# Gas Chromatographic Determination of Free Fatty Acids in Vegetable Oils by a Modified Esterification Procedure

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## ABSTRACT

Free fatty acids in small vegetable oil samples were determined by gas liquid chromatography after a modified $BCl_3$ or $BF_3$/methanol esterification procedure with methylurea. This compound was found to sufficiently reduce triacylglyceride transesterification during free fatty acid esterification with $BCl_3$ or $BF_3$ methanol. Good results were obtained using this new procedure on neutral lipid oils containing known amounts of free fatty acids. Good agreement between the new method and the A.O.C.S. titration method was also demonstrated on commercial and laboratory-extracted crude oils. Because very small oil samples (200 mg) can be analyzed, the new method could be applicable for oilseed physiological studies.

## INTRODUCTION

The determination of free fatty acids in specially processed oils and vegetable oils by titration methods have been described (1,2). Rapid titration and spectrophotometric methods have also been developed to determine free fatty acids in common vegetable oils and oil emulsions (3,4). These procedures are routinely used by commercial oil refiners to monitor free fatty acids during oil extraction and processing, since free fatty acid content is a major index of oil quality (5). Titration procedures have also been used to determine the free fatty acid content in oilseeds for physiological studies (6,7). Only total free fatty acids can be determined by these methods since they are based on indicator color reactions which are independent of fatty acid chain length.

Gas chromatographic columns have been developed which will separate underivatized fatty acids of $C_{14}$ to $C_{20}$ chain length (8). However, repeated direct injection of crude oil on these short columns (1-3 ft.) drastically reduced column life, and therefore limit the use of this procedure for the routine analysis of free fatty acids in oils.

It has been suggested that small amounts of free fatty acids could be esterified directly with $BF_3$/methanol and determined by gas chromatography (9). However, oil containing standard amounts of free fatty acids yielded significantly higher free fatty acid values because some transesterification of oil triacylglycerides occurred. I have found that the addition of methylurea to the esterification reaction significantly reduced transesterification of oil triacylglycerides, and that by adding an internal fatty acid standard, accurate quantitative results on oil free fatty acids could be obtained. Therefore, a modified $BCl_3$/methanol esterification procedure with methylurea was developed and can be used to routinely determine free fatty acid content of small vegetable oil samples by gas liquid chromatography. The new method could be applied to physiological studies with oil seeds for evaluation of individual free fatty acids.

## MATERIALS AND METHODS

### Crude Oil and Neutral Lipid Oil Preparation

Commercial soybeans (Gold Kist, Inc.) were ground with a high speed grinder (10 mesh) and stored in air-tight jars at -15 C. Samples were extracted with petroleum ether (35-60 C, mallinckrodt, Inc.) for 5 hr (soxhlet extraction) and the solvent removed from the crude extract by reduced pressure at 30 C with a rotary evaporator. The extract was flushed with dry nitrogen, stored at -10 C for 30 min, and centrifuged at 23,400 x G for 10 min. The pellet was discarded and the crude oil stored under nitrogen at -15 C. Commercial crude cottonseed oil was also obtained from Gold Kist, Inc. and crude sunflower oil from Cargill, Inc. These oils were not further purified.

A neutral lipid oil was separated from soxhlet extracted soybean oil by silicic acid (BioSil A 100:120 mesh, Bio-Rad, Laboratories) column chromatography. Silicic acid was washed several times with chloroform to remove fines prior to packing the column. Crude oil (10 g) was applied to the column (2.5 x 52 cm) and eluted with chloroform. After the initial void volume was discarded, two void volumes (315 ml) were collected and the solvent was removed by flash evaporation. This fraction contained a neutral lipid oil which was 95% the weight of crude oil initially applied on the column. The neutral lipid oil was composed essentially of triglycerides, since phospholipids, free fatty acids, and other polar lipids were not detected by three-solvent system, two-dimensional thin layer chromatography (10). The absence of free fatty acids in this fraction was also confirmed by A.O.C.S. titration method (2).

### Standard Free Fatty Acid Oil Preparation

Palmitic, stearic, and linolenic acid standards (99.9% pure as determined from TLC and GLC) were dissolved in the neutral lipid oil so that the resulting oil samples would contain known free fatty acid content and composition. These standard oils dissolved in a small amount of hexane could be stored at -15 C for several weeks without deterioration.

### Modified Esterification Procedure and Free Fatty Acid Determination

Methylurea (520-540 mg, Eastman Organic Chemicals), oil (150-300 mg), 2.0 ml heptadecanoic acid/methanol (2.76 mg/ml, internal standard, Nu-Chek-Prep), and 3.0 ml 10% $BCl_3$ or 14% $BF_3$/methanol (Analabs, Inc.) were added to a 15 x 150 mm culture tube. The contents were stirred with a vortex mixer until methylurea had dissolved (ca. 45 sec). A small boiling chip was added, the tube capped with foil, and the mixture heated at 83 C for 5.5 min in a Temp-blok module heater. The reaction mixture was quantitatively transferred to a small separatory funnel (30 ml) with four 1.0 ml portions of hexane. The mixture was shaken and the two liquid phases allowed to separate. Saturated sodium chloride (4.0 ml) was added, the mixture shaken, and the hexane layer collected over solid anhydrous sodium sulfate. The funnel was rinsed with hexane (2.0 ml) and the combined extracts filtered through Whatman No. 4 filter paper. The methyl ester volume was adjusted to 2-3 ml with a slow stream of dry nitrogen prior to chromatography. The quantitation of individual free fatty acids was determined by gas liquid chromatography (GLC) using heptadecanoic acid as internal standard.

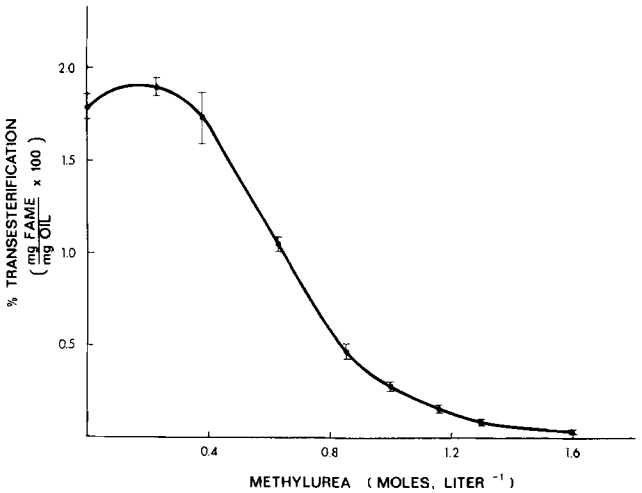


FIG. 1. The reduction of soybean neutral lipid oil transesterification by methylurea during reaction with $BV1_3$/methanol.

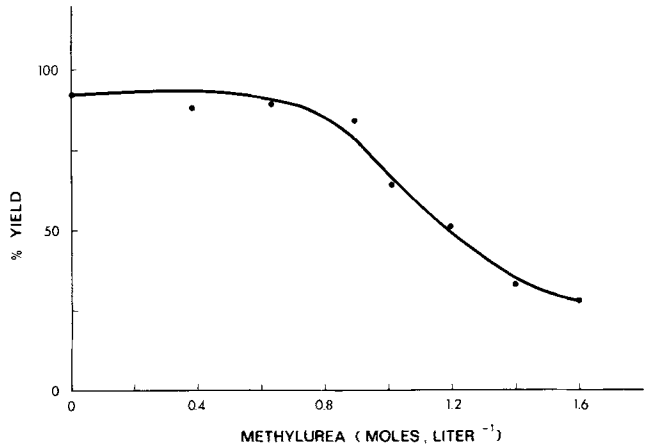


FIG. 2. The effect of methylurea on heptadecanoic acid methyl ester yield. Yields were computed as the ratio of methylheptadecanoate determined from standard curves by GLC to measured amounts of heptadecanoic acid in the reaction.

Gas chromatography was conducted with a Varian Aerograph 1740 equipped with dual flame ionization detectors. Columns, 0.31 (O.D.) x 213 cm stainless steel (premium grade, Supelco, Inc.), were packed with 10% EGSS-X on 100:120 mesh Chromasorb W (AW). Injector and detector temperatures were 225 C and 260 C, respectively. Column oven was operated at 195 C isothermal. Air, hydrogen, and nitrogen flow rates were 245, 25, and 30 ml/min. Individual peak areas of fatty acid methyl esters were determined with an Infotronics CRS-100 digital integrator. Total free fatty acids in crude oils were determined by summation of individual fatty acid methyl esters from GLC and by the A.O.C.S. titration method (2).

## RESULTS AND DISCUSSION

Neutral lipid oil (soybean triglyceride fraction) yielded significant amounts of fatty acid methyl esters when reacted with $BCl_3$/methanol. Because this oil does not contain free fatty acids, transesterification must have occurred. However, transesterification could be reduced by the addition of methylurea to the reaction (Fig. 1). This curve indicates that a concentration range of 1.4-1.6 M methylurea effectively reduced transesterification to very low levels (0.02%-0.05%), while heptadecanoic acid (internal standard) was converted to its methyl ester in 28-33% yield (Fig. 2). Therefore, any long chain free fatty acid should be esterified at about 30% yield under these conditions, while those bound as triacylglycerides would remain virtually unreacted. Thus, using 1.4-1.6 M methylurea per reaction, the following equation was developed to determine % free fatty acid in vegetable oil:

$$\% \text{ FFA} = \frac{1.2\,[H/a]\,\sum_{i=1}^{i=n} b_1 + b_2 + \ldots + b_n}{m} \times 100$$

where 1.2 = Average weight response factor based on experimentally determined values of the weight of each standard acid (mg)/integrator counts for each methyl ester to the weight of heptadecanoic acid (mg)/integrator counts for its methylester under the esterification and GLC conditions described. Standard acids were palmitic, stearic, and linolenic. Most columns tested yielded an average response factor of 1.2; however, a value of 1.1 was obtained with one column. Response factors should be determined for each column used and checked after some usage.

H = Amount of heptadecanoic acid standard (mg) per reaction (2.0 ml x 2.76 mg/ml = 5.52 mg).

a = Peak area for methyl heptadecanoate.

$b_1, b_2, b_n$ = Peak areas for individual fatty acid methyl esters.

m = Weight of oil sample per reaction (mg).

Free fatty acid values obtained with the modified procedure were only slightly higher than known formulation values (Table I). The fatty acid composition in standard oils No. 1 and 2 also indicate good agreement between standard and measured values. Although free oleic and linoleic acids were found in crude oils, these acids were not used in standard oils or in weight response factor determinations because thin layer chromatography (chloroform/methanol/

TABLE I

Determination of Free Fatty Acids in Standard Oils by GLC

| Standard oil | Known formulation | | Analyzed GLC | |
|---|---|---|---|---|
| | Total free acid[a] | Composition[b] | Total free acid | Composition[c] |
| No. 1 | 1.06% | 16:0-17.76 | 1.08% | 16:0-18.91 |
| | | 18-0-25.38 | | 18:0-24.75 |
| | | 18:3-56.85 | | 18:3-56.32 |
| No. 2 | 1.80% | 16:0-12.56 | 1.86% | 16:0-12.18 |
| | | 18:0-27.25 | | 18:0-25.81 |
| | | 18:3-60.18 | | 18:3-6200 |
| No. 3 | 2.48% | 18:3 only | 2.53% | |
| No. 4 | 3.24% | 18:0 only | 3.31% | |

[a]Computed as the ratio of the total weight of free acids to the weight of neutral lipid oil plus total free acids.
[b]Computed as the weight of individual free acid to the total weight of free acids.
[c]Percent of total free fatty acids as area or weight percent.

7.0N ammonium hydroxide, 65:30:4) indicated other lipid components (several spots) in these acids; whereas, palmitic, stearic, and linolenic acids yielded single migrating spots.

The free fatty acid content found in commercial and laboratory-extracted crude oils by the modified and A.O.C.S. titration procedures were compared (Table II). Except for the analysis of crude cottonseed oil, the two methods agreed closely. When methylurea was omitted in the analysis of Sunflower oil A, the free fatty acid content was 1.24%, which was three times the value by the modified and A.O.C.S. methods. The higher value indicates more fatty acid methyl esters were produced in this oil as a result of transesterification.

The free fatty acid content found in commercial cottonseed oil was consistenly 3.5 times the value of the A.O.C.S. method (Table II). There is no explanation for this discrepancy at this time, although, it could be related to refining techniques, or it is possible that the modified procedure is not as effective on cottonseed oil as with sunflower and soybean oils.

One useful feature of the modified procedure is that free fatty acid composition can be determined in a given oil (Table III). The data show significant differences in free fatty acid composition among these oils with linoleic being the major free acid.

The mechanism by which methylurea reduces transesterification is unknown. It has been shown that urea readily forms inclusion complexes with long chain unsaturated and polyunsaturated fatty acid methyl esters (11, 12). Such complex formation may also be possible between methylurea and oil triacylglycerides containing these fatty acids. If such complexes are formed, the configuration of the lipid molecule may be altered sufficiently to reduce transesterification.

Interaction between single free fatty acids and methylurea may also reduce direct esterification by inclusion complex formation as evidenced by the reduction in methyl ester yield as a function of methylurea concentration (Figure 2).

The time required for analysis of most oil samples was 30-40 min, which is slower than titration methods. However, the very small sample size used and the data obtained on individual free fatty acids may well justify the extra analytical time. Chromatographic conditions and reagents used, with the exception of methylurea, are identical to those for the determination of total fatty acids in crude oil (9); therefore, no additional equipment is needed.

A stainless steel column, 0.31 (O.D.) x 213 cm, packed with 10% DEGS, 0.5% $H_3PO_4$ on 100:120 mesh Chromasorb W(AW) was also tested. Resolution and quantitative results were the same for fatty acid methyl esters: however, this column also eluted underivatized free fatty acids which meant that at least a 90 min interval must elapse between sample injections. This problem was not encountered using 10% EGSS-X. For optimum resolution and operation, glass injector inserts must be cleaned regularly. Column life could also be extended by cutting about 3.0 cm from the injector end of the column for about three times.

Soybeans generally contain 21-23% oil (dry basis) with free fatty acid levels of 0.3-0.5% of the oil (6). Therefore, sufficient crude oil (200 mg) could be extracted from four to six mature soybeans and easily analyzed with the modified procedure. Because soybean pods contain three to five beans, variability in total and individual free fatty acid content between pods of the same or different plants could be determined. Similar studies could be conducted with six to eight sunflower seeds, since crude oil content is ca. 53% (dry basis) with oil free fatty acid levels of 0.2-0.4% (7). Because it can analyze small samples of seed for free fatty acid content and composition, the modified procedure should be suitable for physiological studies of different oilseed crops grown and stored under various conditions.

TABLE II

Determination of Free Fatty Acids in Commercial and Extracted Oils by GLC and A.O.C.S. Titration Methods[a]

| Oil | A.O.C.S. | GLC |
|---|---|---|
| Sunflower A[b] | 0.370 ± 0.005 | 0.368 ± 0.005 |
| Sunflower B[b] | 0.712 ± 0.017 | 0.720 ± 0.009 |
| Cottonseed[b] | 0.04 ± 0.10 | 0.140 ± 0.0 |
| Soybean A[c] | 2.22 ± 0.017 | 2.26 ± 0.025 |
| Soybean B[d] | 0.283 ± 0.022 | 0.293 ± 0.009 |

[a]Average of three determinations with standard error of the mean. Values are weight percent.
[b]Commercial crude oil.
[c]Laboratory extracted crude oil from field damaged soybeans (10% splits).
[d]Laboratory extracted crude oil from good soybeans.

TABLE III

Free Fatty Acid Composition in Oils as % of Total Free Fatty Acids

| Fatty acid | Soybean oil[a] | Sunflower oil[a] | Cottonseed oil[a] |
|---|---|---|---|
| 16:0 | 19.89 | 9.98 | 36.87 |
| 18:0 | --- | 5.09 | --- |
| 18:1 | 15.81 | 12.83 | 17.77 |
| 18:2 | 64.29 | 72.08 | 45.35 |
| 18:3 | --- | 0.71 | --- |

[a]Total free fatty acid content was 0.29% (soybean), 0.36% (sunflower), and 0.14% (cottonseed).

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