

The percentages of major components of the original mixture found by GLC are in good agreement with those obtained by PC of the distilled fractions. Some of the results obtained by AI however deviate more markedly. Several minor components, mainly in the distilled fractions, were detected by GLC and/or PC and were tentatively identified. A brief discussion of the results in regard to other analyses of the fatty acids of *Chlorella pyrenoidosa* is found at the end of this report.

## Preparative Procedures

*Analytical Samples.* The stock culture of *Chlorella pyrenoidosa* (No. 7516, American Type Culture Collection) was maintained as described (4). The producing cultures were grown in 24-liter flasks each containing 20 liters of medium. They were placed outdoors at the north side of a building and were supplied with a stream of 5% $CO_2$ in $N_2$. By working up the cultures (4), nearly 10 g. of colorless fatty

TABLE I

Distillation of *Chlorella* Fatty Acid Methyl Esters

| Fraction | Grams | Boiling range, °C., at about 0.6 mm. |
|---|---|---|
| 1 | 0.41 | (109)–120.5 |
| 2 | 0.62 | 120.5–123.0 |
| 3 | 3.52 | 123.0–124.5 |
| 4 | 3.30 | 124.0–127.0 |
| 5 | 0.61 | 127.0–131.0 |
| 6 | 1.53 | 131.0–132.5 |
| 7 | 1.55 | ~132.5 |
| 8 | 5.17 | 132.5–132.0 |
| 9 | 5.04 | ~132.0 |
| 10 | 1.17 | 132.0–133.5 |
| 11 | 1.67 | 133.5–134.5 |
| 12 | 3.84 | 134.5–137.0 |
| Residue | about 4.2 | |

A whirling band column, 5 mm. × 60 cm., was used. After Fraction 11 was taken, the distilling flask was overheated in order to increase the amount of distilled esters. A total of 87.1% was distilled within 8 hrs.

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acid methyl esters were obtained from each flask after 12 to 15 weeks. Aliquots of an accumulated mixture were sealed in ampules, and other portions were distilled in lots of about 30 g. Table I gives an account of the distillation, the fractions of which were utilized in further analyses. The column had been flooded and then equilibrated for 30 min. The occurrence of $C_{18}$ ester in the first fraction (Table II) shows that this procedure is not suitable for obtaining a pure short-chain portion. Each fraction, after thorough mixing, was divided into several aliquots. The usual precautions of handling under $N_2$, sealing in high vacuum, and storing at −20° were taken.

*Reference Lipids.* Authentic esters were needed for standardizations in GLC, for quantitation in some of the PC procedures, and for determining absorption constants to be used in AI. The unsaturated acids of the $C_{18}$ series which occur in *Chlorella pyrenoidosa* had been identified as oleic, linoleic, and linolenic acids. Therefore samples of these acids, as prepared by the Hormel Foundation from olive, safflower, and linseed oil, respectively, could be used. Authentic samples of the unsaturated $C_{16}$ acids were prepared from the stock of *Chlorella* fatty esters by distillation and countercurrent distribution in the same manner as described for the isolation of these acids in radioactive form (4). The substances were pure according to GLC and PC.

*Methyl-$C^{14}$ Esters.* Nonradioactive esters to be analyzed by procedures involving assay of $C^{14}$ were saponified at room temperature and re-esterified with diazomethane-$C^{14}$ (5).

## Analytical Procedures

*Gas-Liquid Chromatography.* A gas chromatograph (Beckman GC-2) was used with a stainless steel column 8 ft. long and 0.25 in. o.d., with wall thickness of 0.02 in. Chromosorb W was purified and impregnated with 15% by weight of Resoflex 446, as described previously (6). The stationary phase was screened to 60 to 80 mesh, and the column was equilibrated for 8 hrs. at 220°. Operating temperatures were 197° for samples containing chain lengths of 16 or less carbon atoms and 218° for all other samples. Helium was the carrier gas at an inlet pressure of 30 p.s.i. and a flow-rate of 60 ml./min. The size of the samples was 5 $\mu$l.

The thermoconductivity cell of the apparatus was calibrated with the pertinent $C_{16}$ or $C_{18}$ methyl esters under the described conditions. The model mixtures contained between 12 and 32% of each of the four components. The standard deviation from the mean was found to be insignificant, permitting single determinations of the analytical samples. Ratios of peak areas were so nearly identical with ratios of component weights as to render superfluous any more elaborate calibration. A small systematic error however is indicated by the fact that all area percentages of palmitate and stearate were slightly high in the calibrations while those of linoleate and linolenate were somewhat low (5 experiments). Although these deviations appear to be significant statistically, they are certainly outweighed by errors connected with the preparation of the esters from the natural material. The calibration values of hexadecatrienoate were around the true value while those of hexadecadienoate were slightly high.

*Paper Chromatography.* All paper chromatograms were developed at 30° in ascending reversed-phase systems. Developing systems [2] were:

1. silicone (Dow-Corning 200, 10 cs.) + aqueous acetic acid, on Whatman No. 1 paper (7,8);
2. silicone (Dow-Corning 200, 10 cs.) + aqueous acetic acid + peracetic acid, on Whatman No. 1 paper (5);
3. mineral oil (heavy liquid petrolatum, USP, viscosity 335/350, b.p. 315–400°) + aqueous acetic acid, on Schleicher and Schuell paper No. 2043b. The authors of this procedure recommend an undecane fraction of petroleum, b.p. 190–220° (9). Modifications of the developing system and of the indicator of PC-3c have been described by several investigators (10,11,12,13).

Indicators [2] were:

a) α-Cyclodextrin + $I_2$ and $I_2$ for saturated and unsaturated esters, respectively (7,8); used with Systems 1 and 2.
b) Methyl-$C^{14}$ esters (5); used with Systems 1 and 2.
c) Copper salts for acids (9,14); used with System 3.

Optical measurements were made with a densitometer and recorded manually. Radioactive esters were measured with a recording chromatogram scanner (Volk Radiochemical Company, Chicago, Ill.). Indicators a) are applicable only when the unknown is investigated together with model mixtures (7,15) and the results are obtained as weight percentages. When Indicators b) and c) were applied to model mixtures of $C_{16}$ or $C_{18}$ esters and acids, respectively, the responses of scanner and densitometer were found to be proportional to the equivalents contained in the samples. Accordingly analyses involving these indicators were made without model mixtures. The average deviation from the mean was smallest in procedure 1b. Analytical samples were run in triplicate with this method whereas six to eight determinations were carried out with PC-1a and 3c. Whenever possible, results obtained in equivalents were converted into weight percentages.

*Alkaline Isomerization.* Analyses were carried out as recently described (16). Weight percentages of acids were calculated by using the absorbencies $k_{233} = 92.0$ for linoleic, and $k_{268} = 79.0$, $k_{233} = 44.7$ for linolenic acids. The constants for hexadecadienoic acid $k_{233} = 100$, and trienoic acid, $k_{268} = 108$, $k_{233} = 46.6$, are averages obtained from six and three measurements, respectively, upon the authentic compounds. All analytical samples were run in duplicate. Comparison of data with those of the other methods required their conversion into ester percentages.

*$C_{20}$ Esters by Dilution.* Methyl-$C^{14}$ arachidate was added to hydrogenated *Chlorella* fatty esters. Three different mixtures were prepared and chromatographed on paper. Method 1a and the chromatogram scanner were used on the same chromatograms for assaying $C_{20}$ ester originally present. The esters of $C_{22}$ and $C_{24}$ acids were also detected in these chromatograms.

## Results

Analyses by GLC and PC depend upon separation. GLC achieved the resolution of all major components

[2] The indices will be used for brevity; for example, PC-1a = paper chromatography in System 1 with indicators a.

TABLE II
Composition of Fraction 1 (Weight Percentages)

| Methyl ester of acid | GLC | PC | | | | | AI[c] |
|---|---|---|---|---|---|---|---|
| | | Hydrogenated | | | 2a[a] | 2b[b] | |
| | | 1a | 1b | 3c | | | |
| <$C_{12}$ | 1.7 | | | | | | |
| $C_{12}$ | 0.8 | | | | | | |
| $C_{14}$ saturated | 12.1 | | | | 1.0 | 7.5 | |
| $C_{14}$ monoene | 7.0 | | | | | | |
| $C_{14}$ diene | 10.0 | | | | | | |
| $C_{14}$ triene | 2.7 | | | | | | |
| Total $C_{14}$ | $\Sigma$=31.8 | 9.8 | 8.8 | 18.6 | | | |
| $C_{15}$ | | 14.3 | 14.4 | | 2.4 | 16.2 | |
| $C_{16}$ saturated | 6.2 | | | | 0.5 | 6.6 | |
| $C_{16}$ monoene | 7.3 | | | | | | |
| $C_{16}$ diene | 34.2 | | | | | | 32.9 |
| $C_{16}$ triene | 14.1 | | | | | | 9.4 |
| Total $C_{16}$ | $\Sigma$=61.7 | 76.0 | 74.8 | 81.4 | | | |
| $C_{18}$ saturated | 4.2 | | 1.7 | | | | |
| Total unsaturated | $\Sigma$=75.3 | | | | | 69.7 | |

[a] Weight ratios of saturated components.
[b] Eq. %.
[c] Calculated with constants of the $C_{16}$ acids; using the constants of $C_{18}$ diene and triene, the values would be 34.1 and 12.4%.
(PC-3c did not distinguish between $C_{15}$ and $C_{14}$ acids whereas in GLC $C_{15}$ ester was interpreted as unsaturated $C_{14}$ ester.)

of the original sample. Because of the superpositions of vinylogous-homologous components (8), resolution by PC is limited to mixtures of uniform chain-length or of saturated compounds. The individual saturated components of a complex mixture are however separated in peroxidic solvent, where the unsaturated compounds are oxidized to substances of higher $R_f$ value (5). AI, when not combined with a previous separation of complex mixtures, can reveal only the total of dienes, trienes, tetraenes, etc. When applied to mixtures of single chain-length, it can measure individual components, provided the occurrence of isomers has been ruled out.

In order to provide a suitable variety of analytical problems and to search for minor components, all fractions and the residue of the distilled *Chlorella* fatty esters (Table I) were analyzed by PC and AI, and Fractions 1, 3, 5, 7 and the residue were analyzed also by GLC. Fractions 1, 5, and the residue were believed to be enriched with minor components, and Fractions 3 and 7 to be of uniform chain-length. Results of these analyses are given in Tables II to VI. The hydrogenated and the original esters were analyzed by all methods; the results are listed in Tables VII and VIII. Tables IX and X are a synopsis of the total composition of *Chlorella* fatty acids by different methods.

Palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids have been identified as components of *Chlorella* fat. Hexadecadienoic acid and -trienoic acids have been characterized (4), but their structures are unknown. The other components have been designated as their chromatographic properties suggest. Some of them have been detected as radioactive methyl esters, which proves that they belong to the acidic moiety of the fat. All components are listed according to increasing chain-length and unsaturation.

TABLE III
Composition of Fraction 3
(Weight Percentages)

| Methyl ester of acid | GLC | PC | | | AI[a] |
|---|---|---|---|---|---|
| | | 1a | 1b | 3c | |
| $C_{16}$ saturated | 29.9 | 28.8 | 29.0 | 38.1 | |
| $C_{16}$ monoene | 12.1 | 12.0 | 13.3 | 12.1 | |
| $C_{16}$ diene | 34.7 | 33.6 | 34.5 | 28.8 | 35.2 |
| $C_{16}$ triene | 23.2 | 25.4 | 23.2 | 21.0 | 23.1 |

[a] Using the constants of $C_{18}$ diene and triene, the values would be 34.6 and 30.3.
(PC-3c agreed better with other PC methods in Fractions 4, and 8 to 12, than in this fraction.)

## Discussion

*Methods.* The analyses of the major components, in particular by GLC and PC, agree within limits which will meet the requirements of many investigations. It is striking that the greatest absolute difference found in Table IX between any of the values of GLC and PC occurs with stearate, which itself represents the smallest relative amount. High values have been encountered with stearyl acetate in GLC of fatty alcohol acetates, and the same phenomenon may prevail here (6).

TABLE IV
Composition of Fraction 5
(Weight Percentages)

| Methyl ester of acid | GLC | PC | | | AI[b] |
|---|---|---|---|---|---|
| | | Hydrogenated | | | |
| | | 1a | 1b | 3c | |
| $C_{16}$ saturated | 43.4 | | | | |
| $C_{16}$ monoene | 8.8 | | | | |
| $C_{16}$ diene | 4.2 | | | | |
| $C_{16}$ triene | 2.6 | | | | |
| Total $C_{16}$ | $\Sigma$=59.0 | 60.0 | 45.3 | 62.2 | |
| $C_{17}$ saturated | 4.5[a] | | 15.6 | | |
| $C_{18}$ saturated | 1.8 | | | | |
| $C_{18}$ monoene | 16.3 | | | | |
| $C_{18}$ diene | 12.2 | | | | 11.9 |
| $C_{18}$ triene | 6.2 | | | | 8.4 |
| Total $C_{18}$ | $\Sigma$=36.5 | 40.0 | 39.1 | 37.9 | |

[a] $C_{17}$ ester appears between hexadecadienoate and -trienoate.
[b] Using the constants of $C_{16}$ acids, the values would be 9.2% dienoate and 6.2% trienoate.
(Although the developing system is identical in PC-1a and 1b and therefore resolutions should be equal, $C_{17}$ ester was detected only by Indicator b. The amount is higher than that found by GLC since isomeric esters are not separated by PC under the conditions applied. GLC separates such isomers as demonstrated with hydrogenated esters (Table VII). One of the $C_{17}$ esters detected may be obscured by an unsaturated $C_{18}$ ester.)

In the PC analyses reported in this paper and on other occasions it has been found that volatilization can lead to loss of short-chain esters. A chromatographed spot of methyl palmitate-$C^{14}$ lost about 40% of its radioactivity within six weeks when the cut-out spot was placed in a diffusion cell over 20% KOH at a distance of approximately 3 mm. Palmitic acid-$C^{14}$ was identified in the alkaline solution. Evaporation, as well as diffusion on the paper, is accelerated by humidity. Therefore all chromatograms were stored over $CaCl_2$ when not measured immediately.

Similarly short-chain and highly unsaturated acids are subject to loss because of their solubility in the staining and rinsing baths of Indicator c). Reportedly the solubility of the Cu salts of laurie and higher acids, including linoleic, is too low to affect their quantitative determination (14). According to another source, the salts of laurie and linolenic acids are markedly soluble (13). Esters or acids shorter than $C_{14}$ have not been detected by any of the PC methods although their presence in small quantity is shown by GLC of Fraction 1 (Table II).

With AI the largest deviations were found for linoleate and linolenate (Table IX). The differences were spread rather consistently over Fractions 7–12, which suggests a systematic error. This is in contrast to the findings of authors who used other versions of AI and compared the results with those by GLC and

TABLE V

Composition of Fraction 7
(Weight Percentages)

| Methyl ester of acid | GLC | PC | | | AI |
|---|---|---|---|---|---|
| | | 1a | 1b | 3c | |
| $C_{16}$, 0, 1, 2, 3, double bonds | 1.7, 1.2 1.1, 0.9 | | | | |
| $C_{18}$ saturated | 1.3 | | | | |
| $C_{18}$ monoene | 47.1 | 50.0 | 59.6[a] | 57.6 | |
| $C_{18}$ diene | 28.0 | 29.8 | 26.8 | 25.8 | 24.6 |
| $C_{18}$ triene | 18.7 | 20.4 | 13.8 | 16.7 | 23.1 |

[a] About 10% palmitate was present, according to PC-2b; on this basis oleate must be corrected to 49.6%, with only minor changes for the other esters.

(PC-1b and 3c measure the sum of oleic and palmitic esters (acids) while PC-1a and GLC measure oleate specifically. Accordingly the values for oleate are lower by the latter methods.)

TABLE VI

Composition of the Residue [a]

| Methyl ester of acid | GLC[b] wt. % | PC Hydrogenated 1a[d] wt.% | 1b eq.% | 1b[b] wt.% | 2a eq.% | AI wt.% |
|---|---|---|---|---|---|---|
| $C_{18}$ saturated | 13.2 | | | | 6.1 | |
| $C_{18}$ monoene | 30.7 | | | | | |
| $C_{18}$ diene | 10.6 | | | | | 2.4 |
| $C_{18}$ triene | 18.5 | | | | | 10.2 |
| Total $C_{18}$ | Σ=(31.5) | 35.1 | 46.3 | (32.4) | | |
| $C_{19}$ | 2.9[c] (1.2) | | | | | |
| $C_{20}$ | 12.0 | | | | | |
| | 6.4 | | | | 1.0 | |
| | 3.8 | | | | | |
| Total $C_{20}$ | Σ=( 9.6) | 3.9 | 6.4 | ( 4.9) | | |
| $C_{22}$ | 0.9 | | | | 0.6 | |
| | 1.0 | | | | | |
| Total $C_{22}$ | Σ=( 0.8) | 1.3 | 3.6 | ( 3.0) | | |
| $C_{24}$ | | 2.5 | 2.8 | ( 2.5) | 2.1 | |
| Oxid.+ polym. | (57) | 57.3 | 40.8 | (57.0) | 90.1 | |

[a] The residue is a brown, semisolid material, which precludes using PC-3c.

[b] Values in parentheses are calculated, assuming 57% oxidized and polymerized material (PC-1a).

[c] $C_{19}$ ester appears between $C_{18}$ dienoate and trienoate.

[d] The defined components were measured by comparison with model mixtures, and the amount of oxidized and polymerized material was calculated by subtraction.

(PC-1a gives the most reliable weight of percentage of the oxidized and polymerized portion. Necessarily the numerical value for weight percentage is higher than for equivalent percentage. The latter is found directly by PC-1b.)

TABLE VII

Composition of the Hydrogenated Total Esters
(Weight Percentages)

| Methyl ester of acid | GLC | PC | | |
|---|---|---|---|---|
| | | 1a | 1b[a] | 3c |
| $C_{16}$ | 27.4 | 27.7 | 26.8 | 23.8 |
| $C_{17}$ | 1.1 | | | |
| | 1.3 | | | |
| $C_{18}$ | 70.0 | 72.3 | 73.2 | 76.2 |
| Oxid. + polym. | | | ( 3.2) | |

[a] Oxidized and polymerized material has been determined as equivalent percentage but was not taken into account when calculating the weight percentages of the other components.

(The retention volumes in GLC of "$C_{17}$ esters" are close to those of $C_{16}$-dienoate and -trienoate. Interpretation as straight and branched compounds appears more correct [see also Table IV].)

PC (1,2,3). The results might have been improved for both acids by using isomerization absorbancy constants of natural linolenic acid rather than of the trienoic acids obtained by debromination of hexabromostearic acid. This fallacy has been avoided in the case of hexadecatrienoic acid by isolating it in the pure natural form.

GLC and PC differ basically in that only the latter allows inspection of the entire path of the sample. Therefore the $C_{24}$ ester and the autoxidized and polymerized portions were detected by PC. The latter undefined material might arise from exposure in preparing unsaturated samples, or oxygenated compounds might even be present originally. In quantitation they are found by PC-1b as ester equivalents, and a factor for conversion into weight must necessarily be arbitrary. Therefore the subtractive method based on weight comparisons in PC-1a appears more reliable.

The harmony of retention volumes and $R_f$ values enhances the certainty of identification of several of the minor components. Morris recently found that certain hydroxy compounds and primary autoxidation products undergo alteration into new compounds under the conditions of GLC (18). The possibility of creating artifacts by one analytical method increases the value of verification by another method. The failure of GLC to detect the $C_{15}$ ester (Table II) might have been corrected by examination of an hydrogenated sample of this fraction. The advantage of such multiple analyses is obvious with $C_{17}$ esters. GLC detected one $C_{17}$ ester in an enriched fraction (Table IV) while analysis of the hydrogenated esters, without enrichment, revealed two esters in the $C_{17}$ region (Table VII). For positive identification of most of the minor components, additional analyses are necessary. Their quantitation must be accepted with reservations since for many of them neither the quantitative response of detector or indicator nor the loss in the chromatographic column or the distillation are known. Moreover not all fractions were analyzed by GLC.

It is obvious that the amount of work required was greatest with PC and that the most information was obtained in the least time with GLC. None of the methods or instruments was fully exploited in the search for unknown minor components. Being now more familiar with the fatty acid sample after the multiple analyses, some suggestions for improved analyses are obvious. They refer mainly to the minor components. The residue should be subjected to an alembic distillation before its analysis. GLC should be applied with a greater variety of temperatures and stationary phases, and it should be applied to hydrogenated samples. PC, with samples containing $C_{14}$ and shorter-chain acids, should use nonvolatile derivatives, for example, the methylanilides (5). The absorbancy constant for linolenic acid used in this particular AI method should be revised.

*The Fatty Acids of Chlorella pyrenoidosa.* The estimates of major components made earlier (4,19) are close to the analytical results reported here. Differences in illumination, $CO_2$ supply, $O_2$ removal, and size of batches between the previous and the present cultures did not markedly influence the composition of fatty acids produced by *Chlorella pyrenoidosa*. The *algae* were grown in a low-nitrogen medium over a prolonged time. This explains the essential difference between our samples of fatty acids and samples from *Chlorella pyrenoidosa* that had been cultivated a shorter time on high-nitrogen media for a high yield of protein. Within the $C_{16}$ or $C_{18}$ series, palmitic or oleic acids are prevalent in one case while, in the other, hexadecatrienoic or linolenic acids are predominant (20,21). Hexadeca- and octadecatetraenoic acids had been found in the more unsaturated type of *Chlorella* fat, as indicated by AI in distilled

TABLE VIII
Analysis of the Original Mixture of Fatty Acid Methyl Esters [a]
(*Equivalent Percentages*)

| Methyl esters of acid | GLC | PC | | | |
|---|---|---|---|---|---|
| | | 2b | | 1b [c] | 3c |
| $C_{14}$ diene | 0.6 | | | | |
| $C_{16}$ saturated | 14.0 | 12.9 | $C_{16}$ sat.+$C_{18}$ monoene | 47.7 | 45.3 |
| $C_{16}$ monoene | 3.4 | | $C_{16}$ monoene+$C_{18}$ diene | 21.3 | 22.6 |
| $C_{16}$ diene | 7.6 | | $C_{16}$ diene+$C_{18}$ triene | 24.0 | 25.0 |
| $C_{16}$ triene | 5.5 | | | 5.0 | 4.4 |
| $C_{17}$ saturated | trace [b] | | | | |
| $C_{18}$ saturated | 3.4 | 2.1 | | 2.0 | 2.7 |
| $C_{18}$ monoene | 33.9 | | | 34.8 [d] | |
| $C_{18}$ diene | 17.3 | | | | |
| $C_{18}$ triene | 14.3 | | | | |
| $C_{20}$ | trace | | | | |
| Total unsat. [e] | $\Sigma$=82.6 | 85.1 | | | |
| Oxid + polym. | | | | (3.6) | |

[a] Results obtained by AI are listed in Table IX.
[b] $C_{17}$ ester appears between $C_{16}$-dienoate and -trienoate.
[c] The value found for oxidized and polymerized material has not been taken into account in calculating the other values.
[d] The value for $C_{18}$ monoene results from subtraction of 12.9 (2b) from 47.7 (1b).
[e] The sum of values obtained by GLC is to be compared with 81.5% by PC-2b, which results from 85.1% when considering the oxidized and polymerized portion.

(PC does not resolve the pairs $C_{16}$ saturated + $C_{18}$ monoene, $C_{16}$ monoene + $C_{18}$ diene, and $C_{16}$ diene + $C_{18}$ triene. The corresponding sums by GLC are 47.9, 20.7, and 21.9%.)

fractions (21). The presence of hexadecatetraenoic acid in lipids obtained from the alga *Scenedesmus obliquus* has also been established (22). None of the methods used in the present investigation revealed these or any other tetraenoic acids.

The chain-length distribution appears to be independent of the changes in unsaturation since the amount of the $C_{16}$ acids is 25 to 30% in all cases. The rest consist mainly of $C_{18}$ acids. The earlier finding of $C_{14}$ and lower acids (21) was supported and made more specific. $C_{12}$ and shorter acids have been found by other authors in the culture medium of *Chlorella pyrenoidosa* (23). They may originate from the short-chain acids found here in the *algae*.

The presence of odd-numbered or branched acids so far has not been reported in *algae* although such acids are rather widely distributed among terrestrial and marine animals and in certain bacteria. The only oils of autotrophic plants from which odd-numbered acids have been isolated are coconut oil containing nonanoic, undecanoic, and tridecanoic acids (24) and tall oil containing (+)-14-methylhexadecanoic acid (25). GLC has, in several instances, indicated the presence of such acids in vegetable oils (26). With the detection of penta-, hepta-, and nonadecanoic acids and a branched acid in *Chlorella* fat the question arises whether they originate from this organism. It has been stated that "healthy" cultures of *Chlorella pyrenoidosa* cannot be obtained without the proper flora of bacteria, which is easily distinguished from an "illegitimate" flora (27). Our cultures were uniform in appearance, and several tests did reveal bacteria, which however never became significant on a weight basis. Presently no convincing reasons are seen for assigning these acids to organisms other than the *algae*.

TABLE IX
Composition of *Chlorella* Fatty Esters: Major Components
(Weight Percentages)

| Methyl ester of acid | Original | | Sum of fractions | | | |
|---|---|---|---|---|---|---|
| | GLC | AI [a] | PC [b] | | | AI [c] |
| | | | 1a | 1b | 3c | |
| $C_{16}$ saturated | 13.6 | | 13.1 | 12.7 | 13.5 | |
| $C_{16}$ monoene | 3.2 | | 2.0 | 2.4 | 2.5 | |
| $C_{16}$ diene | 7.0 | | 5.8 | 6.2 | 5.5 | 6.3 |
| $C_{16}$ triene | 5.1 | | 4.3 | 4.4 | 3.9 | 4.2 |
| $C_{18}$ saturated | 3.5 | | 1.1 | 3.0 | 1.7 | |
| $C_{18}$ monoene | 34.7 | | 33.9 | 33.3 | 35.1 | |
| $C_{18}$ diene | 17.7 | 28.2 | 17.0 | 17.4 | 16.5 | 13.8 |
| $C_{18}$ triene | 14.6 | 23.4 | 14.0 | 13.3 | 14.2 | 17.3 |

[a] Calculated with constants of linoleic and linolenic acids, which is the usual procedure for this method.
[b] Superimposed components of mixed chain-length fractions were estimated according to their ratios in neighboring fractions which were essentially uniform in chain length and permitted resolution. About 0.8 g. of unsaturated components, out of 32.6 g. esters, had to be estimated this way.
[c] The respective constants were used in calculating $C_{16}$ and $C_{18}$ components.

(Results by GLC must be too high because approximately 3.5% of undefined material [Tables VII and VIII] is not taken into account. On the other hand, results by PC must be considered as too low, particularly for the unsaturated components. They are based upon distillation which increased the amount of undefined material from 3.5 to 7.5% [Table VI]. The pattern of high values by GLC and low values by PC is borne out in all but one instance.)

TABLE X
Composition of *Chlorella* Fatty Esters: Minor Components
(Weight Percentages)

| Methyl ester of acid | Detected in | | Detected after distillation | | | |
|---|---|---|---|---|---|---|
| | original GLC | hydrogenated GLC | GLC [b, c] | PC | | |
| | | | | 1a | 1b | 3c |
| $<C_{12}$ | | | 0.02 | | | |
| $C_{12}$ | | | 0.01 | | | |
| $C_{14}$ saturated | | | 0.15 | | | |
| $C_{14}$ monoene | | | 0.09 | 0.14 | 0.11 | 0.25 |
| $C_{14}$ diene | 0.5 [a] | | 0.13 | | | |
| $C_{14}$ triene | | | 0.03 | | | |
| $C_{15}$ | | | | 0.18 | 0.18 | |
| $C_{17}$ | trace | 1.1<br>1.3 | 0.09 | | 0.38 | |
| $C_{19}$ | | | 0.15 | | | |
| $C_{20}$ [d] | trace | | 0.67<br>0.36<br>0.20 | 0.47 | 0.63 | |
| $C_{22}$ [d] | | | 0.05<br>0.06 | 0.17 | 0.39 | |
| $C_{24}$ | | | | 0.32 | 0.32 | |

[a] $C_{14}$ diene might be $C_{15}$ (Table 11).
[b] Only Fractions 1, 3, 5, 7 and the residue have been analyzed.
[c] The amount of undefined material obtained by PC-1a (Table VI) has been introduced in calculating the percentages of $C_{16}$ and higher components.
[d] 1% $C_{20}$ esters was found by radioactive dilution, applying PC-1a and 1b. The genuine occurrence of arachidate and behenate, which had been detected in these chromatograms, was verified in column-partition chromatograms by a method described elsewhere (17).

(Sixteen compounds have been found that occur in amounts of 1% or less. GLC detected three or four in the original, or hydrogenated mixture, and probably 10 additional ones after distillation. PC, after distillation, detected four minor components which could be identified with those already mentioned, and two additional ones in originally saturated and in hydrogenated form, respectively.)

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# Reactions of Polyunsaturated Fatty Alcohols. IX. Molecular-Weight Distribution of Some Conjugated Soybean Vinyl Ether Homopolymers[1]

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The molecular-weight distributions of several conjugated soybean vinyl ether homopolymers were studied by means of the integral fractionation technique. Polymers having number-average molecular weights of 2,200, 4,800, and 10,000 prepared with stannic chloride as catalyst as well as a polymer having a molecular weight of 3,400 prepared with boron trifluoride as catalyst were included in the study. The observed distributions for all the polymers were found to approximate the so-called "most probable" distributions expected theoretically for polymers obtained by simple difunctional polymerization.

THE MOLECULAR-WEIGHT distribution of a polymer is an important characteristic to which many of its physical and chemical properties are related. Polyunsaturated fatty vinyl ether polymers and copolymers (12) have been under investigation at this laboratory because of their promising properties as coatings, especially for metal. Knowledge of their molecular-weight distribution might contribute to a better understanding of their drying behavior and of the properties of their films. Since the functionality of a polyunsaturated fatty vinyl ether polymer molecule towards oxygen varies with the number of monomer units in the chain, knowledge of the molecular-weight distribution would provide information on the maximum and minimum functionalities to oxygen available in the polymer and on the relative amounts of material having these and intermediate functionalities.

This paper reports the results of an investigation of the molecular-weight distribution of several conjugated soybean vinyl ether homopolymers. These were selected to permit comparison of polymers that had different number-average molecular weights or that were prepared with different polymerization catalysts.

## Discussion of Fractionation Methods

Because of the physical and chemical properties of polyunsaturated fatty vinyl ether polymers, special problems are encountered in their fractionation. Reasonably quantitative recovery of fractions is difficult because these polymers are liquid. Because these polymers and the fractions separated from them are sensitive to oxygen and because this sensitivity increases with molecular weight, handling in an inert atmosphere is necessary. Furthermore the molecular weights of the polymers and their fractions lie in ranges such that only very approximate values can be obtained.

In view of these problems, simple fractional precipitation of the polymers was considered impractical. The integral fractionation method (10) and the cumulative volume technique (1,2) appeared more suitable for investigation.

The principle of the integral fractionation method is illustrated by Figure 1. A very dilute solution of polymer is divided into a number of aliquots of equal volume. (For simplicity only five aliquots are shown in the figure; in practice a large number are required.) To each aliquot is added an increasingly large volume of nonsolvent. This results in precipi-

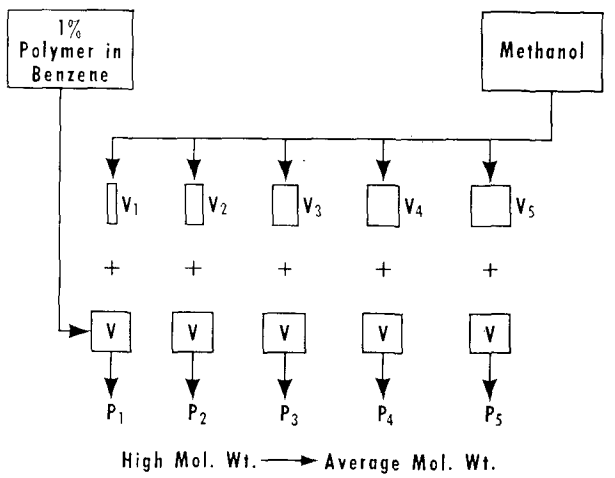


FIG. 1. Simplified representation of the integral fractionation method.

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