

A Rapid and Quantitative Procedure for the Preparation of Methyl Esters of Butteroil and Other Fats¹

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Abstract

A simple and convenient method for the quantitative preparation of methyl esters of fatty acids from glyceride fats and oils is described. The procedure, using potassium methylate as catalyst and a heating interval of 2 min at 65°C in a closed vial, is applicable to fats containing both low and high molecular weight fatty acids such as butteroil. The methyl esters of samples ranging from a few mg to 30 mg are isolated by CS₂ extraction and a TLC technique.

A similar procedure using sulfuric acid in methanol as catalyst is described for the conversion of free fatty acids to methyl esters.

For the routine analysis by GLC of fats and oils such as lard, tallow, soybean, cottonseed oil or butteroil, no isolation of the methyl ester is required. A CS₂ extraction carried out in the reaction vial allows the GLC analysis immediately after the reaction period (2 min).

Introduction

SINCE THE ADVENT of gas-liquid chromatography (GLC) for the analysis of fatty acids, we have been concerned with the conversion of glycerides and other lipids to their fatty acid methyl esters. In the last several years a substantial number of procedures have been published for this conversion (1-3,7,11,12,14,15) but many of these are specialized techniques requiring elaborate equipment and uncommon reagents. In a previous paper (5) we reported conditions for the direct conversion of lipids to their fatty acid methyl esters. However, the procedure is unsuitable for fats such as butteroil which contain lower molecular weight fatty acids. Losses of these acids occur in the refluxing of the reaction mixture, on the solvent extraction of the esters from aqueous phases, and in the removal of solvent. The procedure was more rapid than the classical methods but the manipulations involved made it difficult to adapt to routine analyses of a large number of samples.

To overcome these limitations, a new procedure was devised for the preparation of methyl esters of fatty acids from fats whose composition included both low and high molecular weight acids. The method is rapid, simple, and quantitative and eliminates many error-prone manipulations. It is particularly suitable for the routine GLC analysis of fats where a large number of samples are to be analyzed. The procedure has several variations depending on the nature of the fat sample to be esterified.

Experimental

Preparation of Methyl Esters—All Oils Including Butteroil

Method I. Alkaline Catalyzed Reaction. From 1 to 30 mg of an oil is weighed into a small screw-top

vial (A. H. Thomas No. 9802—15 × 45 mm). In many cases the exact weight of the oil is not required and 2 drops of the oil from a disposable pipette approximates 25 mg. To the sample is added 0.25 ml of 0.4 N potassium methylate in anhydrous methanol (5); in practice, this is 10 to 12 drops of reagent. The vial is flushed with nitrogen, and the cap, with a tinfoil or Teflon liner, is securely applied. The vial is immersed in a water bath at 65°C to a depth of ½ in. and is shaken vigorously for 30 sec. The mixture of fat and reagent is now homogeneous and the conversion is essentially complete.

The heating is continued without shaking for an additional 1½ min. The vial is removed from the bath and cooled to room temperature or lower. It is opened and 0.6 g of a 50/50 mixture of silica gel and calcium chloride is quickly added and stirred thoroughly with a small stainless steel stirring rod. Three milliliters of CS₂ is added, the cap is replaced, and the vial is shaken for 1-2 min either by hand or in a mechanical shaker. We have found a dentist's amalgamator ("Wig L Bug" model 5-a, Crescent Dental Mfg. Co., Chicago, Ill.) modified to hold the small vial to be very useful but any automated shaker could be used.

After the shaking, the vial is placed in a small centrifuge and spun for 1-2 min at 1200 rpm which is sufficient to settle the adsorbent leaving a clear layer of CS₂. For butteroil or other oils containing lower molecular weight acids, an aliquot of the CS₂ can be taken by syringe and injected directly into a chromatograph equipped with a flame ionization detector. Thus there is little possibility for loss of volatile constituents. For other oils, the CS₂ layer may be withdrawn and evaporated under nitrogen, allowing the methyl esters to be isolated.

In most cases the total time for the preparation of the methyl esters is less than 6 min. An alternate procedure for the isolation of methyl esters of the higher weight acids is described later in the text under TLC.

Preparation of Methyl Esters—Oils with High Free Fatty Acid Content (10-50%)

Method II. For oils containing a high content of free fatty acids, a modification of the above procedure is required since the treatment of acids in methanol with alkaline catalysts does not yield ester formation to any appreciable extent. The sample, 1 to 30 mg in the small vial, is first treated with the potassium methylate catalyst including the 2-min heating period and later cooling. At this point the vial is opened and the acidic catalyst, 0.3 ml or 10 drops of boron trifluoride (10% in methanol) (Eastman Organic Chemical No. 3706, Rochester, N.Y.) (8,9,15), is added. The vial is recapped and returned to the water bath for an additional 2 min of heating. The isolation of the methyl esters hereafter follows the details of Method I.

¹ Presented at the AOC Meeting, Philadelphia, October 1966.

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Preparation of Methyl Esters—Fatty Acids

Method III. The conversion of fatty acids to methyl esters is accomplished by a slightly different procedure than that for the glyceride oils. To the sample, 1 to 30 mg in the vial is added 0.1 ml or 3 drops of benzene. The vial and contents are warmed slightly to aid in solubilizing the acids. The reagent, 0.3 ml or 10 drops of 4% sulfuric acid in methanol, is added and the vial is tightly capped and immersed in the water bath at 65°C. The procedure from this point is now identical with that for the glycerides as in Method I.

Thin-Layer Chromatography

Thin-layer chromatographic plates (20 × 20 cm) were coated singly by spreading a mixture of 7 g of acetone washed Silica Gel G (E. Merck, Desaga, Heidelberg, Germany) and 17 ml of distilled water with a spreader designed to give a layer thickness of ca. 275 microns. The plates, after brief air drying, were activated by heating in an oven at 105°C for 2 hr and cooled in a desiccator. The procedure for the isolation of purified methyl esters by TLC is as follows: The CS₂ layer from Methods I, II or III is withdrawn with a disposable pipette and added dropwise on the silica gel plate about 2 cm from the bottom edge. The rapid addition of solvent to the plate produces a sample band of ca. 3 cm in width. It is recommended that the addition of the CS₂-rich sample be carried out in a well-ventilated fume hood. The plate is developed in an ascending manner using petroleum ether, ethyl ether, acetic acid 85:15:1 as the developing solvent. All solvents were redistilled before use. At the end of a 10-min development time the plate is removed from the tank and the residual solvent is allowed to evaporate. The methyl ester zone is readily detected by viewing the plate in diffuse light. When very small amounts of sample are chromatographed and the zone is indistinct, it may be visualized by placing the plate in a tank containing iodine vapor for a few seconds. The band is marked, the area scrapped from the plate, and the silica gel collected. The methyl esters are readily isolated from the silica gel by extraction with ethyl ether.

All of the reaction products from Methods I, II and III were evaluated by TLC for completeness of the conversion. Aliquots of the reaction mixtures were taken immediately after the heating period and spotted on the TLC plates. A volume calculated to give a sample size of ca. 200 μg was selected so that any residual glycerides or fatty acids could be detected. The plate was developed in the ascending manner for a distance of 10 cm and the developing solvent was the same as previously stated.

The CS₂ extract of the methyl ester preparations was also evaluated by this TLC technique.

Gas-Liquid Chromatography

The CS₂ solutions of the extracted methyl esters were injected in an Aerograph 1522 (Varian Aerograph, Walnut Creek, Calif.) dual flame chromatograph equipped with a H₂ flame ionization detector. The column was an 8 ft × 1/8 in. O.D. stainless steel coiled tube packed with 12% stabilized diethylene glycol succinate (DEGS) on 42–60 mesh Chromosorb "W". For butteroil methyl esters, the column was heated isothermally at 100°C for the determination of the 4:0, 6:0, 8:0 and 10:0¹ esters. It was

¹ Number to left indicates carbon number; number to right indicates double bonds.

TABLE I
Fatty Acid Composition^c (wt %)

| Fatty acid | Tallow | | Soybean oil | | Cottonseed oil | |
|------------|----------------|----------------|----------------|----------------|----------------|----------------|
| | A ^a | B ^b | A ^a | B ^b | A ^a | B ^b |
| 14:0 | 5.0 | 5.2 | 0.1 | 0.1 | 0.7 | 0.8 |
| 16:0 | 24.3 | 24.0 | 11.1 | 11.1 | 19.5 | 18.0 |
| 16:1 | 2.8 | 3.0 | | | | |
| 18:0 | 25.0 | 25.2 | 3.7 | 3.5 | 1.4 | 1.9 |
| 18:1 | 40.3 | 40.0 | 24.2 | 24.8 | 16.3 | 17.3 |
| 18:2 | 1.4 | 1.5 | 53.6 | 54.8 | 61.8 | 60.7 |
| 18:3 | 0.8 | 0.8 | 7.3 | 7.8 | | |

^a Methyl esters prepared by Method I.
^b Catalyst Methanol-Sulfuric acid (10).
^c Major components.

then programmed at 4°/min to 176°C to remove the balance of the esters. The sample was also analyzed isothermally at 175°C. The areas under the peaks of individual components were determined by an electronic integrator (Infotronics Model CRS-11HSB, Infotronic Corp., Houston, Texas) coupled to a digital printer. Areas and percentages thus obtained had good agreement with mixtures of methyl esters of known composition. Esters from oils other than the butteroil were run isothermally at 175°C. Analyses were also performed with a GLC apparatus designed and assembled at this Laboratory. It was provided with a four-filament thermal conductivity cell detector. The column was an 8 ft × 1/8 in. O.D. stainless steel coiled tube packed with 25% ethylene glycol succinate (EGS) on 42–60 mesh Chromosorb "W," acid- and base-washed. The column was heated isothermally at 204°C and helium was used as the carrier gas. Peak areas were also determined by an electronic integrator.

To evaluate the effectiveness of the potassium methylate method for preparing methyl esters, a number of commercial fats were subjected to the procedure and then analyzed for fatty acid composition by GLC. For comparison, methyl esters of the same fats were prepared by the procedure reported by The American Oil Chemists' Society (10) and these were also analyzed by GLC. The fats selected for the comparative analysis were beef tallow, cottonseed oil, and soybean oil. The results are shown in Table I.

Butteroil methyl esters were prepared by Method I and analyzed by GLC. Since the AOCS procedure is not suitable for fats with fatty acids of less than 12 carbon atoms, no direct comparison of fatty acid compositions could be made. However, in Table II the fatty acid composition of a butteroil analyzed by the present procedure is compared with the average values for 106 butterfat samples isolated from pooled raw milk (4).

A commercial GLC standard of coconut oil methyl

TABLE II
Fatty Acid Composition of Butteroil (wt %)

| Fatty acid | A ^a | B ^b |
|---------------------|----------------|----------------|
| 4:0 | 4.2 | 3.6 |
| 6:0 | 1.6 | 2.2 |
| 8:0 | 1.3 | 1.2 |
| 10:0 | 2.7 | 2.5 |
| 12:0 | 3.2 | 2.8 |
| 14:0 | 10.9 | 10.1 |
| 14:1 | 1.9 | 1.6 |
| 16:0 | 27.5 | 25.0 |
| 16:1 | 2.4 | 2.6 |
| 18:0 | 12.2 | 12.1 |
| 18:1 | 25.9 | 27.1 |
| 18:2 | 2.0 | 2.4 |
| 18:3 | 1.3 | 2.1 |
| Others ^c | 2.9 | 4.7 |

^a Values from methyl esters prepared by Method I.
^b Average of 106 butterfat samples from pooled raw milk (4).
^c Includes all minor constituents, odd-numbered and branched chain fatty acids.

TABLE III
Fatty Acid Composition of Coconut Oil (wt %)

| Fatty acid | A ^a | B ^b | C ^c | D ^d |
|------------|----------------|----------------|----------------|----------------|
| 8:0 | 7.6 | 7.6 | 8.8 | 8.0 |
| 10:0 | 5.2 | 5.2 | 6.9 | 6.5 |
| 12:0 | 48.9 | 49.0 | 49.0 | 47.6 |
| 14:0 | 15.1 | 15.1 | 17.2 | 17.3 |
| 16:0 | 6.9 | 6.8 | 7.8 | 8.5 |
| 18:0 | 2.8 | 2.7 | 2.2 | 2.7 |
| 18:1 | 11.5 | 11.5 | 5.5 | 6.4 |
| 18:2 | 2.1 | 2.2 | 2.0 | 2.1 |

^a Coconut oil methyl ester standard.
^b Same esters subjected to Method I.
^c Coconut oil; methyl ester prepared by Method I.
^d Same coconut oil; collaborative study (13).

esters was analyzed and these values are compared in Table III with analysis of the same esters subjected to all of the steps of Method I. Included in this table is the comparison of the analysis of coconut oil methyl esters prepared by Method I with the median values for the same oil reported by a number of investigators in a collaborative study (13).

Table IV is a comparison of values obtained in the analysis of a lard. Composition A was determined by the direct GLC analysis of an aliquot of the CS₂ solution resulting from the extraction of the methylation reaction in Method I. Value B was obtained by the GLC analysis of the methyl esters isolated from an aliquot of the same CS₂ solution by the TLC technique. Value C resulted from analysis of the methyl esters also isolated from this CS₂ solution by evaporation of the CS₂.

A cottonseed oil was treated as in Method I and the CS₂ extract was immediately analyzed by GLC. The balance of the CS₂ solution was stored in the refrigerator at 38C for 30 days before analysis. Table V compares the values for the two CS₂ extracts.

A commercial lard was saponified and the fatty acids were recovered. A portion of these acids was converted to fatty acid methyl esters by the procedure of the recent AOCs collaborative study (10). A second portion of the acids was converted to methyl esters by Method III. Table VI shows the GLC analyses of the two methyl ester preparations.

Discussion

From previous studies on the preparation of methyl esters, it was known that the reaction under certain conditions proceeded with great rapidity. The limiting factor in the development of a simple and rapid procedure was obviously the time spent in the variety of manipulations required to isolate the product. The loss of lower molecular weight esters in extraction procedures or from volatility was also to be considered. The simplest procedure would have been to carry out the reaction and to inject some portion of the reaction mixture directly into the chromatograph. The chromatogram of the reaction mixture was found to be unsatisfactory for a quantitative

TABLE IV
Fatty Acid Composition of Lard (wt %)

| Fatty acid | A ^a | B ^b | C ^c |
|------------|----------------|----------------|----------------|
| 10:0 | 0.1 | 0.1 | 0.1 |
| 12:0 | 0.1 | 0.1 | 0.1 |
| 14:0 | 1.2 | 1.3 | 1.3 |
| 16:0 | 25.1 | 26.0 | 25.4 |
| 16:1, 17:0 | 3.5 | 3.4 | 2.8 |
| 17:1 | 0.2 | 0.2 | 0.2 |
| 18:0 | 11.8 | 10.9 | 12.3 |
| 18:1 | 46.4 | 46.2 | 46.0 |
| 18:2 | 11.1 | 11.2 | 10.8 |
| 18:3 | 0.5 | 0.8 | 1.2 |

^a GLC analyses directly on CS₂ solution.
^b Methyl esters isolated by evaporation of CS₂ solution.
^c Methyl esters isolated by TLC.

TABLE V
Fatty Acid Composition of Cottonseed Oil (wt %)
Effect of Storage in CS₂ Solution

| Fatty acid | 6 Min | 30 Days |
|------------|-------|---------|
| 14:0 | 0.7 | 0.6 |
| 16:0 | 19.5 | 19.6 |
| 16:1 | 0.4 | 0.6 |
| 18:0 | 1.4 | 1.3 |
| 18:1 | 16.3 | 16.5 |
| 18:2 | 61.8 | 61.4 |

fatty acid analysis, particularly for butteroil, since the methanol peak of the solvent obscured the peaks of the lower molecular weight esters. Isolation of the methyl esters by extraction was considered and various solvents for this purpose were investigated. CS₂ was a desirable solvent from several considerations but it produced two liquid phases with the reaction mixture and methyl esters were found to be distributed in each phase. However, the treatment of the reaction mixture with silica gel prior to extraction with CS₂ yielded a single liquid phase and permitted sampling of the CS₂ extract for direct GLC analysis. In the analysis of butteroil, the small amount of methanol dissolved in the CS₂ gave a peak which partially overlapped the methyl butyrate peak. The addition of calcium chloride to the silica gel treatment complexed enough of the methanol to substantially decrease this interference.

The comparison in Table I of the analysis of a number of fats indicates the procedure gives data fully comparable to other methods. The time advantage alone may be 20- to 25-fold per sample yet there is no significant difference in any of the compositions reported.

The fatty acid analysis of butterfat is always a difficult analysis and many of the available methods lack speed and simplicity which is particularly desirable where large numbers of samples are to be assayed. The values shown in Table II for fats of this type, while not absolute, indicate the method yields data fully equivalent to other procedures.

The free fatty acids found in unrefined glyceride oils (0.1 to 5.0%) usually reflect the fatty acids of the intact glycerides since they are produced by a relatively nonspecific type of enzymatic hydrolysis in the oil-bearing stock prior to processing. Thus their effect on the fatty acid composition of the sample is negligible but the presence of unesterified acids could produce extraneous peaks in the gas-liquid chromatograms. Method I minimizes this effect as the silica gel adsorption treatment will remove free acids to the extent of 10% of the sample weight.

Method II was designed primarily for the analysis of high acid products such as inedible tallows and greases where the free acids are to be included in the fatty acid composition.

While not shown in the tabular data, we have evaluated Method II. Known amounts of a pure

TABLE VI
Fatty Acid Composition of Lard (wt %)

| Fatty acid | A ^a | B ^b |
|------------|----------------|----------------|
| 12:0 | 0.1 | 0.1 |
| 14:0 | 1.5 | 1.4 |
| 16:0 | 28.2 | 26.9 |
| 16:1, 17:0 | 2.7 | 2.6 |
| 17:1 | 0.3 | 0.3 |
| 18:0 | 13.3 | 12.6 |
| 18:1 | 43.7 | 45.2 |
| 18:2 | 8.5 | 9.2 |
| 18:3 | 1.5 | 1.1 |

^a Methyl esters prepared by Method III.
^b Methyl esters prepared by methanol-sulfuric acid catalyst (10).

fatty acid were added to a pure synthetic glyceride. GLC analysis of the resulting esters accounted completely for the added acid while TLC examination of the product showed no sign of unconverted free acids.

When Method I was applied to a commercial GLC coconut oil methyl ester standard, the esters so treated gave an analysis identical to the untreated esters. These analyses, shown in Table III, columns A and B, demonstrate the CS₂ extraction procedure yields quantitative recovery of all esters. In the same table, columns C and D compare the analyses for a coconut oil with the median value achieved by 24 analysts, each from a different laboratory, each of whom prepared the esters by various procedures.

It is interesting to note that the values for the lard composition shown in Table IV are equivalent for each of the modifications. This means the choice of procedure may depend on the individual requirements at the time of analyses and the methyl esters need not be isolated unless required for other studies.

Table V shows that methyl esters prepared by Method I and stored in the CS₂ extractant for as long as 30 days had little effect on the composition of the fatty acids. This is an important advantage because with rapid methylation, the time controlling factor in the analyses of a large number of samples will be the chromatographic determinations. Storage

of the esters will allow the chromatographic analysis to be performed when most convenient.

For completeness of the procedure, Method III, the conversion of fatty acids to methyl esters, has been included. Table VI gives typical data obtained with this technique but the reaction has not been as extensively studied as the alkaline conversion of the glycerides. TLC evaluation of these esters did not show any unconverted free acids.

REFERENCES

1. Craig, B. M., and N. L. Murty, *JAOCS* **36**, 549-552 (1959).
2. Hyun, S. A., G. V. Vahouny and C. R. Treadwell, *Anal. Biochem.* **10**, 193-202 (1965).
3. Kishimoto, Y., and N. S. Radin, *J. Lipid Res.* **6**, 435-436 (1965).
4. Jensen, R. G., G. W. Gander and J. Sampugna, *J. Dairy Sci.* **45**, 329-331 (1962).
5. Luddy, F. E., R. A. Barford and R. W. Riemenschneider, *JAOCS* **37**, 447-451 (1960).
6. Mason, M. E., and G. R. Waller, *Anal. Chem.* **36**, 533-536 (1964).
7. McGinnis, G. W., and L. E. Dugan, Jr., *JAOCS* **42**, 305-307 (1965).
8. Metcalfe, L. D., and A. A. Schmitz, *Anal. Chem.* **33**, 363-364 (1961).
9. Metcalfe, L. D., A. A. Schmitz and J. R. Pelka, *Anal. Chem.* **38**, 514-515 (1966).
10. Report of Instrumental Techniques Committee AOCs 1964-1965, *JAOCS* **43**, 12A (1966).
11. Roper, R., and T. S. Ma, *Microchem. J.* **1**, 245-260 (1957).
12. Sato, Y., and M. Momotani, *Bunseki Kagaku* **10**, 196-198 (1961).
13. Smalley Committee Report 1967, *JAOCS*, to be published.
14. Smith, L. M., *J. Dairy Sci.* **44**, 607-622 (1961).
15. Tove, S. B., *J. Nutr.* **75**, 361-365 (1961).
16. van Wijngaarden, D., *Anal. Chem.* **39**, 843-849 (1967).