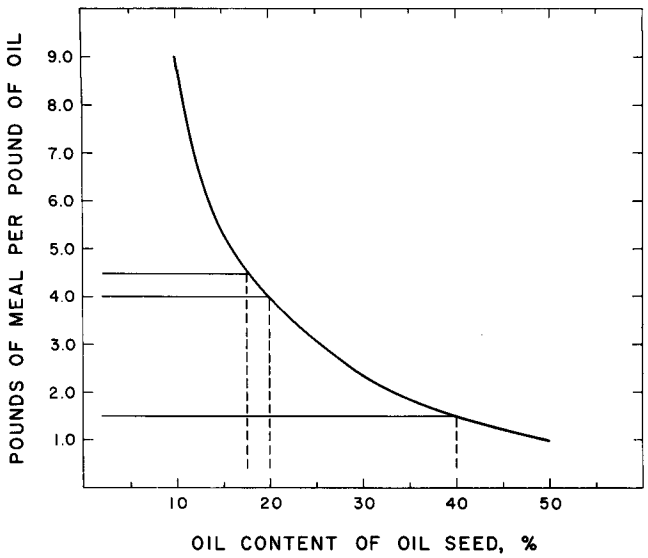


FIG. 4. Ratio of pounds of meal per lb of oil at different levels of oil content in the seed crushed.

displaced then the loss in meal supply amounts to 2.5 lb per lb oil or a total of 30 million lb meal.

Even under present pricing conditions it is evident that for soybeans the meal has become the most valuable product, with the oil the by-product. As higher oil percentages in crops are obtained, and as demand for meal increases faster than for oil, the situation will become increasingly critical.

This brief analysis indicates the importance of developing auxiliary sources of high protein concentrates so that oil and meal supplies can be kept in reasonable balance. It appears uneconomic to process seeds with a lower oil content than soybeans; hence the answer lies in production of a high protein material that can be used directly in feed formulation. Present trends in feeding practice indicate that an oil content of 7–10% in the ration would be an advantage. This suggests that our oil bearing crops might well be adapted to produce high yielding low oil content varieties to supply this market. Such a development would be a constructive step in the solution of a problem that is rapidly becoming critical in the oil seeds industry.

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# Determination of the Glyceride Structure of Fats: Gas Liquid Chromatography of Oxidized Glycerides[1]

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## Abstract

A method has been developed for the determination of glyceride composition of natural fats, which involves oxidation of the fat by permanganate-periodate, esterification of the oxidized glycerides, and subsequent GLC using a flame ionization detector. Quantitative analyses, requiring about 4 hr and 20 mg of sample, are reported. The method gives the distribution of individual saturated acids within the glycerides. Glyceride composition of four vegetable oils has been determined, using the above procedure.

## Introduction

THE QUANTITATIVE analysis of glycerides in a fat has always posed a difficult problem because of the similarity in physical properties between adjacent glycerides in a series. Several methods have been described for the determination of glyceride composition. In some methods fractionation of glycerides is carried out directly on the fat (3,24,25,27–29) and in others after oxidation of unsaturated fatty acid components (5,13,32). The observation (15,26) that pancreatic lipase cleaved the 1,3 positions in a glyceride leaving the fatty acid in the 2 position intact, gave another method (30) of getting information about glyceride structure. Recently (22,23) a micromethod involving ozonization of double bonds, followed by catalytic reduction of ozonides, has been described. The ozonides, as well as the aldehyde cores obtained by the reduction of ozonides, are then separated and quantitatively estimated by TLC.

A successful application of GLC for the separation of glycerides was reported by Fryer et al. (4) who obtained fingerprint chromatograms of various natural fats. Pelick et al. (20), using a temperature of 290–300C, recovered simple triglycerides within 40 min after injection. Huebner (11) achieved good separations of triglycerides from triacetin to tristearin, using temp from 110–370C. Kuksis and McCarthy (14) separated triglyceride mixtures according to their carbon number by GLC, with SE30 as liquid phase, using a flame ionization detector system and with temp from 200–320C. This method was later (16) applied to the determination of the triglyceride composition of molecular distillates of butter oil.

The above methods of glyceride analysis by GLC do not distinguish unsaturated glycerides from the saturated ones having the same carbon number. This could, however, be achieved by oxidizing the mixture which converts the unsaturated glycerides into compounds with smaller carbon numbers, GLC of which

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would then give the amounts of unsaturated glycerides. Using the above concept, the glyceride composition of four vegetable oils, cocoa butter, cottonseed, olive, and soybean oils has been determined. Results have been compared with those obtained by other methods, and also with an earlier method proposed by one of the authors (32).

## Experimental

*Materials.* Samples of synthetic triglycerides were obtained from Canada Packers, Toronto, and purified by silicic acid chromatography (10). About 0.1 g of the material was put on a 6 × 35 cm column of Mallinckrodt Silicic Acid (100 mesh). The triglyceride fraction was eluted with 5% diethyl ether in Skellysolve F, (a hydrocarbon fraction with bp range of 35–58C). TLC was used to check the seperations (1). Triazelain was prepared by oxidizing triolein with permanganate-periodate (31) and purified on a silicic acid column. Twenty g silicic acid was slurried onto a column with 20% ether in benzene. The material obtained by the oxidation of 125 mg triolein was put on the column and eluted with 20% ether in benzene. Triazelain appeared between 35–50 ml eluate. Fractions eluted between 38–47 ml were pooled and the purity of the triazelain was checked by TLC which gave a single spot for triazelain.

Cocoa butter, cottonseed, olive, and soybean oils were commercial samples, and had iodine values of 39,108,83 and 130, respectively.

## Methods

*Oxidation.* The method of oxidation was essentially as given by von Rudloff (31). In a typical oxidation, 20 mg triolein, dissolved in 5 ml tertiary butanol, was added to a mixture of 5 ml oxidant solution, 4 ml distilled water, 1 ml of a solution containing 8 mg potassium carbonate and 10 ml tertiary butanol. The reaction mixture was stirred 2½ hr at 65C, cooled in tap water, and ethylene gas was bubbled in until the pink color disappeared. The tertiary butanol was then removed under reduced pressure (water pump) on a water bath at 70–80C in a rotary evaporator. HCl (2–3 drops) was added to make the contents acidic, and after saturating the aqueous solution with sodium chloride, the oxidized glycerides were extracted with three 25-ml portions of chloroform. The chloroform layer was separated and solvent was removed in a current of air. Diazomethane solution was added to the residue to esterify the free carboxyl groups. The oxidized and methylated esters were taken up in a small volume of chloroform and chromatographed on the GLC unit. The proportion of reagents used for the oxidation was adjusted according to the fatty acid composition. Linoleic and linolenic acids require three and five times, respectively, as much as that required for oleic acid (31).

*GLC Analysis.* GLC was carried out on a F&M model 500 programmed temp gas chromatographic unit with flame ionization attachment. Analyses with several supports and stationary phases were attempted under a variety of conditions. The system described here gave the best separations. A 4-ft–$\frac{3}{16}$ in. diam spiral stainless steel column was packed with Anakrome ABS (an acid washed, base washed and silicone treated flux calcined diatomaceous earth) 60–70 mesh, coated with 2% SE30 (silicone rubber gum). Temp at the injection port and detector block were respectively 385 and 355C. Flow rates for air, helium, and hydrogen were 400, 100, and 30 ml/min, respectively. The oxidized, esterified glycerides from 20 mg fat were made up to 0.2 ml with chloroform and a sample of 1–3 $\mu$l was injected. Attenuation was kept at 800. The column was programmed 260–325C at 3°/min, after which it was run isothermally till all the glycerides were eluted. Peak areas were measured by drawing tangents to the curves and using the height of the triangle times the width of the base. All compositions were converted to mole percentages.

*Analysis of oils by the method of Youngs* (32). This method has been described earlier (32), the only alteration being in the methanolysis of oxidized fat, to prevent small losses of dimethyl azelate during the removal of solvent after esterification. The oxidized fat (20 mg) was taken up in 2 ml methanol containing 3% anhydrous hydrogen chloride by weight. Esterification was carried out in a sealed tube, by heating the contents for ½ hr in a boiling water bath. The tube was cooled, and a sample was injected directly onto the GLC unit. In this case a 6-ft, ¼-in. diam stainless steel column, packed with Chromosorb W (40–60 mesh) coated with 16% by weight of QF 1 (commercial fluorinated silicone) was used. Temp was commenced at 150C and a programming rate of 5.6°/min was used.

## Results and Discussion

Figure 1 shows the separation of esters of oxidized synthetic glycerides, triazelain ($A_3$), palmitodiazelain ($PA_2$), dipalmitoazelain ($P_2A$), distearoazelain ($S_2A$), tripalmitin ($P_3$), stearodipalmitin ($P_2S$), distearopalmitin ($PS_2$), and tristearin ($S_3$), which were obtained by oxidizing a mixture of triolein, palmitodiolein, dipalmitoolein, distearoolein, tripalmitin, stearodipalmitin, distearopalmitin, and tristearin. The identity of the peaks was determined by comparison with individually oxidized glycerides. $S_2A$ and $P_3$ were found to emerge at the same time. Taking a carbon number of 18 for stearic and 16 for palmitic, azelaic must have a relative carbon number of 12 to give a total of 48 (omitting the 3 carbons of the glycerol moiety common to all) for each of these two glycerides. This is consistent with the finding that dimethyl azelate and methyl laurate emerged together on a SE30 column. On this basis carbon numbers were assigned to each peak as shown in Figure 1. As pure samples of stearodiolein and palmitostearoolein were not available, they could not be included in the mixture. However, taking into consideration the emergence time in relation to carbon numbers, stearodiazelain ($SA_2$) with a carbon number of 42 would be expected to be eluted between $PA_2$ and $P_2A$; similarly, palmitostearoazelain (PSA) between $P_2A$ and $S_2A$. This is borne out by the results for cocoa butter. From the above, it is apparent that 10 glyceride types can be determined in fats containing glycerides of palmitic, stearic, and unsaturated fatty acids (oleic, linoleic, and linolenic) which give rise to azelaic acid on oxidation. Further, no peaks could be found for diglycerides on GLC, thereby showing that no hydrolysis had taken place during oxidation.

For quantitative analysis using the flame ionization detector system, it is necessary to know the relative response of the compounds under investigation. McCarthy, Kuksis, and Beveridge (16) found that peak area with medium and long chain saturated triglycerides approximately represented the weight composition, but stated that the shorter chain triglycerides, as in butter oil, would be expected to show

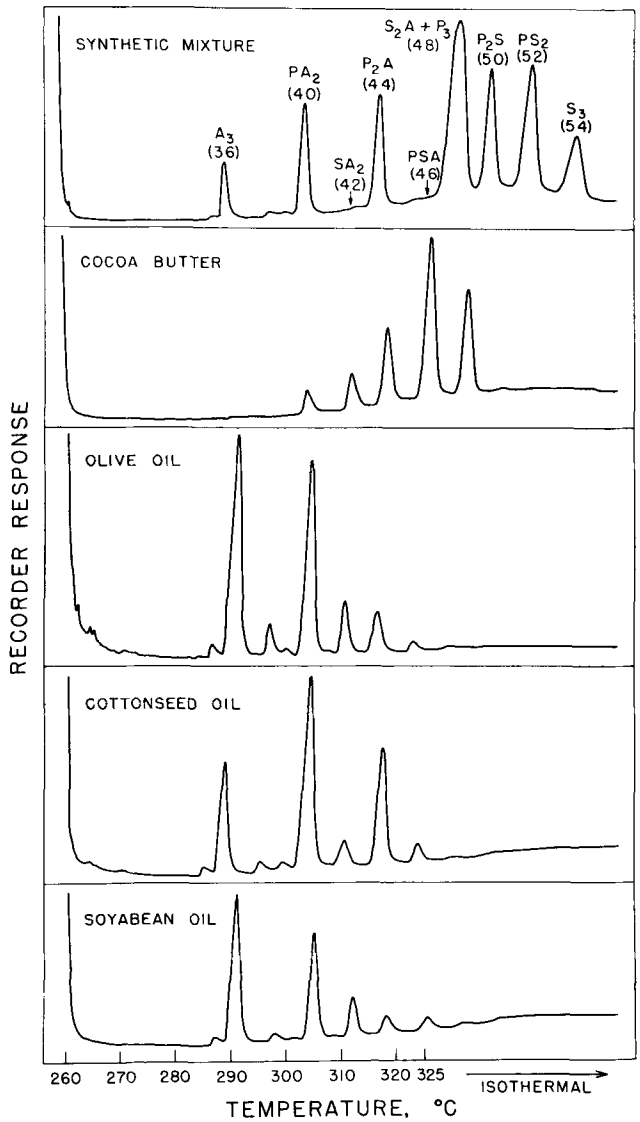


FIG. 1. GLC charts of oxidized methylated glycerides.
Column—4 ft–3/16 in. in S.S.
Packing—2% SE30 on Anakrom A.B.S.
Helium flow rate—100 ml/min.
Hydrogen flow rate—30 ml/min.
Air flow rate—400 ml/min.
Temperature program—260–325C @ 3°/min.
Inj. port temperature—385C.
Block temperature—355C.
Attenuation—800.
Chart speed—2 min/in.

lower responses per unit of weight in this detector system. McWilliam and Dewar (17) have shown that the molar response is related to the carbon number. (The carbon numbers used in quantitative calculation using the flame ionization detector system are not necessarily the same as those previously used to predict the emergence time of individual glycerides on a silicone column.) Ongkiehong (19) found that with oxygenated compounds an approximate response factor may be obtained if the carbon number is taken as the number of carbon atoms remaining after as many $CO_2$ groups as possible have been split off. Perkins et al. (21), while determining the relative response per mole of some esters in the flame ionization detector found that the relative response of esters as compared to hydrocarbons effectively diminished by one carbon atom per molecule for mono esters. Thus *n*-pentyl propionate with 8 carbon atoms had an effective number of 7 carbon atoms in the molecule.

TABLE I
Relative Response of $A_3$ and $P_3$ in Flame Ionization Detector

| | Actual wt % | Area % obtained | Actual mole % | Area % corrected for carbon number |
|---|---|---|---|---|
| $A_3$ | 51.4 | 43.5 | 56.8 | 57.8 |
| $P_3$ | 48.6 | 56.5 | 43.2 | 42.2 |

In the present investigation, a mixture of purified $A_3$ and $P_3$ was separated by GLC, and the relative response was calculated by both the above methods (Table I). Results calculated on the basis of area percentage corresponding to weight percentage showed poor agreement, while those calculated after correcting area percentage on the basis of carbon number, showed good agreement with the actual mole percentage. The carbon numbers used were obtained as follows. Trimethyl triazelain ($A_3$), having 6 ester groups and 33 actual carbon atoms, will have effective carbon atoms of 27; tripalmitin ($P_3$), which has 3 ester groups and 51 actual carbon atoms, will have an effective number of carbon atoms of 48. Therefore, in all subsequent calculations, peak areas were divided by the appropriate effective carbon numbers to give mole percentages.

Next, attempts were made to analyse a mixture of $P_3$ and $S_3$. $P_3$ started emerging at the maximum temp used (325C). $S_3$ emerged after an additional 7 min at this temp. The amount of $S_3$ determined by this procedure was always lower than the amounts taken. In another run, programming was continued beyond 325C at 3°/min until $S_3$ was eluted at 340C. Even then there was a low recovery of $S_3$. However, when $S_3$ was added to oxidized cocoa butter and run, quantitative recovery of $S_3$ was obtained. As the amount of $S_3$ in most natural fats is very small, this error in the quantitative determination of $S_3$ is negligible.

Using the procedure described above, glyceride compositions of cocoa butter, cottonseed, olive, and soybean oils were determined (Fig. 1), and the results summarized in Table II. Cocoa butter contains predominantly disaturated glycerides. GLC analysis has given distinct peaks for $P_2U$, PSU, and $S_2U$ giving values of 19.1,39.9, and 22.7% respectively. Meara (18) had worked out the composition by a crystallization procedure, giving values of 4,57 and 22 for the above glycerides. These values are at variance with regard to $P_2U$ and PSU and agree in the case of $S_2U$. Olive and soybean oils have predominantly triunsaturated glycerides with some $PU_2$, while in the case of cottonseed oil, there is more $PU_2$ than $U_3$. Fatty acid compositions of the fats obtained by GLC analysis agree very well with those calculated from the glyceride compositions; see Table III. This close agreement between calculated and observed values also indicates the general accuracy of the use of carbon numbers with respect to relative response per mole, when using the flame ionization detector system.

To differentiate UUS from USU, and SSU from SUS (U, unsaturated; S, saturated), analysis of these fats was also carried out by the method of Youngs (32) with slight modifications as outlined in the experimental section. Results show in Table IV. It can be seen that results obtained by the present GLC method and those of Youngs' method agree very well to within ±1%. In Youngs' method the oxidized glycerides were separated into two groups, one containing $GS_3$ and $GS_2U$, and the other $GSU_2$ and $GU_3$. After esterification, they were analysed separately by GLC and the fractions contained only the expected

TABLE II

Analysis of Triglycerides by GLC
Mole %

| | $U_3$ | $PU_2$ | $SU_2$ | $P_2U$ | PSU | $S_2U$ | $P_3$ | $SP_2$ | $S_2P$ | $S_3$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Cocoa butter | 0.8 | 6.2 | 8.8 | 19.1 | 39.9 | 22.7 | ..... | 1.4 | 1.1 | ..... |
| Olive oil | 59.6 | 29.8 | 5.8 | 3.8 | 1.0 | ..... | trace | ..... | ..... | ..... |
| Cottonseed oil | 29.1 | 42.4 | 6.5 | 18.0 | 3.0 | ..... | 1.0 | ..... | ..... | ..... |
| Soybean oil | 55.5 | 28.0 | 9.2 | 4.8 | 2.3 | 0.2 | ..... | ..... | ..... | ..... |

glycerides, which showed the effectiveness of the partition chromatographic separation. The individual composition of the two fractions when added together agreed well with the composition determined on total glycerides.

The results obtained in the present investigation by these two methods are compared with those previously reported, (Table V) in terms of $GS_3$,$GS_2U$, $GSU_2$, and $GU_3$. In the case of cocoa butter values obtained by the GLC method agree closely with those of Meara (18), Jones and Hammond (12) and Privett and Blank (23), and also with that of Kartha's hypothesis (13). Olive oil containing nearly 60% triunsaturated glycerides had no $GS_3$. This is in agreement with the results of Privett and Blank (23) and with Kartha's hypothesis (13). The values given by Hilditch and Maddison (7) show more of $GSU_2$ than $GU_3$. In the case of cottonseed oil, $GU_3$ is lower than calculated by Kartha's hypothesis (13), by about 15%, while $GS_2U$ and $GSU_2$ are slightly higher. Hilditch and Maddison (6) reported 58.4% $GSU_2$. The countercurrent method of determination of glyceride structure of soybean oil (29) gave 57.7% $GU_3$ and the present figures agree with this. $GS_3$ was absent in soybean oil.

The present method allows a quick, convenient and accurate determination of glyceride composition of natural fats, especially in those cases where very small samples of triglycerides are available; e.g., in the case of lipids from body fluids and glycerides labelled with $C_{14}$. Although 20 mg of sample has been used for analysis, it has been found that glyceride composition can be determined with only 5 mg of sample. The complete analysis is carried out in ca. 4 hr. The present method gives the distribution of individual saturated acids within the glycerides. All the unsaturated acids are, however, estimated together in one group as azelaoglycerides. To obtain the distribution pattern of the unsaturated fatty acids as well, it would be necessary to separate the original fat into different groups on the basis of unsaturation.

TABLE III

Fatty Acid Composition of Triglycerides
Mole %

| | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | Total unsat'd |
|---|---|---|---|---|---|---|---|
| Cocoa butter | | | | | | | |
| Calc.[a] | 29.1 | .... | 32.8 | ..... | ..... | .... | 38.1 |
| Original[b] | 28.1 | .... | 32.9 | 35.3 | 3.0 | .... | 38.3 |
| Olive oil | | | | | | | |
| Calc.[a] | 12.7 | .... | 2.3 | ..... | ..... | .... | 85.0 |
| Original | 12.6 | 1.1 | 2.6 | 76.3 | 6.8 | .... | 84.2 |
| Cottonseed oil | | | | | | | |
| Calc.[a] | 28.5 | .... | 2.8 | ..... | ..... | .... | 67.7 |
| Original[c] | 29.8 | 0.8 | 2.4 | 16.6 | 49.4 | .... | 66.8 |
| Soybean oil | | | | | | | |
| Calc.[a] | 13.3 | .... | 4.0 | ..... | ..... | .... | 82.7 |
| Original | 12.2 | .... | 4.4 | 26.1 | 50.1 | 7.2 | 83.4 |

[a] Values calculated from Table II.
[b] Also 20:0–0.7%.
[c] Also 14:0–1.0%.

TABLE IV

Analysis of Triglycerides by Youngs' Method
Mole %

| | UUU | UUS | USU | SSU | SUS | SSS |
|---|---|---|---|---|---|---|
| Cocoa butter | 1.7 | 14.8 | 1.9 | 14.0 | 67.6 | .... |
| Olive oil | 59.6 | 34.6 | .... | ..... | 5.8 | .... |
| Cottonseed oil | 29.6 | 49.2 | .... | 5.2 | 14.0 | 2.0 |
| Soybean oil | 55.7 | 35.5 | 2.8 | 1.4 | 4.6 | .... |

TABLE V

Comparison of Analysis of Triglycerides
Mole %

| | Ref. | S | $GS_3$ | $GS_2U$ | $GSU_2$ | $GU_3$ |
|---|---|---|---|---|---|---|
| Cocoa butter | 18 | 61 | 2 | 77 | 21 | 1 |
| | 12 | 54.7 | 0.9 | 83.9 | 15.2 | ..... |
| | 23 | 57.2 | 2 | 81 | 17 | ..... |
| | 13 | 61 | 2 | 80 | 17 | 1 |
| | *30* | 59.9 | 7.1 | 67.5 | 23.3 | 2.1 |
| | Youngs' method | 61.9 | .... | 81.6 | 16.7 | 1.7 |
| | Present method | 61.9 | 2.5 | 81.7 | 15.0 | 0.8 |
| Olive oil | 9 | ..... | .... | ..... | 45.3 | 54.7 |
| | 7 | ..... | .... | ..... | 61.4 | 38.6 |
| | 13 | 15 | .... | 6 | 32 | 62 |
| | 23 | 11.6 | .... | 4.6 | 33.5 | 62.1 |
| | Youngs' method | 15.2 | .... | 5.8 | 34.6 | 59.6 |
| | Present method | 15.2 | .... | 4.8 | 35.6 | 59.6 |
| Cottonseed oil | 6 | 28 | .... | 13.2 | 58.4 | 28.4 |
| | 13 | 31 | .... | 25 | 44 | 31 |
| | Youngs' method | 32.3 | 2.0 | 19.2 | 49.2 | 29.6 |
| | Present method | 32.3 | 1.0 | 21.0 | 48.9 | 29.1 |
| Soybean oil | 8 | 17 | .... | 2 | 55 | 43 |
| | 29 | 13.7 | not determined | | | 57.7 |
| | 30 | 12.8 | .... | 3.7 | 31.0 | 65.3 |
| | Youngs' method | 16.5 | .... | 5.9 | 38.3 | 55.7 |
| | Present method | 16.5 | .... | 7.3 | 37.2 | 55.5 |

Separation of the triglycerides by the method of DeVries (2) followed by analysis of different groups by the present method should give the distribution of both saturated and unsaturated fatty acids. Such a procedure is being investigated in this laboratory.

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