notes on methodology

Artifacts produced during acid-catalyzed methanolysis of sterol esters

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Summary Sterol esters were transesterified within 1 hr using either acid or base catalysts. Acid-catalyzed methanolysis of sterol esters with HCl, H_2SO_4, or BF_3 leads to the formation of two artifacts derived from the sterol portion of the molecule; they were identified as dehydrated and methoxylated derivatives of sterols. These two artifacts were not produced using a base-catalyzed methanolysis with $NaOCH_3$.

Supplementary key words dehydrated sterol · methoxylated sterol

Complete methanolysis of sterol esters is known to be more difficult than that of glycerol-derived esters. Therefore, to assure complete transesterification, longer reaction times (1) or a combination of acid and base catalysis (2) have been recommended. Luddy, Barford, and Riemenschneider (3) reported yields of 92–93%, with 4% unchanged cholesterol esters remaining, with acid-catalyzed methanolysis; base-catalyzed reactions were believed to be quantitative, producing only small amounts of free fatty acids. Morrison and Smith (4) reported two artifacts during the methanolysis of cholesterol esters using BF_3 as catalyst; these were identified as dehydrated and methoxylated cholesterol.

During our studies on the quantitative determination of sterols from sterol esters of rapeseed oils, we consistently observed lower sterol levels using HCl-catalyzed methanolysis compared to using $NaOCH_3$–methanol as catalyst. In addition, two unidentified spots were observed on examining the TLC chromatograms of the acid-catalyzed reaction products. Therefore, we investigated the quantitative methanolysis of sterol esters using acid (HCl, H_2SO_4, and BF_3) and base ($NaOCH_3$) as catalysts. BF_3–Methanol was used for comparison since two artifacts had already been identified using this catalyst (4).

The sterol esters used in this study were isolated from rapeseed oil cultivar Tower by column chromatography on acid-treated Florisil (5), and were purified by TLC using hexane–diethyl ether 90:10. To determine independently the sterol composition of these sterol esters, a portion was reacted with ethyl magnesium bromide; the free sterols were isolated by TLC, converted to acetates, and analyzed by GLC on columns packed with 5% SP-2401. The relative concentrations of the three major sterols were brassicasterol 5%, campesterol 40%, and β-sitosterol 55%.

Sterol esters (2 mg in 0.5 ml benzene) were transesterified for 1 hr at 80°C in culture tubes fitted with a Teflon-lined screw cap by adding 2 ml of anhydrous methanol containing 5% HCl gas, 2% conc. H_2SO_4, 10% BF_3 (Eastman Kodak Co., Rochester, N. Y.), or 10% $NaOCH_3$ (Applied Science Laboratories, State College, Pa.). Examination of the hexane extracts by TLC using 1,2-dichloroethane as solvent showed that all starting material had reacted after 1 hr, and that the products included methyl esters (R_f 0.56) and free sterols (R_f 0.08). All acid-catalyzed methanolysis reactions showed two additional spots designated X (R_f 0.78) and Y (R_f 0.37). Morrison and Smith (4) reported finding similar spots during BF_3-catalyzed methanolysis. In addition the color change of spots X and Y observed after spraying with H_2SO_4–ethanol 1:1 and heating were the same as reported by these authors (4).

In separate experiments, free sterols (cholesterol, stigmasterol, and β-sitosterol), as well as their acetates, were subjected to the same conditions of transesterification. Every acid-catalyzed methanolysis of sterols or their acetates resulted in the formation of artifacts X and Y as judged by TLC, while neither of these spots were observed using $NaOCH_3$–methanol. Therefore, it was concluded that the formation of artifacts was not specific to sterol ester methanolysis, but may also occur with free sterols.

Artifacts X and Y were isolated using 1,2-dichloroethane as developing solvent. As indicated by the respective R_f values in **Table 1,** use of the common solvent system hexane–diethyl ether–acetic acid 90:10:1 caused the methoxylated sterols, dimethyl acetals (derived from plasmalogenic lipids) and methyl esters to cochromatograph. No improvement in the separation of these three lipid classes was obtained using hexane–diethyl ether 95:5 and developing the chromatogram twice in the same direction (6). When using this solvent, methyl esters and dimethyl acetals were separated by degree of unsaturation, and thus

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry.

TABLE 1. R_f values of some lipid classes and artifacts produced during methanolysis of sterol esters

Lipid Classes	Solvent: Hexane–Diethyl Ether–Acetic Acid 90:10:1	Solvent: 1,2-Dichloroethane
Dehydrated sterol	0.76	0.78
Sterol ester	0.73	0.72
Methyl ester	0.46	0.56
Triglyceride	0.35	0.39
Dimethyl acetal	0.43	0.28
Methoxylated sterol	0.42	0.37
Free sterol	0.04	0.08

Separations were carried out using TLC plates coated with silica gel G, 0.25 mm in thickness.

methyl arachidonate had the same migration rate as a saturated dimethyl acetal. The use of benzene as a solvent to separate the two artifacts from methyl esters (4) has the disadvantage of not distinguishing dimethyl acetals from methoxylated sterols. The use of 1,2-dichloroethane as a solvent gives a clear separation of all possible products of methanolysis.

Analysis of artifacts X and Y by GLC (glass column 2.7 m × 2 mm; packing 10% SP-222-PS, Supelco Inc., Bellefonte, Pa.; operating temperature 200°C) showed that bands X and Y each consisted of a mixture of three components. Those for band X emerged after the methyl ester with relative retention times (methyl hexadecanoate = 1.0) of 9.7, 11.0, and 12.9, respectively. The three components of band Y emerged last with relative retention times of 18.0, 20.5, and 24.2, respectively. In every acid-catalyzed transesterification reaction, the relative concentration of the components in bands X and Y were very similar to the relative concentrations of the three major sterols present in the original sterol esters.

Since the GLC column (10% SP-222-PS) resolved methyl esters and artifacts X and Y, it permitted a comparison between the artifacts produced and total methyl esters. The relative abundance of artifacts X and Y in the total transesterification mixtures are shown in **Table 2.** No attempt was made to establish

TABLE 2. Extent of artifacts produced during methanolysis of sterol esters

Components	Percent Artifacts: HCl	H_2SO_4	BF_3	$NaOCH_3$
Dehydrated sterol	4–8	2–5	3–6	None
Methoxylated sterol	2–3	3–5	5–7	None

The range from three separate experiments with each catalyst is given. Percent artifact is determined by expressing in percent the total area of each artifact to the total area of methyl esters from GLC chromatographs.

the quantitative recovery of methyl esters, but as judged by TLC, sterol ester hydrolysis was complete and no sterol-containing artifacts other than X and Y were observed. The sensitivity of detection of sterols was 0.5 μg per TLC spot. Under normal circumstances transesterification reactions with freshly prepared catalysts using reasonable reaction times result in quantitative recovery of methyl esters (1, 4), with the exception of BF_3–methanol, which may yield artifacts from unsaturated fatty acids even under ideal conditions (7). The data in Table 2 would indicate that slightly more artifacts were produced when HCl was used as catalyst than when either H_2SO_4 or BF_3 was used. Dehydration and methoxylation of the sterol appeared to proceed to the same extent whether H_2SO_4 or BF_3 was used, but dehydration predominated when using HCl.

Artifacts X and Y were each examined by GLC–MS, using a 1.5 m column (2 mm ID) packed with 3% OV-17 and operated at 240°C, in conjunction with a Finnigan 3100D quadrupole mass spectrometer. The molecular ion peaks of the three components in mixture X were at *m/e* 380, 382, and 396, which correspond to the molecular weights of the dehydrated products of brassicasterol, campesterol, and β-sitosterol, respectively. Each spectrum contained peaks indicating the loss of a methyl (M − 15) and a propyl (M − 43) group. In addition, a prominent ion occurred at *m/e* 255, which corresponds to the loss of the complete isoprenoid side chain. The molecular ion peaks of the three components in mixture Y were at *m/e* 412, 414, and 428, which correspond to the molecular weights of the methoxy derivatives of brassicasterol, campesterol, and β-sitosterol, respectively. Each of the three spectra contained peaks that were identified as loss of methyl (M − 15), methanol (M − 32), methyl and methanol (M − 47), propyl and methoxy (M − 74), loss of the complete isoprenoid side chain (*m/e* 287), and loss of methanol plus the isoprenoid side chain (*m/e* 255).

These experiments demonstrate that methanolysis of sterol esters is complete in 1 hr. Not only BF_3–methanol (4) but also common acid catalysts, such as HCl or H_2SO_4, lead to substantial formation of dehydrated and methoxylated products from the sterol moiety. Base-catalyzed methanolysis of sterol esters using $NaOCH_3$–methanol is therefore recommended for the quantitative analysis of sterols.

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REFERENCES

1. Christie, W. W. 1973. Lipid analysis. Pergamon Press, Oxford, England. 85–90.
2. Kates, M. 1972. Techniques of lipidology: isolation, analysis and identification of lipoids. *In* Laboratory Techniques in Biochemistry and Molecular Biology. T. S. Work and E. Work, editors. North Holland Pub. Co. Inc. Amsterdam, Holland. 361–364.
3. Luddy, F. E., R. A. Barford, and R. W. Riemenschneider. 1960. Direct conversion of lipid components to their fatty acid methyl esters. *J. Amer. Oil Chem. Soc.* **37:** 447–451.
4. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
5. Carroll, K. K. 1963. Acid-treated Florisil as an adsorbent for column chromatography. *J. Amer. Oil Chem. Soc.* **40:** 413–419.
6. Mangold, H. K. 1969. Aliphatic lipids. *In* Thin-layer Chromatography. E. Stahl, editor. Springer-Verlag, Heidelberg, Germany. 373.
7. Fulk, W. K., and M. S. Shorb. 1970. Production of an artifact during methanolysis of lipids by boron trifluoride–methanol. *J. Lipid Res.* **11:** 276–277.