

Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol

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SUMMARY Fatty acid methyl esters and dimethylacetals suitable for gas chromatographic analysis were prepared by treatment of lipids with boron fluoride–methanol (140 g BF_3 per liter of methanol). This reagent is stable and easy to handle. Reaction conditions were investigated for triglycerides, diglycerides, monoglycerides, free fatty acids, sterol esters, phosphatidyl ethanolamines, phosphatidyl serines, phosphatidyl cholines, monophosphoinositides, monogalactosyl glycerides, phosphatidyl cholines (choline plasmalogens), digalactosyl glycerides, and sphingomyelins. The methyl esters and dimethylacetals were readily purified by thin-layer chromatography, and yields were quantitative. There were few undesirable side reactions, and they did not affect the validity of the method. The procedure developed is simple, rapid, and generally applicable to lipids.

A METHOD FOR preparing fatty acid methyl esters for gas–liquid chromatography (GLC) should ideally be simple, rapid, and quantitative, and should give rise to no unwanted structural changes or side reactions. Methods in which free fatty acids are liberated and then converted to methyl esters require several manipulative steps, and are more liable to error than single step methanolysis methods. Methanolysis of lipids is normally carried out with an alkaline (1–3) or an acidic (4–9) catalyst. Numerous catalysts have been patented for the alcoholysis of fats and oils, including boron fluoride (10). Boron fluoride has been used as a catalyst for the esterification of acids (11, 12) in the form of its coordination complex with methanol, and recently it has been used in an empirical manner for conversion of bacterial lipids to fatty acid methyl esters (13). Since boron fluoride

alcoholates behave like strong acids (14) they would be expected to promote methanolysis of lipids in a manner similar to HCl or H_2SO_4 added to methanol (4–9), with the added advantages conferred by the extreme electropolarity of the boron fluoride (Ref. 14, p. 27). This paper describes a study of the preparation of methyl esters and dimethylacetals from the major classes of lipids with boron fluoride–methanol, and shows that it is a useful reagent for this purpose.

MATERIALS AND METHODS

Materials

Boron fluoride–methanol (140 g BF_3 per liter of methanol) was obtained from Applied Science Laboratories, Inc., State College, Pa. Pentane and hexane were purified by passing through silica gel (15). All other solvents were of reagent grade and were used without further purification. Lipids were obtained from the following sources.

National Institutes of Health standard methyl ester mixtures E and F, methyl linoleate, methyl linolenate, 2-hydroxymyristic acid, and 12-hydroxystearic acid were from Applied Science Laboratories, Inc. Methyl ricinoleate and C_8 to C_{20} saturated methyl esters were from Calbiochem, Los Angeles, Calif. The methyl ricinoleate was purified by silicic acid chromatography.

Palmitaldehyde dimethylacetal was prepared from palmitaldehyde (K & K Laboratories, Inc., Jamaica, N. Y.) by treatment with boron fluoride–methanol, and was purified by thin-layer chromatography (TLC).

Triglycerides (tricaprylin to tristearin), fatty acids, and cholesterol were from Eastman Organic Chemicals, Rochester, N. Y. Dipalmitin from Sigma Chemical Co.,

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St. Louis, Mo. and monostearin from K & K Laboratories, Inc. were purified by Florisil chromatography (16).

Cholesteryl stearate was from Nutritional Biochemicals Corp., Cleveland, Ohio. Cholesteryl methyl ether was a gift from Dr. W. E. Thiessen, Chemistry Dept., University of California, Davis, Calif.

Dipalmitoyl phosphatidyl ethanolamine and phosphatidyl serines were from Mann Research Laboratories, New York, N. Y.; dipalmitoyl phosphatidyl choline from Sigma Chemical Co.; inositol phosphatides from Nutritional Biochemicals Corp.; beef brain sphingomyelins from Mann Research Laboratories; and beef heart lecithins (for choline plasmalogens) from Sylvania Chemical Co., Orange, N. J. per Arnel Products Co., New York, N. Y.

Lysophospholipids were obtained by hydrolysis of the corresponding diacyl phospholipids with snake venom phospholipase A.

Mono- and digalactosyl glycerides were extracted from whole wheat flour (17) and purified by TLC.

Preparation of Methyl Esters and Dimethylacetals

An aliquot of lipid solution (dried with anhydrous sodium sulfate if necessary) was evaporated to dryness under nitrogen in a centrifuge tube provided with a Teflon-lined screw cap. Boron fluoride-methanol reagent (Table 3) was added *under nitrogen*, in the proportions 1 ml reagent per 4–16 mg of lipid, and the tube was closed with the screw cap. The tube was then heated in a boiling water bath for the requisite time, cooled, and opened. The esters were extracted by adding 2 volumes of pentane, then 1 volume of water, shaking briefly, and centrifuging until both layers were clear. This gave 97–99% extraction of esters. No further extractions were made unless hydroxy esters were present, in which case two or three additional extractions with pentane were necessary.

To recover dimethylacetals, the extraction was modified to prevent reversion of acetals to aldehydes. The acetals (and esters) were extracted by adding to the methanolysate 4 volumes of pentane, then 1 volume of 5 N sodium hydroxide—all at 0°—shaking and centrifuging as above. The reaction of alkali with boron fluoride-methanol is strongly exothermic, and saponification of methyl esters might occur if the mixture is not cooled.

For some kinds of routine work purification of methyl esters may be unnecessary. However, in the presence of compounds which may interfere with gas-liquid chromatography (GLC) of long-chain methyl esters (e.g., some sterol derivatives), and for precise work in general, contaminants should be removed by TLC (see Results; Purification of Methyl Esters and Dimethyl Acetals).

Analytical Methods

Lipids were separated by TLC on plates coated with Merck Silica Gel G. Developing solvents are given below and in every case proportions are by volume. Spots were detected with Rhodamine 6G in 0.25 M dipotassium hydrogen phosphate (18), with iodine, with 2',7'-dichlorofluorescein, or by charring with 50% sulfuric acid. Amines were detected with ninhydrin (19), and choline with modified Dragendorff reagent (20). Methyl esters and dimethylacetals, after detection with 2',7'-dichlorofluorescein, were recovered from TLC plates by scraping off the spots into filter funnels plugged at the end of the stem with glass wool, and eluting with chloroform or diethyl ether.

Fatty acid methyl esters and dimethylacetals were analyzed by GLC in a Loenco 15A gas chromatograph with a flame ionization detector. The column was 6 ft × 1/4 inch o.d. packed with 20% LAC-2R-446 and 2% phosphoric acid on 60–80 mesh Chromosorb W, and was operated at 196°. In later work, a 4 ft column packed with 12% stabilized diethylene glycol succinate polyester on 60–70 mesh Anakrom A, operated at 186°, was used since it gave shorter retention times and improved resolution. A 4 ft nonpolar column of 15% silicone rubber SE-30 on 60–80 mesh Chromosorb W, operated at 224°, was also used. In later work this was replaced by a 3 ft column of 12% Apiezon L on 60–70 mesh Anakrom AS, operated at 220°. Relative peak areas were measured as retention distance × peak height, and weight percentage compositions calculated by applying correction factors obtained from chromatograms of National Institutes of Health methyl ester mixtures E and F (the correction factors were unity over the range C₁₂–C₂₀). Yields of methyl esters were determined by adding known amounts of a standard methyl ester to lipid methanolysates before the pentane extraction and subsequent GLC. Dimethylacetal yields were determined by adding a known amount of standard methyl ester to the purified acetals before GLC. A correction factor was applied to compensate for the lower ionization detector response to acetals relative to corresponding weights of esters. This was determined from known weights of palmitaldehyde dimethylacetal (the main acetal in the lipids studied) and methyl palmitate.

Ultraviolet spectra were obtained in isooctane over the range 210–320 m μ with a Beckman DB recording spectrophotometer. Infrared spectra were recorded from thin films or carbon tetrachloride solutions of lipids with a Beckman IR-5 spectrophotometer. Phosphorus was determined by a method for 1–10 μ g amounts (21), and aliquots of samples were taken accordingly. The vinyl ether content of plasmalogens was estimated by the revised method of Gottfried and Rapport (22).

RESULTS

Triglycerides

Methanolysis of triglycerides containing medium or long-chain fatty acids necessitated the addition of a solvent such as benzene to dissolve the triglycerides in the boron fluoride-methanol. Figure 1 shows the effects of different concentrations of boron fluoride-methanol and benzene on the conversion of tripalmitin to methyl palmitate. Because the results were obtained by TLC, a technique of limited sensitivity, the points at which all tripalmitin disappeared could not be determined precisely. Methanolysis of tripalmitin was complete in 25 min, using 25% boron fluoride-methanol, 20% benzene, and 55% methanol. This result was confirmed by GLC using methyl myristate as standard: the yield of methyl palmitate was $100.0 \pm 1.7\%$ of theory (Fig. 2).

The complex nature of Fig. 1 is only partially due to the increased solubility of tripalmitin with increasing amounts of benzene in methanol. Complete solubility (within 10 min at 100°) was attained only in the 20 and 30% benzene series. Presumably other factors are responsible for the decreased reaction rates observed at higher boron fluoride concentrations.

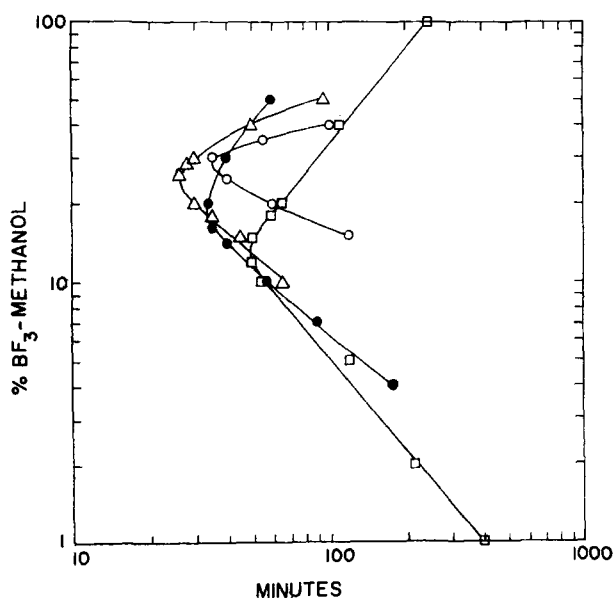


FIG. 1. The effect of different concentrations of benzene and boron fluoride-methanol on the rate of conversion of tripalmitin to methyl palmitate. Methanol was used as diluent as required. Four milligrams of tripalmitin per milliliter of reagent were used, and the lipids were extracted into 2 ml of pentane. Aliquots (30 μ l) were applied to TLC plates, which were developed with ether-hexane, 30 : 70, and spots were detected with Rhodamine 6G. The time for complete conversion to methyl palmitate was taken as the time after which no residual tripalmitin could be detected. \square = no benzene; \bullet = 10% benzene; Δ = 20% benzene; \circ = 30% benzene.

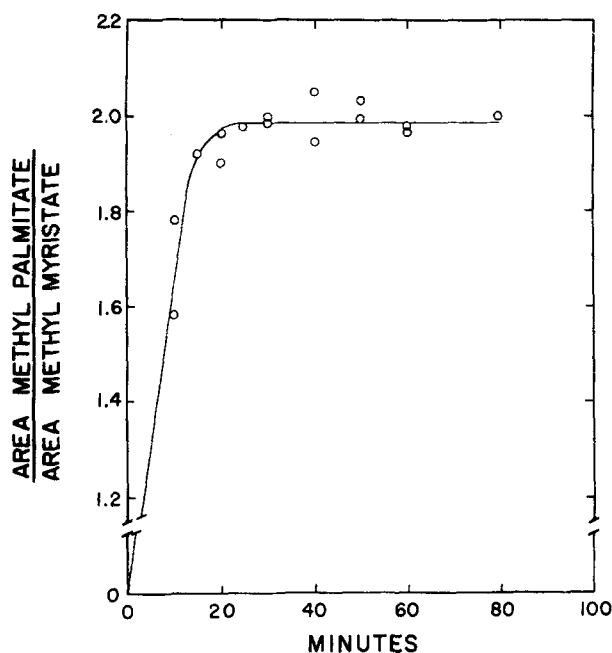


FIG. 2. The amounts of methyl palmitate obtained from tripalmitin after various methanolysis times (25% BF_3 -methanol, 20% benzene, 55% methanol), compared with standard amounts of methyl myristate. Amounts were measured as the ratio of the areas of the corresponding ester peaks on gas chromatograms. Theoretical ratio, 1.991; ratio from 25 to 60 min, 1.992 ± 0.033 .

Methanolysis of simple triglycerides containing C_8 - C_{18} fatty acids was shown by GLC to proceed at the same rate (being complete in 25 min), whereas in the absence of benzene the rate increased with decreasing fatty acid chain length and with increasing unsaturation. When conditions were anhydrous, no free fatty acids could be detected by TLC, but small amounts of water ($>0.5\%$) gave rise to detectable amounts of free fatty acids in the unpurified methyl esters. Pentane, hexane, diethyl ether, and ethyl chloride were all found to be satisfactory substitutes for benzene.

Diglycerides and Monoglycerides

Diglycerides and monoglycerides were detected as intermediate products in the conversion of triglycerides to methyl esters, and were converted to methyl esters under the same conditions. However, they are much more soluble in hot methanol, and were found to be completely converted to methyl esters in 6-7 min by undiluted boron fluoride-methanol (TLC as for triglycerides). Yields of methyl esters were presumed to be quantitative, since this was the case with triglycerides.

Free Fatty Acids

The esterification of free fatty acids was not studied in detail since this topic has been adequately covered elsewhere (11, 12, 23). It was observed that free fatty

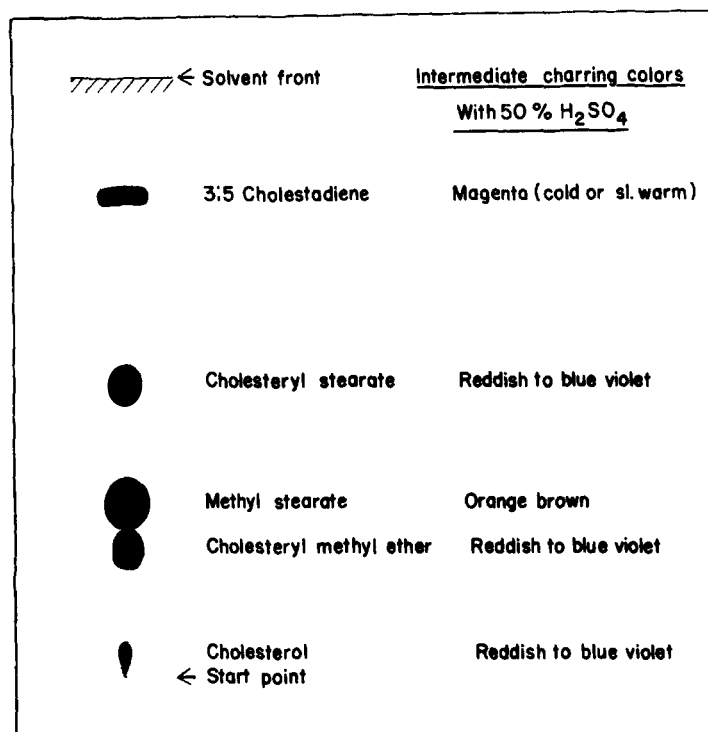


FIG. 3. TLC of products from cholesteryl stearate after partial methanolysis with boron fluoride-methanol. TLC plates were developed with benzene-hexane, 20 : 80, and charred with 50% sulfuric acid.

acids were rapidly esterified under any of the conditions described in this paper. Palmitic acid was also quantitatively esterified in 6 min at room temperature by undiluted boron fluoride-methanol. As a general procedure this has the disadvantage that higher fatty acids are insufficiently soluble in methanol at room temperature, and would not be fully esterified.

Sterol Esters

Since sterol esters are almost insoluble in methanol, benzene was added to obtain reasonable methanolysis times. Optimal conditions, although similar to those for triglycerides, were not the same. The fastest rate of conversion of cholesteryl stearate to methyl stearate was obtained with 35% boron fluoride-methanol, 30% benzene, and 35% methanol. Residual cholesteryl stearate could not be detected after 43 min by TLC (details below). Methanolysis was found to be complete in 45 min by GLC and the yield of methyl stearate was $102.4 \pm 1.6\%$ of theory. Methanolysis of wheat sterol esters was found (by TLC) to be complete under the same conditions. It was also found that methanolysis of triglycerides was complete under these conditions, and thus the fatty acid composition of sterol and glycerol esters can be determined together if required.

The cholesterol liberated during the methanolysis of cholesteryl stearate is attacked by boron fluoride-

methanol. Figure 3 shows the separation by TLC of cholesteryl stearate and its derivatives after partial methanolysis with boron fluoride-methanol. After being sprayed with 50% sulfuric acid, a compound with R_F 0.8 gave a bright magenta color with little or no warming, whereas cholesteryl stearate (R_F 0.4), another unknown compound (R_F 0.2), and cholesterol (R_F 0.05) gave typical bluish-violet colors only during charring. The compound with R_F 0.8 was identified as 3,5-cholestadiene (found mp $79-79.5^\circ$, λ_{max} 228, 235, 243 $m\mu$; infrared showed absence of $-\text{OH}$, $-\text{OCH}_3$, $-\text{C}=\text{O}$, but strong absorption at 1640 cm^{-1} due to conjugated diene). The compound with R_F 0.2 was identified as cholesteryl methyl ether (found mp $82.5-84.5^\circ$, λ_{max} a broad peak at 228 $m\mu$; infrared spectrum identical to that of an authentic specimen, with strong absorption at 1100 cm^{-1} due to $-\text{OCH}_3$).

Phosphatidyl Ethanolamine, Serines, and Choline

Methanolysis of dipalmitoyl phosphatidyl ethanolamine proceeded rapidly in undiluted boron fluoride-methanol. The course of the reaction was followed by TLC of the methanolysate (not extracted with pentane) on plates developed with chloroform-methanol-water, 80:25:3, and charred. Methanolysis was complete between 5 and 10 min. Similarly, methanolysis of dipalmitoyl phosphatidyl ethanolamine was shown by GLC to be com-

plete after 7.5 min, and the yield of methyl palmitate was $101.8 \pm 1.3\%$ of theory. Lysophosphatidyl ethanolamine was detected as an intermediate product, and its methanolysis was equally rapid.

Methanolysis of phosphatidyl serines and lysophosphatidyl serines was found (by TLC and GLC) to proceed at the same rate as with phosphatidyl ethanolamine. The yield of esters was not determined since the purity of the sample was uncertain.

Methanolysis of dipalmitoyl phosphatidyl choline and lysophosphatidyl choline was also found (by TLC and GLC) to proceed at the same rate as with phosphatidyl ethanolamine. The yield of methyl palmitate was $100.2 \pm 2.9\%$ of theory.

Monophosphoinositides

Monophosphoinositides are rapidly attacked by aqueous 2 N hydrochloric acid to yield free fatty acids, diglycerides, monoglycerides, phosphatidic acids, glycerol, glycerophosphate, inositol, and inositol phosphate (24). When impure monophosphoinositides were treated with undiluted boron fluoride-methanol for 5 min at 100° , the pentane extract was found to contain methyl esters, diglycerides, and monoglycerides, indicating a comparable breakdown. Further heating was found to convert the glycerides to methyl esters, and methanolysis was found (by GLC) to be complete in less than 10 min. Yields were not calculated since the purity of the sample was unknown.

Sphingomyelins

The amide bond linking fatty acids to sphingosine-type bases is relatively difficult to split, and it is usual to treat such materials with methanolic HCl or H_2SO_4 for 4–16 hr under reflux. Boron fluoride-methanol was found to attack sphingomyelins comparatively rapidly. TLC of pentane extracts, on plates developed with chloroform-methanol-water 80:25:3, showed that a number of ninhydrin-positive products, together with methyl esters and unaltered sphingomyelin, were present in the early stages of methanolysis. After 90 min of methanolysis only one ninhydrin-positive spot was detected, which migrated just behind the methyl esters (R_F 0.9). Paper chromatography (25) showed that no sphingosyl phosphoryl choline was present, so the final ninhydrin-positive material presumably consisted only of sphingosine and related bases (26). Unpurified extracts from sphingomyelins after 90 min of methanolysis were chromatographed on TLC plates developed in ether-hexane, 10:90 and 50:50. All ninhydrin-positive material was retained at the baseline, and no hydroxy fatty acid esters were detected (27, 28).

The course of the methanolysis of sphingomyelins was followed by determining the decrease of pentane-

soluble phosphorus, and by GLC of the methyl esters produced. Both methods showed the reactions to be complete after 75 min. The mean molecular weight of the esters was 332, calculated from GLC analyses. Yields of purified esters were 101–104% of theory by gravimetric estimation, and $99.1 \pm 1.1\%$ of theory by GLC. The absence of amide absorption in the infrared spectrum of the esters, together with the agreement between the above ester yields and theoretical values, showed that significant amounts of ceramide were not present. Higher methyl esters tended to be insoluble in cold methanol before the extraction stage, but were soluble in the pentane phase. Interfacial material was found occasionally during ester extraction, but this did not contain esters and was rejected.

Phosphatidal Cholines (Choline Plasmalogens)

The beef heart lecithin used in this study contained phosphatidal cholines ($0.736 \mu\text{mole}$ of vinyl ether per mg total lipid), phosphatidyl cholines, and trace amounts of sphingomyelins (too little to interfere with the results). Since the plasmalogens were attacked by undiluted boron fluoride-methanol much more slowly than the diacyl phospholipid, it was possible to follow the methanolysis of the plasmalogens (TLC as for phosphatidyl ethanolamine). During the first 15 min of treatment, unaltered plasmalogens were recovered by adding chloroform and water to the digest to obtain the proportions chloroform-methanol-water, 8:4:3, as in a Folch wash (29). The phosphorus content of the chloroform phase reached a minimum in 23–30 min.

Dimethylacetals and methyl esters were recovered by the modified pentane extraction procedure, and were separated on, and recovered from, TLC plates developed with benzene. A methyl ester standard was added to the separated dimethylacetals on the TLC plate, before the band was scraped off for elution of the acetals. During GLC, the dimethylacetals were partially decomposed on the diethylene glycol succinate column (cf. Refs. 30, 31), but were satisfactorily chromatographed on the SE-30 column. Yields of acetals and esters both reached a maximum after 22–25 min treatment with boron fluoride-methanol. Theoretical yields of dimethylacetals and methyl esters were 21.4 and 53.8%, respectively, when calculated from the vinyl ether content of the original lipid and from mean molecular weights, obtained from GLC analyses of the acetals and esters. Values found by GLC with methyl ester internal standards were $22.8 \pm 2.9\%$ and $51.7 \pm 1.5\%$, respectively.

Galactosyl Glycerides

Wheat galactosyl glycerides were separated by TLC on plates developed in chloroform-methanol-water 80:25:3, and were detected by charring. Mono- and

digalactosyl glycerides were the main components of the mixture, but there was an appreciable amount of steryl glucoside and at least three other compounds, one of which was probably a cerebroside (17). All the above compounds were cleanly separated, and pure mono- and digalactosyl glycerides were prepared by detecting the spots with iodine and eluting them with methanol. Unaltered galactolipids and methyl esters were recovered from methanolysates in the chloroform layer of a Folch wash (29). Both galactosyl glycerides were found (TLC as above) to be rapidly attacked by undiluted boron fluoride-methanol. The reaction presumably involves splitting of the fatty acid-glycerol ester bonds only, since no mono- or diglycerides were detected. Methanolysis of monogalactosyl glycerides was found by GLC to be complete after 10 min, and the yield of methyl esters was $90.7 \pm 5.1\%$ of theory. Since the method of preparing the galactosyl glycerides was such that significant amounts of silica or sugar impurities were likely to be present, this probably represents a quantitative ester yield. Methanolysis of digalactosyl glycerides was not studied in detail, but results showed maximum ester yields after 20–30 min.

Purification of Methyl Esters and Dimethyl Acetals

If the range of fatty acid methyl ester chain lengths is not large, and in the absence of short-chain esters, distillation in vacuo is a suitable purification technique (e.g., Refs. 4, 32), but the method is not flexible with regard to ester load. There is also the possibility of contamination of esters with steroids during distillation, especially with 3,5-cholestadiene, but this was not studied in the present work. Steroids do not present a serious problem in the GLC of methyl esters if late peaks or drifting baselines on chromatograms can be tolerated. On the Apiezon L column used for GLC of methyl esters 3,5-cholestadiene had a retention time corresponding to 23:0–24:0 methyl esters, and cholesteryl methyl ether had a retention time corresponding to a 25:0 methyl ester. On the diethylene glycol succinate polyester column 3,5-cholestadiene corresponded to a 26:0 methyl ester, and cholesteryl methyl ether to a 28:0 methyl ester. Cholesterol would be expected to have a longer retention time than cholesteryl methyl ether on either column. It is thus possible to distinguish steroid peaks from methyl ester peaks on chromatograms when methyl esters of carbon number 24.0 and above are absent. Nevertheless, the authors recommend purification of methyl esters by TLC before GLC, particularly when minor components are being studied. When dimethylacetals are present in methyl esters, they can be tentatively identified by GLC retention data, but the authors prefer to separate them from methyl esters before GLC. This separation may be done by the method of

TABLE 1 R_F VALUES* OF SOME LIPID DERIVATIVES ON MERCK SILICA GEL G TLC PLATES DEVELOPED WITH VARIOUS SOLVENTS

| Lipid Derivative | Solvent | | |
|----------------------------|-----------------------|-----------------------|---------|
| | Ether-Hexane 10:90 | Ether-Hexane 50:50 | Benzene |
| 3,5-Cholestadiene | 0.70 | 0.85 | 0.80 |
| Methyl esters (nonhydroxy) | 0.40 | 0.75 | 0.45 |
| Dimethylacetals | 0.35 | 0.70 | 0.25 |
| Cholesteryl methyl ether | 0.30 | 0.75 | 0.23 |
| Hydroxy methyl esters | 0.05 | 0.55 | 0.05 |
| Cholesterol | 0.05 | 0.25 | 0.05 |

* R_F values are approximate, since they vary with the degree of activation (or deactivation) of the silica gel thin layer, with temperature, and with the relative concentration of solvents in the developing tank atmosphere.

Farquhar (31), or by chromatography. Column chromatography, using heat-activated silicic acid and solvents similar to those described below for TLC, may be employed but is much less convenient than TLC, and for most purposes TLC is preferred.

Generally, the complete spectrum of lipid derivatives will not be present in the methanolysate extracts, and TLC on plates developed with benzene will resolve most of the derivatives. Difficulties may arise when hydroxy esters and sterols are present. Clean separations of all lipid derivatives can be made by two-dimensional chromatography (or rechromatography), using two of the solvent systems given in Table 1. Methyl esters and dimethylacetals were eluted routinely from TLC plates with recoveries of 95–100% and 84–95%, respectively.

Side Reactions

A feature of rapid esterification reagents seems to be that they produce by-products and possibly promote undesirable side reactions (23, 33). This aspect of the use of boron fluoride-methanol was therefore studied in detail. The reagent will react to form dimethyl ether (Ref. 14, pp. 31, 267), which will not interfere with the analysis of methyl esters. No polymers were detected in boron fluoride-methanol even after several hours at 100°, and this is in keeping with the observation that the reagent is stable for several months (12). Decomposition to boric acid, hydrogen fluoride, and other derivatives (Ref. 14, pp. 31, 267) does not seem to occur during methanolysis.

Lough (34) has recently shown that boron fluoride-methanol can cause serious losses of unsaturated esters, and that oleic acid gives methoxy methyl stearate isomers in high yield. This is contrary to general experience, and seems to be due to the abnormally high boron fluoride concentration used (50%, w/v, as opposed to the more

TABLE 2 RECOVERIES OF UNSATURATED ESTERS AFTER TREATMENT OF METHYL ESTERS FOR 90 MIN AT 100° WITH METHANOLIC BF₃, HCl OR H₂SO₄

| Treatment | No. of Samples | Recovery | | |
|--|----------------|-------------|-------------|-------------|
| | | 18:1 | 18:2 | 18:3 |
| % | | | | |
| Controls* | 31 | 100.0 ± 3.4 | 100.0 ± 3.5 | 100.0 ± 3.8 |
| 14% BF ₃ -MeOH† | 16 | 95.9 ± 2.7 | 91.7 ± 3.6 | 91.8 ± 6.3 |
| 3 N HCl-MeOH‡ | 12 | 95.6 ± 3.2 | 92.4 ± 3.3 | 90.5 ± 3.4 |
| 5% H ₂ SO ₄ -MeOH§ | 16 | 95.2 ± 4.6 | 91.8 ± 4.5 | 90.8 ± 5.5 |

* Controls consisted of untreated esters, and esters mixed with each of the reagents without heat treatment, which were then extracted as usual.

† 14% (w/v) boron fluoride-methanol, approx 2 M.

‡ Anhydrous 3 N hydrochloric acid-methanol.

§ 5% (v/v) sulfuric acid-methanol, approx 1.8 N.

usual 12.5–14%, w/v). In the present work, Lough's findings were confirmed (by TLC, GLC, and infrared spectra) when 50% (w/v) boron fluoride-methanol was used to treat oleic acid samples. The study was then extended to compare the effects of commonly used methanolysis reagents on unsaturated esters. A mixture of peroxide-free methyl esters (16:0, 18:1, 18:2, 18:3) was prepared, with methyl palmitate as an internal standard. Methyl myristate was used as an external standard, and was added after the heat treatment stage, immediately before extraction of the esters. Aliquots of the ester mixture were treated for 90 min with 14% (w/v) boron fluoride-methanol, anhydrous 3 N HCl-methanol, or 5% (v/v) H₂SO₄-methanol. The amounts of recovered esters were determined by GLC and compared with appropriate controls (Table 2). Comparable losses of each unsaturated ester were found with all reagents, the polyunsaturated esters showing greater losses. In other experiments it was found that losses increased with duration of treatment and with increasing catalyst concentration. Since the conditions employed (90 min at 100°) represent maximal recommended treatment for methanolysis of sphingomyelins with boron fluoride-methanol, but not necessarily with the other reagents, it is concluded that boron fluoride-methanol causes no greater losses of unsaturated esters than do the other reagents studied, when each is used to obtain complete methanolysis. Losses of unsaturated esters are negligible when the treatment time with boron fluoride-methanol is short, as it is with most lipids (Table 3), and any artifacts are removed during TLC purification of methyl esters and dimethyl acetals.

It must be emphasized that if a methanolysis tube leaks during heating, there will be an increase in boron

fluoride concentration due to preferential loss of methanol, and destruction of unsaturated esters may become significant. Leaks may be minimized by grinding the rims of the tubes flat. As a substitute for Teflon liners, some types of synthetic rubber liners (cleaned by previous boiling in methanol or boron fluoride-methanol) may be used without giving rise to spurious results, if the esters are purified by TLC.

Although the hydroxyl groups in cholesterol and related sterols were found to be attacked by boron fluoride-methanol, those in hydroxy fatty acids apparently have different properties. 12-Hydroxystearic acid, 12-hydroxyoleic acid (ricinoleic acid), and 2-hydroxymyristic acid were all readily converted to the corresponding methyl esters, but even after prolonged treatment no evidence (TLC and infrared spectra) was found for alteration of the hydroxyl group. Likewise, the 2-hydroxymyristic acid did not form a lactide. These findings confirm and supplement those of Metcalfe and Schmitz (12), who found that 12-hydroxystearic acid and 12-ketostearic acid could be esterified with boron fluoride-methanol. In the present study, hydroxy esters were separated from nonhydroxy esters by TLC on plates developed with ether-hexane, 50:50 (Table 1), but the 2-hydroxy ester could not be separated from other hydroxy esters. It is of interest to note that the 2-hydroxy acid was readily separated from other hydroxy acids by TLC on plates developed with ether-acetic acid, 50:1. The 2-hydroxy acid had an *R_F* of about 0.4, whereas other hydroxy acids had an *R_F* of about 0.6. This method may prove to be a convenient alternative to the copper chelate method (35) for separating 2-hydroxy acids.

Hydroperoxide and hydroxyl groups adjacent to double bonds in fatty acid methyl esters are unstable and are dehydrated during GLC to the corresponding nonhydroxy ester with one more double bond conjugated to the original unsaturation (32, 36). It has been shown by one of us (32) that freshly prepared linoleate hydroperoxides are similarly dehydrated by boron fluoride-methanol, but the present study showed that old autoxidized samples yielded additional products, and developed yellow to dark brown colors after prolonged treatment. No attempt was made to identify these additional products, since the colored material was not present in purified esters. A study of the breakdown of partially autoxidized linoleate with increasing time of boron fluoride-methanol treatment showed that the amounts of conjugated products were relatively constant after 10 min (ultraviolet spectrum did not alter), and the amount of unoxidized methyl linoleate remained constant within the experimental error of GLC. These results indicate that the presence of some autoxidized lipids does not lead to significant losses of unoxidized esters,

and this has been confirmed by similar studies with natural products containing highly unsaturated fatty acids. Experiments with peroxide-free methyl linoleate showed that it did not become conjugated (no alteration of ultraviolet spectrum) in boron fluoride-methanol under the preparative conditions recommended, and became only very slightly conjugated under limited aerobic conditions (sealed tube). This does not, however, exclude the possibility of small losses giving rise to nonconjugated products (see above, and Table 2).

To determine the effect of boron fluoride-methanol on conjugated unsaturation, experiments were carried out with stillinga oil and with methyl stillingate (methyl deca-*trans*-2-*cis*-4-dienoate). The infrared spectrum of methyl stillingate, prepared by methanolysis of stillinga oil—see *Triglycerides*—and by repeated fractional distillation in vacuo, was identical to that of the *trans*-2-*cis*-4 isomer in the 850–1300 cm^{-1} region (37). Furthermore, methyl stillingate has a strong absorption maximum at 265 μ which was unaffected by treatment with boron fluoride-methanol for 1 hr, showing that *cis-trans* isomerization did not occur, since this would have caused a slight shift in wavelength and intensity of the absorption maximum (37).

The use of solvents such as benzene, diethyl ether and ethyl chloride during methanolysis of triglycerides and sterol esters increases the risk of explosion or of the formation of artifacts. Ethyl chloride would only be used when volatile esters such as methyl butyrate are to be recovered. Despite the high vapor pressure of ethyl chloride at 100°, the screw cap tubes which were used did not burst, and the routine use of less volatile solvents does not, therefore, seem unduly hazardous. No evidence for the presence of aromatic compounds could be found in esters prepared in the presence of benzene (infrared spectra), and no ethyl esters could be detected by GLC when ethyl chloride and diethyl ether were present in the boron fluoride-methanol. However, when ethanol and propanol were present, significant amounts of ethyl or propyl esters were formed, suggesting that there was an equilibrium between the boron fluoride alcoholates. These findings are consistent with the reactive species being represented by $[\text{CH}_3\text{OBF}_3]^- \text{H}^+$ (14, 38), i.e., the boron fluoride is coordinated throughout the reaction and does not interchange with relatively nonpolar compounds.

Comparison of Boron Fluoride and Boron Chloride as Catalysts

Boron fluoride and boron chloride at equimolar concentrations in methanol were compared as catalysts for the preparation of methyl esters from three classes of lipid. When 25% boron halide-methanol, 20%

TABLE 3 CONDITIONS FOR THE PREPARATION OF METHYL ESTERS AND DIMETHYLACETALS FROM VARIOUS LIPIDS WITH BORON FLUORIDE-METHANOL*

| Class of Lipid | Reagent† | Heating Time at 100° |
|--|--|----------------------|
| | <i>% by volume</i> | <i>min</i> |
| Triglycerides | 25% Boron fluoride-methanol, 20% benzene, 55% methanol | 30 |
| Triglycerides } Sterol esters } | 35% Boron fluoride-methanol, 30% benzene, 35% methanol | 45 |
| Fatty acids | Boron fluoride-methanol | 2 |
| Monoglycerides } Diglycerides } Phosphatidyl ethanolamines } Phosphatidyl serines } Phosphatidyl cholines } Monophosphoinositides } Monogalactosyl glycerides } Phosphatidyl cholines } (choline plasmalogens) } | Boron fluoride-methanol | 10 |
| Digalactosyl glycerides } Sphingomyelins } | Boron fluoride-methanol | 30 |
| | Boron fluoride-methanol | 90 |

* 140 g BF_3 per liter of methanol.

† 1 ml reagent per 4–16 mg of lipid.

benzene, and 55% methanol was used, it was found (by TLC) that methanolysis of tripalmitin was slower in the boron chloride system. Appreciable quantities of diglyceride isomers were found with the boron chloride system, so presumably methanolysis of diglycerides is also slower. Dipalmitoyl phosphatidyl ethanolamine and sphingomyelins were also found (by TLC) to be attacked at a slower rate by undiluted boron chloride-methanol.

Summary of Results

Fatty acid methyl esters and dimethylacetals were prepared in quantitative yields by methanolysis of lipids with boron fluoride-methanol at 100°. The ratio of reagent to lipid was generally 1 ml per 4 mg, but 1 ml per 16 mg was used without needing to increase the reaction time or the volume of reagent. The conditions recommended for various types of lipids (including a safety factor in the heating time) are summarized in Table 3. When lipids of very different types are present (e.g., triglycerides, sterol esters, and sphingomyelins) the mixture can be treated as for sphingomyelins. After cooling, benzene and methanol can be added as for sterol esters, and the methanolysate reheated for 30 min. A simpler procedure would be to heat the lipids in undiluted boron fluoride-methanol for 4–5 hr, but this would increase the losses of unsaturated esters.

DISCUSSION

Boron fluoride is the most electropolar of the boron halides (Ref. 14, p. 27) and is extremely reactive toward many types of organic compounds. When coordinated with methanol it becomes a useful reagent for the preparation of fatty acid methyl esters and dimethylacetals. Since the presence of ethanol and propanol in boron fluoride-methanol gives rise to unwanted ethyl and propyl esters, it is necessary to remove such alcohols from lipids before boron fluoride-methanol treatment. Traces of other common lipid solvents do not present this problem.

Boron chloride-methanol has been claimed to be superior to boron fluoride-methanol for the methanolysis of triglycerides (39), but the system used in that investigation contained excessive amounts of benzene for optimal boron fluoride catalysis. In the present study equimolar concentrations of boron chloride and boron fluoride were compared, and the results showed that boron fluoride was the better catalyst.

Experience with natural lipids (e.g., beef heart plasmalogens and lecithins, and beef brain sphingomyelins) has shown that methyl esters and dimethylacetals prepared with boron fluoride-methanol, as described in this paper, always gave TLC and GLC analyses consistent with negligible side reactions. GLC analyses of methyl esters, including highly unsaturated esters, from a variety of natural lipids were consistently in good agreement with published values. On the basis of the results presented in this paper, it is concluded that boron fluoride-methanol is a satisfactory, generally applicable, methanolysis reagent.

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