Isolation and characterization of an esterified form of steryl glucoside

MARIUS LEPAGE

Food Research Institute, Canada Department of Agriculture, Ottawa, Canada

SUMMARY A hitherto unreported esterified form of steryl glucoside has been isolated from potato tuber lipids and soybean phosphatides. It is composed of sterol, glucose, and fatty acid in the molar ratio 1:1:1. Four components of the sterol moiety have been detected; they include β-sitosterol and stigmasterol (identified by gas–liquid chromatography). Fatty acids are palmitic, stearic, oleic, linoleic, and linolenic acids. As demonstrated by infrared analysis and periodate oxidation, the fatty acid is located on C_6 of the sugar portion.

In a previous paper (1) the presence of free and esterified forms of steryl glucosides in potato lipid extracts was reported. The occurrence of free steryl glucosides in various plants has been described in several reports (2–6). The esterified steryl glucoside (ESG) whose isolation and characterization are described here was not mentioned, however, in any of these publications. This lipid has been identified in a variety of plant tissues: potato leaves and tubers, peas, soybeans, alfalfa, and wheat (7), and appears to be widely distributed in nature. In the sources so far examined, esterified and nonesterified steryl glucosides are present in about equal amount.

EXPERIMENTAL METHODS AND RESULTS

Proof of Existence of a New Homogeneous Lipid Class

Extraction of Lipids

Total lipids were extracted from potato tubers by homogenizing the tissue in hot 80% ethanol for 2 min, filtering, and twice extracting the residue with chloroform–methanol 2:1 (v/v) for 30 min. The combined extracts were filtered and concentrated in vacuo. The lipid residue was taken up in chloroform and purified by partition between chloroform and water.

A commercial source of soybean phospholipids, containing appreciable quantities of esterified steryl glucoside (ESG), was also used as starting material.

Aliquots of the chloroform-soluble material were subjected to silicic acid thin-layer chromatography (TLC), using diisobutyl ketone–acetic acid–water 80:50:10 (DKAW) as developing solvent. After development, the chromatoplates were sprayed with 20% aqueous perchloric acid (1) and the spots were identified as shown in Fig. 1. ESG constituted about 3.5% of potato lipids.

Isolation of Esterified Steryl Glucoside

ESG was separated from a lipid mixture (10 g) by chromatography on a 5.5 cm o.d. column filled to a height of 14 cm with silicic acid (Mallinckrodt, 100 mesh, analytical reagent). The column was washed according to the scheme in Table 1. TLC of the eluates showed that ESG was eluted quantitatively in fraction III along with traces of triglyceride. Further purification was achieved on a 1.8 × 13 cm column of silicic acid (Bio-Rad, 325 mesh) using 2% methanol in chloroform as eluting solvent, after washing the column with 50% ether in hexane.

After elution, fractions containing ESG were pooled and solvents were evaporated in vacuo. The residual oil (ESG) could not be crystallized, but ran as a single spot of R_F 0.65 in DKAW. It was very soluble in pyridine and ether, slightly soluble in chloroform, but insoluble in hexane, methanol, and ethanol.

Deacylation

Aliquots of the oily residue, of purity established by TLC, were deacylated in 0.1 N methanolic potassium hydroxide for 20 min at 38°. The products of this reaction, as

revealed by TLC, were: fatty acid methyl esters (R_F 0.63 in hexane–diethyl ether–acetic acid 90:10:1 (HEA)), and the deacylated product, which had the same R_F value as steryl glucoside (0.46 in DKAW). This derivative was purified by column chromatography as above, but using 5% methanol in chloroform (Table 1).

To prepare larger quantities of steryl glucoside, a 10-g sample of commercial soybean phosphatides (Nutritional Biochemicals Co.) was deacylated with 0.1 N methanolic potassium hydroxide for 30 min at 40°. Methanolysis was stopped by the addition of 0.1 N acetic acid and the lipids were recovered by repeated chloroform extraction. The combined extracts were concentrated and the lipids were taken up in hexane and placed on the 5.5 cm column used previously. Elution was begun with diethyl ether–hexane 1:1 and continued with chloroform until all neutral lipids (free sterols, free fatty acids, and partially hydrolyzed triglycerides) were completely removed, as shown by TLC. Chloroform–methanol 19:1 then eluted the steryl glucoside. No galactolipids were present. The eluate was evaporated in vacuo and the product was crystallized from pyridine by addition of ethanol. It was identified as steryl glucoside, mp 292–295° (reported mp (8): 293–294°).

Acid Hydrolysis

A 50-mg sample of purified ESG was hydrolyzed with 1 ml of 5% anhydrous HCl–methanol in a sealed tube at 100°. After 4 hr, aliquots were removed and tested by TLC. The main products were identified as free sterols (R_F 0.04 in HEA), fatty acid methyl esters (R_F 0.63 in HEA), and glucose. Smaller quantities of methyl sterols (R_F 0.40 in HEA) and sterol esters (R_F 0.73 in HEA) were also present. These last two compounds were observed to be formed on heating free sitosterol and palmitic acid under these conditions.

The fatty acid methyl esters and freed sterols were analyzed by gas–liquid chromatography (GLC) according to McKillican and Sims (9). A Research Specialties Series 600 gas chromatograph employing a 4 ft × $^3/_{16}$ in. column of 10% (w/w) polyvinyl acetate (AYAL 8285, Union Carbide) on 60–80 mesh Gas Chrom P, with its flash vaporizer at 252°, column at 176°, and argon detector at 210° was used with an argon flow of 45 ml/min for the methyl esters analysis. The sterols were analysed as their trimethyl silyl ethers with the same gas chromatograph operating with a 2 ft × $^3/_{16}$ in. column

TABLE 1 FRACTIONATION OF POTATO LIPIDS

Fractions	Solvents	Volume	Lipids
		liters	
I	Hexane	0.5	Sterol esters, pigment
II	Hexane–ether 1:1	1.0	Triglycerides, fatty acids, sterols
III	Hexane–ether 3:7	0.5	Esterified steryl glucosides
IV	Chloroform	0.5	
V	Chloroform–methanol	0.5	Steryl glucosides and galactolipids

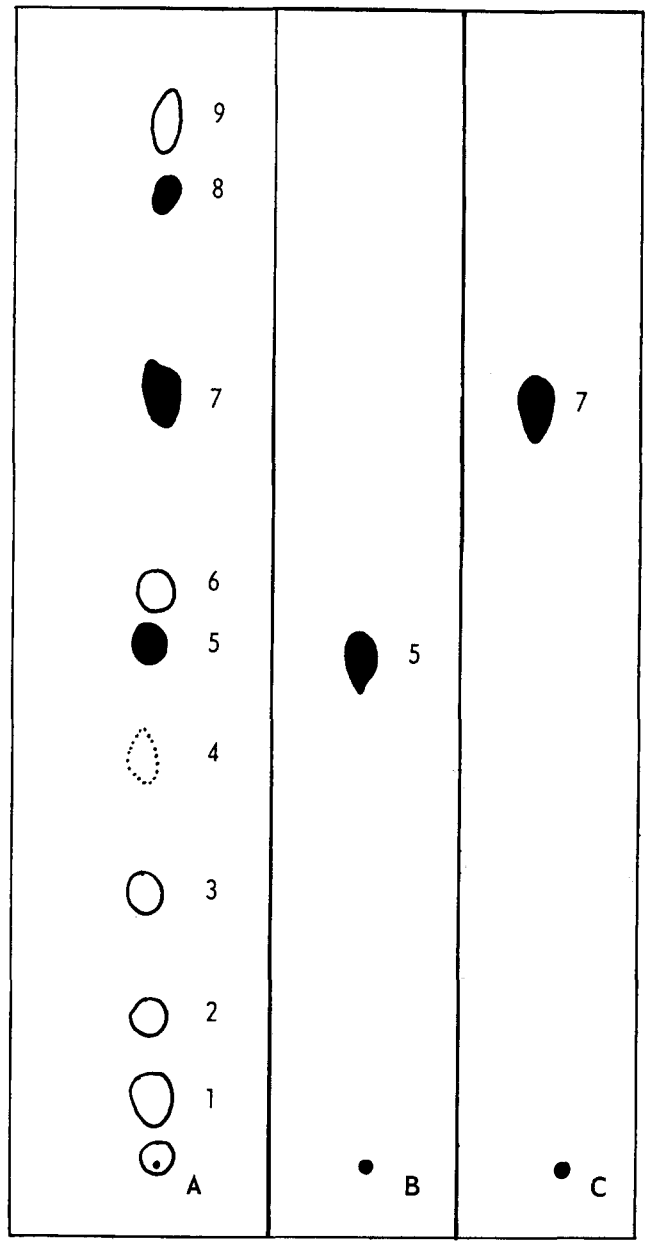

FIG. 1. Tracing of a thin-layer chromatogram of phospholipids and glycolipids. *A*, lipids from potato tubers; *B*, purified steryl glucoside; and *C*, purified esterified steryl glucoside (ESG). The chromatogram was developed in diisobutyl ketone–acetic acid–water 80:50:10 and sprayed with aqueous 20% perchloric acid. Spots were identified as follows: *1*, phosphatidyl inositol and phosphatidyl choline; *2*, digalactolipid; *3*, phosphatidyl ethanolamine; *4*, phosphatidyl glycerol; *5*, steryl glucoside; *7*, monogalactolipid; *7*, esterified steryl glucoside; *8*, free sterol; and *9*, triglycerides, sterol esters, and pigments.

of 3% (w/w) SE-30 silicone on 80–100 mesh Gas Chrom P, a flame ionization detector, its flash vaporizer at 265°, column at 222°, and a nitrogen flow of 83 ml/min. A National Institute of Health test mixture was used as a reference standard for the quantitative determination of the methyl esters, and operating conditions were adjusted daily to produce results in agreement with the supplied composition. Derivatives of pure cholesterol (a gift from Applied Science Laboratory), pure stigmasterol (Sigma Chemical Co.), and at least 90% pure β-sitosterol (Mann Research Laboratories, Inc.) were used as reference standards. Single derivatives were used for measurements of retention time and column and detector efficiencies; mixtures of pure derivatives were used to

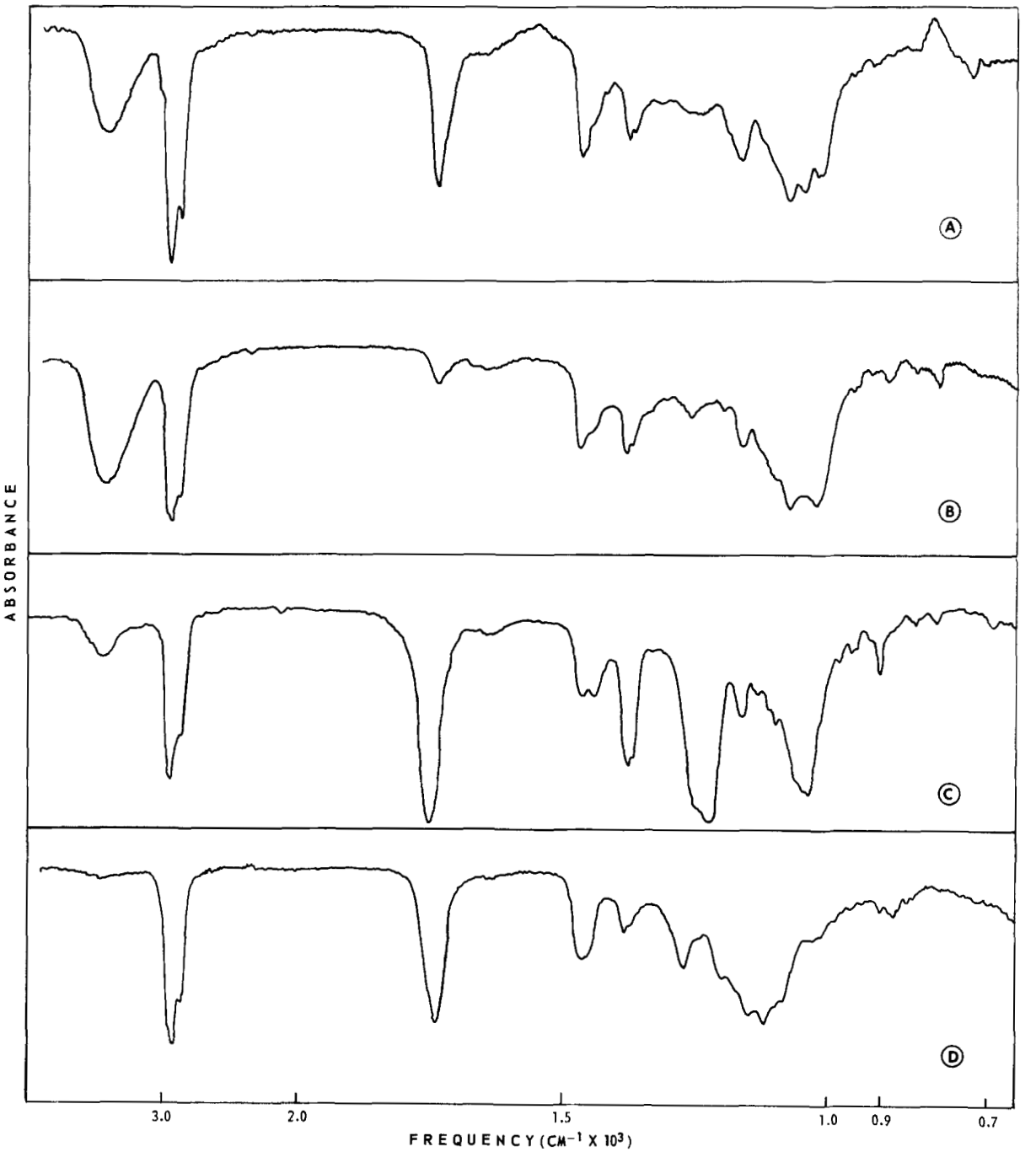

FIG. 2. Infrared spectra of steryl glucosides and derivatives. The esterified steryl glucoside (*A*) was examined in carbon tetrachloride (10.9 mg per 140 μl); the steryl glucoside (*B*) and the steryl glucoside tetraacetate (*C*) in potassium bromide pellets; and the (liquid) methylated esterified steryl glucoside (*D*) between salts.

check on separating power. Glucose was detected by paper chromatography using *n*-butanol–acetic acid–water 4 : 1 : 5 as developing solvent and spraying the chromatograms with aniline hydrogen phthalate.

Tests for Functional Groups

Infrared spectra of ESG (Fig. 2, A) revealed a broad OH group absorption at 3440 cm^{-1}, which was absent from the spectrum of methylated ESG (Fig. 2, D); strong CH_2 absorptions at 2940 and 2860 cm^{-1}; absorptions at 1467, 1445, 1385, and 1370 cm^{-1} attributed to vibrations of methyl and methylene groups in the sterol molecule and the fatty acid chain; and broad absorptions in the region of 1125–1000 cm^{-1} characteristic of sugar. The spectra showed neither amide NH nor CO absorptions at 1640 and 1550 cm^{-1} nor PO absorption at 1650 cm^{-1}, eliminating sphingolipids and phospholipids as possible components of the residue. The main characteristic was a very strong CO absorption at 1740 cm^{-1}, which was also present in the spectra of steryl glucoside tetraacetate (Fig. 2, C) and methylated ESG (Fig. 2, D), which suggested strongly that the unknown steryl glucoside (Spot 5, Fig. 1), was an esterified form of the known steryl glucoside (Spot 5, Fig. 1).

ESG gave negative tests for phosphorus (ammonium molybdate), for amino groups (ninhydrin), and for reducing sugars (aniline hydrogen phthalate); it gave, however, a positive test with periodate–benzidine, periodate–Schiff, anthrone reagent, Liebermann-Burchard reagent, and bromophenol blue, indicating the presence of sugar, sterol, and fatty acids.

TABLE 2 MOLAR RATIO OF THE ESG COMPONENTS

	Sterol	Sugar	Ester Group
Expected	1.0	1.0	1.0
Found	1.0	1.037	1.045

Quantitative determinations of sterol content by the Liebermann-Burchard reaction (10), the sugar by the anthrone reaction (11), and the ester group by the hydroxamic acid test (12) resulted in the establishment of a 1 : 1 : 1 molar ratio as shown in Table 2.

PROOF OF THE STRUCTURE OF ESG AND INDICATION OF HETEROGENEITY WITHIN THE CLASS

Acetylation

A sample (50 mg) of the purified ESG was tested for hydroxyl groups by acetylation with acetic anhydride in pyridine solution. After 30 min at 100° the reaction mixture was diluted with water and extracted with diethyl ether. The ether extract was concentrated and purified by TLC. A major product, R_F 0.89 in DKAW (higher than the intact ESG), was unchanged after further acetylation. It crystallized from hot ethanol and melted at 124–125°.

When a sample of deacylated ESG (potato source) was acetylated under the same conditions, a product of R_F 0.93 in DKAW was obtained, which after crystallization from ethanol had mp 172–173°. Another sample (from soybean phospholipid) treated under the same conditions yielded a product melting at 166–167° [reported mp for steryl glucoside tetraacetate: 164–165° (2) and 171° (4)].

Benzoylation

A 100-mg sample of purified ESG was treated with benzoyl chloride in pyridine solution for 30 min at 100° (4). The mixture was then diluted with water and extracted with dietyl ether. The ether extract was evaporated to dryness and the product obtained had a mp of 35–40°. Thin-layer chromatography showed a single component of R_F 0.07 in HEA and R_F 0.91 in DKAW. Aliquots of this product were treated with 0.1 N methanolic potassium hydroxide for 15 min at 37° and fatty acid methyl esters (R_F 0.67 in HEA) and a derivative of same R_F as steryl glucoside were shown by TLC.

Methylation

The purified ESG was methylated with iodomethane and silver oxide in dioxane (13). The reaction mixture was filtered and evaporated to dryness. The methylated ESG, R_F 0.92 in DKAW, was taken up in chloroform and purified by column chromatography. This derivative displayed on infrared analysis a strong absorption at 1740 cm^{-1} due to its ester group, but the broad OH absorption was absent.

Phenylcarbamoylation

A sample of ESG was treated with phenylisocyanate in dimethyl formamide (14). After heating under reflux for 3 hr, the reaction mixture was diluted with water and the voluminous precipitate filtered, washed with methanol, and dried in vacuo. The main product had R_F 0.93 in DKAW. After purification by column chromatography, it melted at 204–206°.

Analysis: calc for $C_{72}H_{105}O_{10}N_3$ (steryl glucoside palmitate)
C, 73.73; H, 9.03; N, 3.58
calc for $C_{74}H_{107}O_{10}N_3$ (steryl glucoside oleate)
C, 74.00; H, 9.15; N, 3.50
found C, 73.34; H, 9.54, N, 3.78
73.71; 8.55

Hydrolysis of the purified derivative in 0.1 N methanolic potassium hydroxide liberated three products of

R_F 0.70, 0.58, and 0.47 in DKAW corresponding to mono-, di-, and tricarbamoyl derivatives. Fatty acid methyl esters, R_F 0.63 in HEA, were also observed by TLC. In contrast, acid hydrolysis liberated fatty acid methyl esters, free sterols, and a product of R_F 0.00 in DKAW, which was probably the liberated glucose urethan.

Periodate Oxidation

A 10-micromole sample of ESG was oxidized with 0.2 M sodium periodate in 50% aqueous acetone. The acid liberated was continuously titrated by 0.01 N sodium hydroxide with the aid of an automatic titrator.

After 20.5 hr at room temperature, 1.03 moles of formic acid per mole of ESG were produced. This indicated that the fatty acid was attached to C_6 of the sugar portion. If the fatty acid had been attached to any of the other unsubstituted OH groups, i.e., C_2, C_3, or C_4, no formic acid would have been expected.

Sterol Composition

The sterols liberated by acid hydrolysis were analyzed by GLC and tentatively identified by their relative retention times as "Sterol A," 0.78; stigmasterol, 0.85 (15); β-sitosterol, 1.00; and "Sterol B", 1.13. As shown in Table 3, the late-appearing Sterol B was present in potato ESG but not in soybean ESG, whereas with Sterol A, the early-appearing sterol, the converse was observed. β-Sitosterol was the main sterol in both ESG and soybean ESG. McKillican (7) has found only β-sitosterol and Sterol A in wheat ESG.

Fatty Acid Composition

After alkaline hydrolysis, fatty acid methyl esters were purified by TLC and analysed by GLC. The main fatty acids are given in Table 4. The ESG from potato tubers contained approximately equal amounts of saturated and unsaturated fatty acids, whereas the ESG from soybean "lecithin" had a larger proportion of linoleic acid. As these results demonstrate, ESG is very heterogeneous within the class.

DISCUSSION AND CONCLUSIONS

This study has demonstrated the effectiveness of thin-layer chromatography in detecting a previously unnoticed class of plant sterols. This novel lipid class is a mixture of esterified steryl glucosides, each composed of sterol, glucose, and fatty acid in 1:1:1 molar ratio. In spite of demonstrated heterogeneity within the class, discrete ESG could not be resolved even by silicic acid–silver nitrate TLC.

TABLE 3 STEROL COMPOSITION (WT %)*

Source of ESG	Sterol			
	Sterol B	β-Sitosterol	Stigmasterol	Sterol A
Potato tubers	22.6	68.4	9.0	
"Soybean lecithin"		58.7	18.9	22.4

* Percentages are calculated from peak areas using the following factors (unpublished results): 1.1 for β-sitosterol and Sterol A, and 0.9 for stigmasterol and Sterol B.

This new lipid class has been isolated by column chromatography, and its structure established as follows:

1. ESG was nonreducing, contained no phosphorus or amino groups, but gave positive periodate–benzidine, periodate–Schiff, and anthrone reactions, which indicated the presence of sugar bonded through its C_1.

2. Acetylation, benzoylation, methylation, and phenylcarbamoylation were consistent in showing the presence of free hydroxyl groups in the ESG molecule and demonstrating heterogeneity within the class.

3. Release of formic acid by periodate oxidation established the presence of at least three vicinal unsubstituted hydroxyl groups, which could only be the C_2, C_3, and C_4 hydroxyl groups of the sugar portion. This indicated that the fatty acid was linked to C_6 of the sugar. Mycolic acid of "Cord Factor" found in bacteria (16) is also attached to C_6 of sugar.

4. Mention has already been made of the β-configuration of the steryl glucoside (17). Our attempts to hydrolyse the ESG with β-glucosidase, maltase, and amylase were unsuccessful. The general insolubility of ESG made the use of organic solvents necessary and these may have denatured the enzymes. It is also possible that the fatty acid group on C_6 of the sugar interfered with the enzyme action.

On the basis of these observations, we may conclude that the plants so far examined contain free and esterified steryl glucosides, composed of different sterols, fatty acids, and glucose. The sterol is attached directly to C_1 of the sugar, whereas the fatty acid is on C_6 of the sugar part. A steryl 6-acyl-D-glucoside structure is therefore assigned to the new glycolipid class.

The author wishes to express his appreciation to Dr. R. P. A. Sims for advice during the course of this reaearch. He is also

TABLE 4 FATTY ACID COMPOSITION OF ESG

Source of ESG	Fatty Acid					
	16:0	16:1	18:0	18:1	18:2	18:3
Potato tubers	50.7	2.2	9.0	1.2	23.9	4.6
Soybean lecithin	33.7	0.9	7.0	8.8	47.4	2.2

indebted to Messrs. G. Larose and J. Mes for assistance in the preparation of chromatoplates and GLC analyses and to Dr. D. A. Shearer of the Analytical Chemistry Research Service for infrared spectra.

This paper is Contribution No. 16 of the Food Research Institute, Canada Department of Agriculture.

Manuscript received March 19, 1964; accepted June 1, 1964.

References

1. Lepage, M. *J. Chromatog.* **13:** 99, 1964.
2. Matlack, M. B. *J. Am. Pharm. Assoc.* **18:** 24, 1929.
3. Wright, H. E., W. W. Burton, and R. C. Berry. *J. Org. Chem.* **27:** 918, 1962.
4. Swift, L. J. *J. Am. Chem. Soc.* **74:** 1099, 1952.
5. Aylward, F., and B. W. Nichols. *Nature* **181:** 1064, 1958.
6. Carter, H. E., K. Ohno, S. Nojima, C. L. Tipton, and N. Z. Stanacev. *J. Lipid Res.* **2:** 215, 1961.
7. McKillican, M. E. *J. Am. Oil Chemists' Soc.* In press.
8. Kallianos, A. G., F. A. Shelburne, R. E. Means, R. K. Stevens, R. E. Lax, and J. D. Mold. *Biochem. J.* **87:** 596, 1963.
9. McKillican, M. E., and R. P. A. Sims. *J. Am. Oil Chemists' Soc.* **40:** 108, 1963.
10. Carter, H. E., R. A. Hendry, and N. Z. Stanacev. *J. Lipid Res.* **2:** 223, 1961.
11. Bouveng, H. O. *Acta Chem. Scand.* **15:** 87, 1961.
12. Stadman, T. C. In *Methods in Enzymology*, edited by S. P. Colowick and N. O. Kaplan. Academic Press Inc., New York, Vol. 3, 1957, 392.
13. Morris, D. L. *Science* **107:** 254, 1948.
14. Antonis, A. *J. Lipid Res.* **1:** 485, 1960.
15. Eneroth, P., K. Hellström, and R. Ryhage. *J. Lipid Res.* **5:** 245, 1964.
16. Noll, H., H. Bloch, J. Asselineau, and E. Lederer. *Biochim. Biophys. Acta* **20:** 299, 1956.
17. Nielson, B. E., and H. Kofod. *Acta Chem. Scand.* **17:** 1167, 1963.