A new method for the analysis of component mono-, di-, and triglycerides

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SUMMARY

A new micromethod is described for the determination of component mono-, di-, and triglycerides. The basic procedure involves ozonization of the double bonds and catalytic reduction of the ozonides followed by separation and quantification of the glyceryl residues by thin-layer chromatography. The potentialities of the method are demonstrated by the analysis of soybean oil and lard as well as standard mixtures of synthetic mono-, di-, and triglycerides. Procedures for the analysis of the four monoglyceride types, six of the seven possible diglyceride types, and four of the six possible triglyceride types are demonstrated.

Present methods for the determination of component triglycerides involve separation of the fat into fractions of simple composition. The analysis of the constituent fatty acids of these fractions forms the basis for calculating component triglyceride composition. Hilditch and Lea (1) and Kartha (2, 3) carried out the fractionation after oxidation with permanganate. The separation also has been accomplished directly by fractional crystallization (4 to 7) and countercurrent distribution methods (8 to 11). Lipase hydrolysis has been used to obtain information on glyceride structure (12, 13), and may be used in conjunction with the latter methods to provide more detailed information on glyceride composition.

At a recent meeting of the American Oil Chemists' Society two other methods for the determination of glyceride structure were reported. One employed thermal gradient crystallization (14), and the other, an oxidation procedure in which the oxidized fat was separated into two fractions and analyzed by lipase hydrolysis and gas-liquid chromatography (15).

Methods for the determination of mono- and diglycerides are many and varied. However, except for the determination of 1- and 2-mono- and diglycerides (16, 17, 18), these methods do not normally distinguish between the different types of these compounds.

Described here is a general procedure which may be applied on a microscale to the analyses of the individual types of mono-, di-, and triglycerides. The method involves the quantitative fission of the double bonds to yield aldehydes, followed by analysis of the glyceryl residues by the elegant technique of thin-layer chromatography applied to lipids by Mangold and Malins (19) and Mangold (20), and will be referred to in the text of this paper as the ozonization-reduction-thin-layer chromatographic method of analysis.

MATERIALS

The following reference compounds were prepared in highly purified form: 1-monoolein (m.p. 34.9°-35.2°), 1,3-diolein (m.p. 25.0°-25.8°), triolein (I.V. 85.7; Sap. Val. 189.9), 1-monopalmitin (m.p. 76.5°-77°), 1,3-dipalmitin (m.p. 69.8°-70.2°), tripalmitin (m.p. 64.9°-65.1°; Sap. Val. 208.8°), glyceryl-2-oleate-1,3-dipalmitate (I.V. 30.2°; m.p. 34.5°-34.8°), and glyceryl-1-palmitate-2,3-dioleate (I.V. 58.6°; m.p. 19.8°-20.0°). In addition to these, 1,3-distearin (m.p. 79.5°-79.9°) and 1,2-distearin (m.p. 69.5°-70.0°) were obtained from A. de Freitas, of the Department of Biochemistry, University of Minnesota, and 2-monopalmitin was obtained from Dr. F. H. Mattson, of the Procter and Gamble Company, Cincinnati, Ohio. The distearins contained only traces of impurities. The sample of 2-monopalmitin contained 9.9% of the 1-isomer as an impurity, as determined by periodate analysis (21).

The 1-mono- and diglycerides were synthesized by the method of Fischer (22). The crude products were recrystallized several times and then chromatographed.

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on a silicic-acid column (17) until they were homogeneous as determined by thin-layer chromatography (19, 20). Periodate analysis (21) indicated they were pure 1-monoglycerides.

The 1,3-diglycerides were prepared by the trityl synthesis (23, 24) and purified in a similar manner.

A mixed unsaturated-saturated diglyceride also was prepared by reacting oleyl chloride (prepared by reacting oleic acid with oxa!yl chloride, and purified by distillation) with 1-monopalmitin by the Hartman procedure (25). Impurities were removed by silicic-acid column chromatography (17). This preparation (m.p. 37.5°-38.5°) consisted of one major component (84.4%) and one minor component (15.6%) by thin-layer chromatographic analysis. Presumably the major component was glyceryl-1-palmitate-3-oleate and the minor component glyceryl-1-palmitate-2-oleate (25).

Tripalmitin and tripalmitin were prepared by transesterification of the corresponding pure methyl esters with triaecetin, using sodium methoxide as a catalyst (26). Most of the excess of methyl ester and monoglyceride impurities was removed from the crude triglyceride by several extractions of a Skellysolve F solution of the product with 90% ethanol. The bulk of the diglyceride impurity was removed by low temperature fractional crystallization. Traces of impurities still remaining were separated by silicic-acid column chromatography (17).

The preparation of glyceryl-2-oleate-1,3-dipalmitate and glyceryl-1-palmitate-2,3-dioleate was carried out by reaction of oleyl chloride with 1,3-dipalmitin and 1-monopalmitin, respectively. This reaction was carried out according to the procedure described by Hartman (25), except that the chloroform solution of the reactants was refluxed vigorously for 6 hours, during which the chloroform was removed gradually by distillation. The crude products were purified by silicic-acid column chromatography (17) until they were homogeneous by thin-layer chromatography.

Alkali-refined soybean oil and a commercial sample of prime steam lard were obtained from Archer-Daniels-Midland Co., Minneapolis, and Geo. A. Hormel & Co., Austin, Minnesota, respectively. The minor constituents of these fats were removed by adsorption on a column of silicic acid.

**GENERAL PROCEDURE**

A small amount of sample is dissolved in about 30 ml of methylene chloride and ozonized at −60° to −70°, with a mixture of about 3% ozone in oxygen generated in a laboratory ozonizer similar to that described by Bonner (27). The mixture of oxygen and ozone is bubbled through the solution at about 100 ml per minute. Under these conditions the ozonization is complete in about 5 minutes. The reaction is stopped when the solution turns faintly blue, and pure nitrogen is bubbled through the solution to remove all the dissolved oxygen and ozone. Following this, hydrogen is bubbled through the solution at room temperature and maintained at a slight positive pressure by using a small bore outlet tube. About 50 mg of Lindlar catalyst (28) is added to the reaction mixture; stirring is effected by means of a magnetic stirrer.

After allowing about 30 minutes to complete the reduction of the ozonides, the catalyst is removed by filtration through a sintered glass funnel, and washed 2 or 3 times with 5 ml amounts of methylene chloride, which are added to the filtrate. With very small samples the volume of the filtrate is reduced to about 0.5 ml by evaporation under reduced pressure at room temperature, otherwise the solution may be analyzed directly.

A volume of the solution containing about 50 μg of glyceryl residues is spotted with a microsyringe on the base of one or more glass plates (2″ × 8″) having a thin layer of silica gel G prepared according to Stahl (29, 30). The plates are placed in a small glass jar containing the appropriate solvent system, and developed in ascending manner. Usually a developing time of only 10 to 20 minutes is sufficient to provide a separation of the components. After the plates are developed they are dried at room temperature, sprayed with 50% aqueous sulfuric acid, and heated on a hot plate to locate the position of the spots (31).

The spots were measured with a densitometer (Photovolt Corporation, 52-C and 521A) with a stage attached to it for semiautomatic plotting of curves. The slit size of the densitometer was 1 × 5 mm and readings were taken at each millimeter of travel over the length of the plate. No filter was used. Usually there was only a slight difference in the background density from one end of the plate to the other; it varied linearly over the plate and had no effect on the accuracy of the measurements.

The areas under the densitometer curves were found to be directly proportional to the amount of sample for the saturated triglycerides and the glyceryl residues of the unsaturated triglycerides (Fig. 1). However, the area given by compounds of one type of structure may not be the same as that given by the same amount of compounds containing other structures, even though the carbon densities are essentially the same. This is demonstrated in Figure 1 from a comparison of the curves given by tripalmitin, tripalmitin, and the glyceryl residue of tripalmitin obtained by the
ozonolysis and reduction procedure. The significance of this observation is that standard curves must be prepared for the analysis of compounds that do not give spots of the same densitometrically-determined response. Since the standard curve given by the glyceryl residue obtained from triolein (after correction for carbon lost by fission of the double bonds) and tripalmitin were essentially identical, it was not necessary to prepare standard curves for the analysis of mixtures of triglycerides.

At present we have no explanation for the above observations but, under the conditions employed, unsaturation had a pronounced effect on the size and intensity of the spots developed on the plates.

Analysis of monoglycerides. There are four possible types of monoglycerides, viz.:

U E E S E

U and S in these models denote saturated and unsaturated fatty acid constituents. Alpha, i.e. (1-isomer) and beta, i.e. (2-isomer) monoglycerides can be determined by application of periodate analysis before and after perchloric acid isomerization (16). The relative proportions of the 1- and the 2-types also may be determined by applying silicic-acid column chromatographic analysis before and after oxidation with periodic acid (17), and by gas-liquid chromatography according to the procedure described by McInnes et al. (18).

Although compounds with small differences in polarity can be separated by thin-layer chromatography (19, 20), α- and β-monoglycerides cannot be separated from each other by direct application of this technique. However, all four monoglyceride types may be determined by applying the analysis described here in conjunction with periodate oxidation. The general procedure is to conduct a periodate oxidation on an aliquot of the methylene chloride solution of the sample. The α-monoglycerides are oxidized to the corresponding glycol aldehyde esters by this reagent; the β-monoglycerides remain unchanged (17). This reaction may be carried out by any one of several procedures (17, 18, 32, 33). In this investigation the procedure for the analytical determination of α-monoglycerides was used (21). After the reaction was completed the solution was washed with distilled water, dried, and analyzed by thin-layer chromatography. The separation of 2-monopalmitin and glycol aldehyde palmitate obtained from 1-monopalmitin is shown in Figure 2 as an example of this analysis.

The relative proportions of unsaturated and saturated β-monoglycerides may be determined by application of the ozonization-reduction-thin-layer chromatographic procedure to this solution. The determination of unsaturated and saturated monoglycerides by this method is demonstrated in Figure 3 by the analysis of a mixture of 1-monoolein and 1-monopalmitin.
mitin. The analysis of unsaturated and saturated $\beta$-monoglycerides would give similar results.

It may be noted that an analysis of the fatty acids of the glycol aldehyde esters and the 2-monoglyceride fractions recovered from a duplicate plate of the chromatographed periodic acid-oxidized fraction (Fig. 2) also would permit a complete characterization of each monoglyceride type.

Analysis of Diglycerides. These compounds may exist in seven different types, viz.:

\[
\begin{array}{cccccccc}
  & S & S & S & E & E & E & E \\
 I & I & I & I & S & S & S & S \\
 II & U & U & U & S & S & S & S \\
 III & E & E & E & S & S & S & S \\
 IV & S & S & S & S & S & S & S \\
 V & E & E & E & E & E & E & E \\
 VI & U & U & U & U & U & U & U \\
 VII & E & E & E & E & E & E & E \\
\end{array}
\]

$S$ and $U$ in these models also denote saturated and unsaturated constituents, respectively.

Application of the ozonization-reduction-thin-layer chromatographic method of analysis to these compounds gives a series of products that differ greatly in their polarity. The glyceryl residue of the unsaturated diglycerides (II and VI) are the most polar. The fully saturated diglycerides (I and V) remain unchanged and are the least polar; the mixed types (III, IV, and VII) have polarities intermediate between the fully-saturated types and the glyceryl residue of the fully unsaturated types.

The saturated diglyceride types (I and V) were analyzed by thin-layer chromatography with a solvent system consisting of 40% ethyl ether in Skellysolve F. With this solvent system the glyceryl residues from diglycerides containing unsaturated fatty acids remain at the base of the plates, and the simple aldehydes obtained from the fission of the double bonds migrate with the solvent front. The component saturated diglycerides (types I and V) may be analyzed with this solvent system, as demonstrated by the separation of 1,2-distearin from 1,3-distearin (Fig. 4).

The glyceryl residues from the unsaturated diglycerides may be analyzed by thin-layer chromatography with 35% Skellysolve F in ethyl ether. With this solvent system the saturated diglycerides and the aldehydes produced through fission of the double bonds migrate with the solvent front. Figure 5 shows the thin-layer chromatography of the glyceryl residues obtained from 1,3-diolein, glyceryl-1-palmitate-2-oleate, and glyceryl-1-palmitate-3-oleate representing the separation of diglyceride types VI, III, and VII, respectively.

Positional isomers like types III and VII have different polarities; the former, having a primary hydroxyl, is more polar than the latter, which has a secondary hydroxyl. These may be separated directly by thin-layer chromatography with the same solvent system used to separate 1,2-distearin from 1,3-distearin (Fig. 4). However, in order to avoid interference with the analysis of the fully saturated diglyceride types, the unsaturated linkages are converted to aldehydes by ozonization and reduction. The separation of the glyceryl residues of these types (III and VII) (Fig. 5) was not as good as the separation of 1,2- and 1,3-
distearin, but very likely this could be improved by the use of a slightly different solvent composition or by increasing the distance of the migration of the spots on the chromatographic plate, or both.

A sample of type II was not available for analysis but, in view of the difference in polarity between diglycerides containing a secondary and a primary hydroxyl group, no difficulty in separating type II from type VI was anticipated. Since type II would be more polar than type VI, it would have a lower $R_f$ value and would migrate slower under the conditions of analysis used for the separations in Figure 5.

Types III and IV would have essentially the same polarity, regardless of whether or not they were ozonized and reduced, because each has one primary hydroxyl group and one unsaturated fatty acid constituent. Thus separation of these types from each other would not be possible. Nevertheless, it is evident that these types together can be separated from the other five types, giving, all told, the potentiality of analyzing six of the seven diglyceride types by the technique described.

Analysis of Triglycerides. Figures 6 and 7 show the results of the application of the ozonization-reduction-TLC method of analysis to a mixture of triglycerides of known composition consisting of tripalmitin ($G_S^3$), glyceryl-1-palmitate-2,3-dioleate ($G_{S1}U_2$), glyceryl-2,3-dioleate-1,3-dipalmitate ($G_{S2}U_1$), and triolein ($G_U^3$). The glyceryl residues of the $G_S^3$, $G_{S1}S_1$, and the $G_{S1}S_2$ were developed on one plate (Fig. 6) with a mixed solvent of 35% ethyl ether in Skellysolve F. The $G_S^3$ and aldehydes fissioned from the constituent unsaturated fatty acids migrated with the solvent front with this solvent system. The $G_S^3$ and the glyceryl residue of the $G_{S1}S_2$ were developed on another plate (Fig. 7) with 15% ethyl ether in Skellysolve F. The glyceryl residues of the $G_S^3$ and the $G_{S1}S_2$ did not move from the base line with this solvent system.
than 2 percentage units from the individual analyses and from the known composition.

### TABLE 1. ANALYSIS OF A MODEL MIXTURE OF TRIGLYCERIDES

<table>
<thead>
<tr>
<th>Triglyceride Component</th>
<th>Type</th>
<th>Per Cent Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found</td>
</tr>
<tr>
<td>Tripalmitin</td>
<td>GS₁</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>GS₂U₁</td>
<td>24.6</td>
</tr>
<tr>
<td>Glyceryl-1,3-dipalmitate-2-oleate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceryl-1-palmitate-2,3-oleate</td>
<td>GS₂S₂</td>
<td>30.2</td>
</tr>
<tr>
<td>Triolein</td>
<td>GU₁</td>
<td>21.8</td>
</tr>
</tbody>
</table>

The analyses of lard and soybean oil are compared with values obtained by others, in Table 2. Some divergence in analysis may be expected because of natural variations in the composition of these oils. Since our results are in the same range of values found by others, they demonstrate that natural fats and oils such as these can be analyzed by this method.

### TABLE 2. ANALYSIS OF THE TRIGLYCERIDE TYPES OF LARD AND SOYBEAN OIL

<table>
<thead>
<tr>
<th>Triglyceride Type</th>
<th>Lard</th>
<th>Soybean Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Literature Values</td>
</tr>
<tr>
<td></td>
<td>per cent</td>
<td>(4)</td>
</tr>
<tr>
<td>GS₁</td>
<td>6.7</td>
<td>2.4</td>
</tr>
<tr>
<td>GU₃S₂</td>
<td>34.6</td>
<td>28.0</td>
</tr>
<tr>
<td>GU₃S₁</td>
<td>42.7</td>
<td>40.1</td>
</tr>
<tr>
<td>GU₁</td>
<td>16.0</td>
<td>29.5</td>
</tr>
</tbody>
</table>

### DISCUSSION

One of the most important basic principles involved in this method is that the reduction of the ozonides to aldehydes by the Lindlar catalyst (lead-poisoned palladium) is quantitative; other common catalysts, platinum and palladium, for example, give mixtures of alcohols and aldehydes. Since many of the separations are based on relatively fine differences in polarity, production of a mixture of products would be undesirable.

Column chromatographic methods of analysis such as those described by Borgström (17) and Hirsch and Ahrens (35) can also be used for the analysis of the products of ozonization-reduction instead of thin-layer chromatography. Our initial studies were carried out with silicic-acid columns, and quantification was comparable to that obtained with thin-layer silica gel chromatography. However, column chromatographic methods become unwieldy and very time-consuming for the analysis of multiple samples.

The possibility of transesterification, isomerization, or other spurious reactions occurring during the various stages of the procedure were fairly well eliminated by the clear-cut separations and the lack of streaking of the pure standards and mixtures thereof on the chromatographic plates. Furthermore, identical results were obtained on rechromatographing material recovered from duplicate plates. Recovery of fractions that migrate with the solvent front, for example, for reanalysis with another solvent system is common practice.

Borgström (17) found that 2-monoglycerides were partially isomerized to the 1-isomers during passage through a column of silicic acid, although di- and triglycerides remained unchanged. His report and our inability to separate α- and β-monoglycerides by thin-layer chromatography gave us reason to suspect that perhaps some change occurred during the thin-layer chromatography of these compounds. However, no α-isomer could be detected (21) in the sample of 2-monopalmitin (oxidized with periodic acid to remove the 1-isomer originally present) recovered from a chromatographic plate after being subjected to a normal chromatographic analysis similar to that demonstrated in Figure 2. Thus it seems unlikely that there is any alteration of the structure of monoglycerides during thin-layer chromatography. The inability to separate α- and β-monoglycerides indicates that these compounds have essentially the same degree of polarity. The observation that they have the same partition coefficient (36) is further evidence to this effect. Although the β-isomer contains two primary hydroxyl, and presumably would be more polar than the α-isomer, which contains one primary and one secondary hydroxyl, this is probably nullified, according to Dr.

L. J. Morris, by the effect of hydrogen bonding, as demonstrated below:

\[
\text{\(\alpha\)-Monoglyceride}
\]

\[
\text{\(\beta\)-Monoglyceride}
\]

It may be seen from these structures that each compound has only one free primary hydroxyl group. The bound hydroxyl groups obviously contribute to the polarity of these compounds, but apparently in the bound state the primary and secondary hydroxyl groups do not differ much in their contribution to the total polarity.

At present the method has not been extended beyond the analysis of component mono-, di-, and triglycerides of simple fatty acid composition. However, since a host of fats and oils fall into this category, the method should be valuable to many studies of lipid metabolism and plant lipid chemistry, especially since it offers features not common to other procedures. These may be enumerated as follows: (a) It can be carried out on a microscale with an accuracy and precision comparable to existing methods; (b) it is based on the separation and analysis of single components, an important feature for further characterization of individual compounds, especially those containing labeled atoms; (c) it is relatively fast and simple; (d) it permits the estimation of four of the six possible triglyceride types, six of the seven possible diglyceride types, and the four possible monoglyceride types, analyses not now readily performed.

For more general application, the method should be extended to include the determination of the isomers of \(\text{GU_2S_1}\) and \(\text{GU_3S_0}\), and to glycerides varying widely in their fatty acid constituents, both with regard to chain length and degree of unsaturation. It also would be desirable to combine the above procedure with methodology for the determination of the constituent fatty acids of the separated components. Further development of the method along these lines is in progress.

The authors wish to express their thanks to Mr. Anthony de Freitas, of the Department of Agricultural Biochemistry, University of Minnesota, for the samples of 1,2- and 1,3-distearin, and to Dr. F. H. Mattson, of the Procter and Gamble Company, Cincinnati, Ohio, for the sample of 2-monopalmitin.

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**References**