notes on methodology

Identification and quantitation of lipid-bound short-chain diols

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Summary Procedures are described for the hydrolysis of neutral lipid fractions containing long-chain esters and alk-1-enyl ethers of short-chain diols, and for the identification and quantitation of the constituent diols as long-chain cyclic acetals using gas–liquid chromatography in combination with mass spectrometry.

Supplementary key words diol lipids · diol esters · diol alk-1-enyl ethers · cyclic diol acetals · barium hydroxide · boron trifluoride · acetalation · gas–liquid chromatography · mass spectrometry

Small amounts of diol-derived lipids have been found in a variety of animal (2–5) and plant (3, 5) tissues and in microorganisms (3, 5–7). The constituent diols usually were detected after acidic or alkaline hydrolysis of neutral ester and alk-1-enyl ether lipids and silylation or, better, acetylation (5).

We have encountered considerable difficulties in the hydrolysis of lipid fractions containing minute quantities of lipid-bound diols, especially of diols bearing tertiary hydroxy groups.[1] Acid-catalyzed methanolysis involves extended exposure of the lipid mixture to an acidic medium which can cause elimination of water from polyhydric alcohols and other side reactions. Hydrochloric acid-catalyzed methanolysis also leads to exchange of reactive hydroxy groups with chlorine, as is evident from the formation of appreciable amounts of 1-chloropropanediol during methanolysis of triglycerides.[1] Methanolysis catalyzed by boron trifluoride is known to be incomplete (8), whereas alkaline saponification does not cleave alk-1-enyl ether linkages. The isolation and analysis of minute quantities of short-chain diols as acetyl derivatives proved to be quite difficult, as has been experienced by other investigators (5). Considerable losses occur during purification of short-chain diol diacetates due to their solubility in both aqueous media and organic solvents, and due to their volatility.

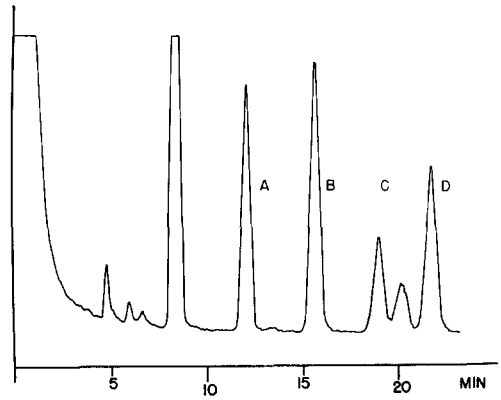

Fig. 1. Gas-liquid chromatography of cyclic diol acetals. *A*, 2-pentadecyl-4,4-dimethyl-1,3-dioxolane (R_T 12.0 min, recovery 69%); *B*, 2-pentadecyl-1,3-dioxolane (15.7 min, 93%); *C*, *cis*- and *trans*-2-pentadecyl-4-isopropyl-1,3-dioxolane (19.0 and 20.3 min, 65%); *D*, 2-pentadecyl-1,3-dioxane (21.8 min, 71%).

The mass spectra of the acetates of diols usually do not exhibit parent ion peaks, and a great number of fragmentations and rearrangements are observed, especially with branched diol isomers, which are not easily predictable.

The purpose of this note is to describe a novel procedure for the hydrolysis of neutral lipid fractions and for the derivatization and analysis of small amounts of short-chain diols bound to long-chain acyl and alk-1-enyl ether groups. Virtually complete cleavage of both types of neutral diol lipids is accomplished by treating the lipids with barium hydroxide in boiling methanol and then with methanolic boron trifluoride at reflux temperature. Barium hydroxide cleaves triacylglycerols completely within 1 min and acyl groups linked to tertiary hydroxy groups within 30 min. Boron trifluoride in methanol accomplishes conversion of the barium salts of fatty acids to methyl esters within 2–3 min and, at the same time, cleaves alk-1-enyl ethers. Excess boron trifluoride is then precipitated from the reaction mixture by complexing it with sodium fluoride. The hydrolysis mixture is subsequently reacted with long-chain aldehyde in the presence of *p*-toluenesulfonic acid in boiling benzene (9) to form cyclic acetals of the polyhydric alcohols. The reaction produces long-chain cyclic acetals of 1,2- through 1,5-diols in good yields. Excess aldehyde and fatty acid methyl esters are finally converted to alcohols by lithium aluminum hydride reduction without affecting the diol cyclic acetals, and the latter are separated from the bulk of polar by-products by adsorption chromatography. Finally, the diol cyclic acetals are resolved and identified by combination of gas–liquid chromatography and mass spectrometry.

The reliability of the procedures is demonstrated for a standard mixture containing trihexadecanoylglycerol and 1% of each of the dihexadecanoates of 2-methyl-1,2-

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[1] Baumann, W. J., and E. Schupp. Unpublished results.

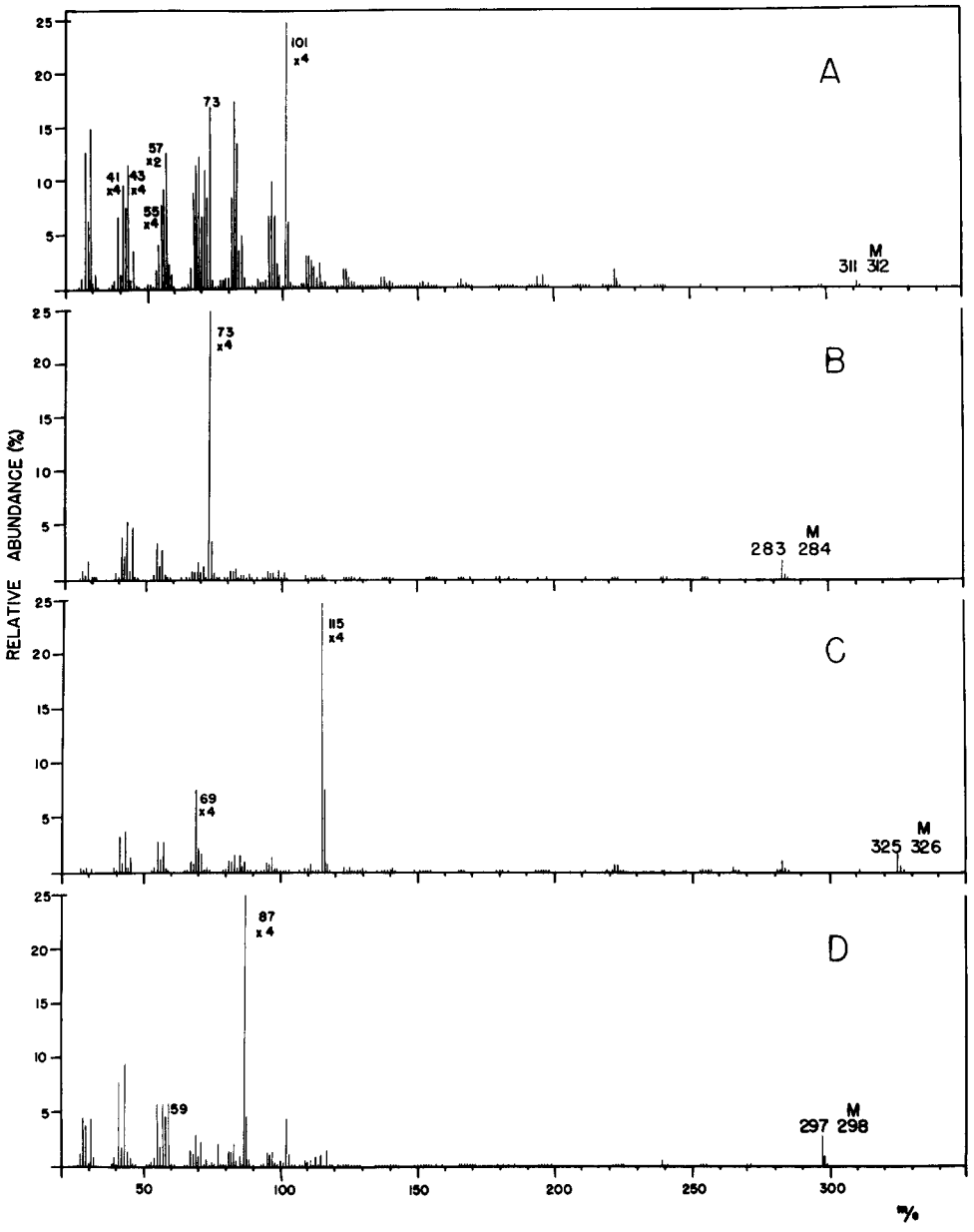


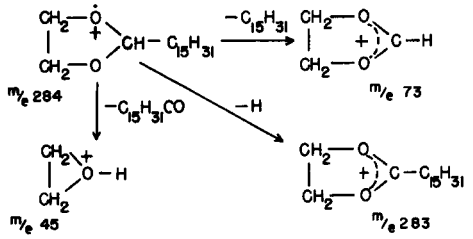
FIG. 2. Mass spectra (70 ev) of the cyclic acetals of hexadecanal and 2-methyl-1,2-propanediol (*A*), 1,2-ethanediol (*B*), 3-methyl-1,2-butanediol (*C*), and 1,3-propanediol (*D*).

propanediol, 1,2-ethanediol, 3-methyl-1,2-butanediol, and 1,3-propanediol.[2] Alkaline and acidic hydrolyses followed by acetalation, as described below, produced cyclic diol acetals *A–D* (Fig. 1), respectively. The yields were determined by gas–liquid chromatography on a polar liquid phase and comparison of peak areas with those of an external 1,3-propanediol acetal standard. Considering the low levels of diol diesters used and the fact that the diol lipids chosen varied widely in structure and stability, the yields (65–93%) are quite satisfactory. If greater accuracy is desired, use of the specific deuterated diol diesters as internal standards and analysis by combined gas–liquid chromatography–mass spectrometry is practical.

The mass spectra of long-chain cyclic acetals of diols are very characteristic and are uniquely suited for the identification of the diol moieties (see Fig. 2). Under

[2] 1,2-Ethanediol and 1,3-propanediol were from Matheson Coleman & Bell, Norwood, Ohio. 2-Methyl-1,2-propanediol and 3-methyl-1,2-butanediol were prepared by lithium aluminum hydride reductions of methyl 2-hydroxyisobutyrate (Aldrich Chemical Co., Milwaukee, Wis.) and 2-hydroxyisovaleric acid (Koch-Light Laboratories, Colnbrook, England), respectively. Long-chain diesters were prepared by procedures similar to those described previously (10).

electron impact, the long-chain cyclic acetal of 1,2-ethanediol, for example, produces *m/e* 73 in an abundance of 65% of the *total ionization* (Fig. 2, *B*). This very prominent ion $[M-C_{15}H_{31}]^+$ is of great diagnostic value, as it contains the intact diol moiety. Favored formation of resonance-stabilized cyclic acetal ions is also reflected in the high abundance of $[M-H]^+$ (1.18%), which is higher than that of the parent ion M^+ (0.28%). Another characteristic ion occurs at *m/e* 45 in 3.03% abundance due to $[M - C_{15}H_{31}CO]^+$.



Its formation involves fragmentation of the ring and, hence, is more likely to be formed from the acetals of branched and nonvicinal diols (see Fig. 2, *A* and *C*). These unique spectral characteristics, especially the prominence of $[M - C_{15}H_{31}]^+$, permit the identification of diol acetals, even when the latter are not completely resolved by gas–liquid chromatography. Quantitations with internal deuterated diol standards are preferentially based on this ion.

The procedure is used routinely in our laboratory for the analysis of lipid-bound diols in biological materials. The method of diol analysis is also of practical significance for the determination of short-chain diols produced from polyunsaturated aliphatic moieties by reductive ozonolysis (11).

(*a*) *Methanolysis.* A mixture of 300 mg of neutral lipids and 15 ml of distilled dry methanol is placed in a 100-ml three-necked flask equipped with nitrogen inlet tube, reflux condenser with drying tube, dropping funnel, and magnetic stirrer. The heterogeneous mixture is heated to reflux temperature, 200 mg of $Ba(OH)_2 \times 8\ H_2O$ (Fisher Scientific, Fairlawn, N.J.) is added, and saponification is continued for 30 min. Then, 9 ml of a methanolic BF_3 solution (14%, w/v) is added to the hot reaction mixture. The reaction is completed within 2–3 min at reflux temperature. After cooling and addition of 1.5 g of NaF (Merck, Rahway, N.J.), stirring is continued for 10 min. Dilution with 75 ml of distilled diethyl ether and stirring for 30 min causes precipitation of most of the inorganic material which is then filtered off on a sintered glass funnel. The salt is washed with 25 ml of a mixture of diethyl ether–methanol 3:1. The solvent is removed under vacuum in a 250-ml three-necked flask that will be used in the subsequent acetalation step in order to avoid losses of polyhydric alcohols.

(*b*) *Acetalation.* Dry benzene, 125 ml, 100 mg of *p*-toluenesulfonic acid, and half of a solution of 300 mg of hexadecanal, prepared by dimethyl sulfoxide oxidation of hexadecyl methanesulfonate (12, 13), in 50 ml of distilled dry benzene are added to the transesterified lipid mixture. The flask is equipped with magnetic stirrer, nitrogen inlet tube, dropping funnel, water separator or distillation head, and condenser, and the mixture is heated to reflux temperature. The second portion of the aldehyde solution is added dropwise over a period of 30 min, then the reaction is continued for another 10 min. During the reaction a total of approximately 50 ml of benzene is distilled off. The mixture is cooled in an ice bath, transferred into a separatory funnel containing an excess of cold aqueous 2% potassium carbonate solution, and extracted with a total of 250 ml of diethyl ether. The organic phase is washed twice with small amounts of water, the solvent is removed under reduced pressure, and the residue is dried carefully in vacuo.

(*c*) *Lithium aluminum hydride reduction.* A mixture of 350 mg of $LiAlH_4$ and 150 ml of anhydrous diethyl ether placed in a 250 ml three-necked flask is refluxed under stirring for 30 min. The products of acetalation, dissolved in 50 ml of anhydrous diethyl ether, are added slowly through a dropping funnel, and the mixture is refluxed for 2 hr. Excess $LiAlH_4$ is destroyed by addition of water-saturated diethyl ether, then a few milliliters of water are added to precipitate the inorganic hydroxide. The ether solution is decanted, the hydroxide is washed with a few portions of fresh ether, and the combined organic phases are washed with water and evaporated to dryness under reduced pressure.

The residue is taken up in hexane and fractionated on six chromatoplates (20 × 20 cm) coated with 3-mm layers of silica gel H (Merck A.G., Darmstadt, Germany), using hexane–diethyl ether 90:10 (v/v) as developing solvent (14). The least polar fraction is eluted with water-saturated diethyl ether and the solvent is evaporated. The residue was further resolved into individual diol acetals and quantified by gas–liquid chromatography using a Hewlett-Packard 5750 instrument with flame ionization detector. The aluminum column, 300 × 0.2 cm I.D., packed with 10% SP-1000 on Gas-Chrom P, 80–100 mesh (Supelco, Bellefonte, Pa.), was operated at 210°C using helium (20 ml/min) as carrier gas.

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