notes on methodology

Improved determination of glycerol and fatty acids in glycerides and ethanolamine phosphatides by gas–liquid chromatography

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SUMMARY The fatty acid and glycerol content of glycerides were estimated by a simplified hydrogenolysis–acetylation procedure and GLC. When fatty acids interfered in the analysis, glycerol alone was estimated by saponification–acetylation and GLC. Preliminary acetolysis was necessary for the estimation of glycerol in ethanolamine phosphatides by these methods.

The fatty acid composition and ester-to-glycerol ratio of glycerides were recently estimated by hydrogenolysis–acetylation followed by the separation of fatty alcohol acetate and glyceryl triacetate reaction products by gas–liquid chromatography (GLC) (1). Ratios were occasionally high, presumably because of some loss of glyceryl triacetate during the isolation of products. In the present study, simplified procedures are reported which increase the reliability and extend the application of the determination. Glyceryl triacetate and fatty alcohol acetates were isolated directly as the residue after evaporation of a xylene solution under high vacuum. This eliminated refluxing with ethanol and an aqueous wash which were used previously to remove excess acetic anhydride and acetic acid. An internal standard, eicosanyl acetate was used to estimate the absolute glycerol and fatty acid content. A second procedure, saponification–acetylation, was developed. This procedure yields glyceryl triacetate and long-chain fatty acids; the latter remain on the GLC column. It employs hexadecanyl acetate as an internal standard and may be used when only the glycerol content is required; when fatty alcohol acetates which elute with glyceryl triacetate are formed in the hydrogenolysis–acetylation procedure; and when the glyceride contains eicosanoic acid. As an extension of the method, ethanolamine phosphatides were subjected to preliminary acetolysis (2, 3) and their glycerol content was obtained by saponification–acetylation.

Materials. Methyl eicosanoate was purchased from Lachat Chemicals Inc. (Chicago, Ill.). Chimyl dipalmitate and batyl distearate were purchased from California Corporation for Biochemical Research (Los Angeles, Calif.). L-α-Cephalin (chromatographically pure) was purchased from General Biochemicals Inc. (Chagrin Falls, Ohio). A C_8–C_{20} saturated methyl ester test mixture was purchased from Applied Science Laboratories (State College, Pa.). Hexadecanyl acetate was synthesized from methyl palmitate. Other materials were described previously (1). Test samples were analyzed by GLC and the following impurities noted: methyl eicosanoate, 2.2 mole % stearate; distearin, 4.2 mole % palmitate; chimyl dipalmitate, 7.3 mole % stearate; batyl distearate, 12.2 mole % palmitate. All solvents were reagent grade. Egg yolk triglycerides and ethanolamine phosphatides were prepared from a lipid extract (4) of two egg yolks by the method of Horning et al. (5). A 4-cm column packed with 85 g Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pa.) was eluted as described (5). The 60% benzene in hexane fraction was rechromatographed to give triglycerides which were homogeneous as judged by thin-layer chromatography (TLC) (6). Elution was continued with benzene and chloroform and then a 20% methanol in chloroform fraction was isolated. This fraction was rechromatographed on Unisil and diethylaminoethyl cellulose in the acetate form (7) to give ethanolamine phosphatides, which were identified and judged homogeneous by TLC (8). Fatty acid ester (9) and phosphorus (10) were determined.

Gas-Liquid Chromatography. Analyses were obtained with an Aerograph A-350-B gas–liquid chromatograph[1] equipped with a Wheelco Type A electronic integrator.[2] A 10-ft stainless steel column, 0.25 inch i.d., containing 10% ethylene glycol succinate polyester (EGS) on 60-80 mesh Gas Chrom P[3] was used; temperatures, 170–200°; carrier gas, helium; flow rate, 100 ml/min. Peak areas were corrected for molar response relative to hexadecanyl acetate taken as 100 (11). Since a different chromatograph and higher flow rates were used in this study, response values for fatty alcohol acetates and glyceryl triacetate were redetermined. The results were in agreement with previous data (1, 11) with the exception of glyceryl triacetate (relative molar response, 71). This suggests that response data within a homologous series may be applied with different instruments and under different conditions. The relative response between other compounds may be altered. The relative molar response for commercial methyl eicosanoate, 102, was lower than

[1] Wilkens Instrument and Research, Inc., Walnut Creek, Calif.
[2] Barber-Colman Co., Rockford, Ill.
[3] Applied Science Laboratories, State College, Pa. The relative retention time for glyceryl triacetate varied for different batches of EGS.

TABLE 1 THE FATTY ACID AND GLYCEROL CONTENT OF GLYCERIDE SAMPLES ESTIMATED BY HYDROGENOLYSIS–ACETYLATION AND GLC*

Sample†	14:0		16:0		18:0		Glycerol		Ester/ Glycerol‡
	Known	Found	Known	Found	Known	Found	Known	Found	
				μmoles§					
Monopalmitin			254	266 ± 8	8	10 ± 2	254	256 ± 10	1.0
Monostearin					274	276 ± 16	268	263 ± 20	1.0
Dimyristin	268	292 ± 18			Not measured		134	130 ± 8	2.2
Dimyristin	212	214 ± 6			15	12 ± 4	106	103 ± 5	2.1
Dipalmitin			377	388 ± 19	Not measured		188	177 ± 8	2.2
Dipalmitin–dimyristin	340	356 ± 14	322	334 ± 18	Not measured		331	321 ± 14	2.1
Trimyristin	246	266 ± 5			12	11 ± 2	82	82 ± 8	3.2
Tripalmitin			401	389 ± 10	11	15 ± 3	134	122 ± 6	3.2
Chimyl dipalmitate			146	132 ± 4	20	20 ± 3			
Batyl distearate			18	18 ± 1	134	133 ± 5			

* Methyl eicosanoate was the internal standard.
† Sample and internal standard impurities were allowed for in the calculation of "known lipid content."
‡ 18:0 impurity from internal standard omitted.
§ Mean ± standard deviation.

that calculated (1, 11), while a pure laboratory sample gave the expected value. Thus commercial samples should be purified or calibrated before they are used as standards.

Hydrogenolysis–acetylation. From 30 to 100 mg of glyceride and a known amount of methyl eicosanoate were dissolved in 20 ml of dry ether and placed in a 100 ml round-bottomed flask. About 200 mg of lithium aluminum hydride was dissolved in 30 ml of dry ether. The residue was allowed to settle and the solution transferred by pipette, 1 ml at a time, to the glyceride solution until boiling stopped. A volume excess of this solution was then added and the mixture refluxed for 60–90 min. Acetic anhydride was added dropwise to decompose excess lithium aluminum hydride, followed by 25 ml of acetic anhydride and 30 ml of xylene (bp 138.5–140°). Ether was removed by boiling with the flask open. When the vapor temperature rose to 110–115°, the flask was closed and the contents were refluxed for 6 hr. The mixture was filtered and the filtrate evaporated at 55° for 60 min using first a water-pump and then high vacuum. The residue was dissolved in dry ether for GLC. If early extraneous peaks were found to interfere in GLC, xylene was added, and vacuum evaporation repeated.

TABLE 2 DUPLICATE ANALYSES OF EGG YOLK TRIGLYCERIDE BY HYDROGENOLYSIS–ACETYLATION AND GLC*

Component	Sample A		Sample B	
	μmoles/ml	mole %	μmoles/ml	mole %
16:0	3.3 ± 0.2†	26.0	3.3 ± 0.1†	25.6
16:1	0.3 ± 0.1	2.4	0.4 ± 0.1	2.9
18:0	1.3 ± 0.2	10.0	1.3 ± 0.1	10.2
18:1	6.9 ± 0.3	54.1	6.8 ± 0.2	52.6
18:2	0.9 ± 0.1	7.5	1.1 ± 0.1	8.7
Glycerol	4.3 ± 0.2		4.2 ± 0.2	
Total ester	12.7		12.9	
Ester/Glycerol	3.0		3.1	
Total ester‡	13.1 ± 0.8		13.1 ± 0.8	

* Methyl eicosanoate was the internal standard.
† Mean ± standard deviation.
Colorimetric analysis (9).

Hydrogenolysis–acetylation of a C_8 through C_{20} methyl ester test mixture gave quantitative results with C_{10} through C_{20} acetates even after prolonged vacuum evaporation, while octyl acetate was recovered in low yield. Mean ester-to-glycerol ratios for five samples of monoglycerides, three of diglycerides, and four of triglycerides were 1.04, 2.12, and 3.07, respectively. Data obtained with an internal standard on the fatty acid and glycerol content of glyceride and diacyl glyceryl ether samples are summarized in Table 1. Typical mean recoveries in this series were 97 and 101% for glyceryl triacetate and hexadecanyl acetate respectively. Duplicate analyses of an egg yolk triglyceride solution are shown in Table 2. The alcohol acetate data were similar to methyl ester data for egg yolk triglycerides (12). Minor peaks with longer retention times, presumably polyunsaturated fatty alcohol acetates, were observed in our study; however, they were not identified or included in the calculations. The total ester content obtained with GLC agreed with total ester estimated by a colorimetric method.

Saponification–Acetylation. From 30 to 100 mg of glyceride and a known amount of hexadecanyl acetate were dissolved in 30 ml of methanol and placed in a round-bottomed flask. About 25 mg of sodium was dissolved in 10 ml of methanol and added to the solution. After refluxing for 2 hr, methanol was evaporated using a water-pump. Water (5 ml) was added and the mixture was refluxed for 60 min. Acetic anhydride (35 ml) and 35 ml of xylene were added and the acetates prepared and isolated as described above. A larger volume of acetic anhydride

TABLE 3 GLYCEROL CONTENT OF GLYCERIDE AND PHOSPHOLIPID SAMPLES ESTIMATED BY SAPONIFICATION–ACETYLATION AND GLC*

Sample	Glycerol		Recovery
	Known	Found	
	μmoles	*μmoles*	%
Monopalmitin	303†	314 ± 16‡	104
Tripalmitin	125	129 ± 4	103
Cephalin	75	77 ± 4	103
Cephalin	105	109 ± 4	104
Ethanolamine phosphatide	69	65 ± 4	94
Ethanolamine phosphatide	69	65 ± 6	94

* Hexadecanyl acetate was the internal standard.
† Estimated from weight or phosphorus content.
‡ Mean ± standard deviation.

was used here to remove water from the saponification mixture.

Results obtained by saponification–acetylation are summarized in Table 3. Only glyceryl triacetate and the alcohol acetate standard were eluted in GLC. Recovery data were similar to those obtained by hydrogenolysis–acetylation.

Quantitative glycerol data were not obtained with phospholipids analyzed by hydrogenolysis–acetylation or saponification–acetylation. Glyceryl triacetate yields were low and variable. Commercial cephalin and egg yolk ethanolamine phosphatides were, therefore, subjected to acetolysis (2, 3). From 60 to 100 mg of phospholipid and a known amount of hexadecanyl acetate were refluxed with 10 ml of acetic acid–acetic anhydride (4 : 1, v/v) for 8 hr and the products isolated by vacuum evaporation. Quantitative glycerol data were then obtained by saponification–acetylation (Table 3). In several experiments, choline phosphatides (egg yolk) were found to give low glycerol recoveries with acetolysis followed by saponification–acetylation.

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REFERENCES

1. Horrocks, L. A., and D. G. Cornwell. *J. Lipid Res.* **3:** 165, 1962.
2. Bevan, T. H., D. A. Brown, G. I. Gregory, and T. Malkin. *J. Chem. Soc.* 127, 1953.
3. Hoefnagel, M. A., A. Van Veen, and P. E. Verkade. *Rec. Trav. Chim.* **81:** 461, 1962.
4. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
5. Horning, M. G., E. A. Williams, and E. C. Horning. *J. Lipid Res.* **1:** 482, 1960.
6. Malins, D. C., and H. K. Mangold. *J. Am. Oil Chemists' Soc.* **37:** 576, 1960.
7. Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller, and J. S. O'Brien. *J. Am. Oil Chemists' Soc.* **38:** 544, 1961.
8. Horrocks, L. A. *J. Am. Oil Chemists' Soc.* **40:** 235, 1963.
9. Stern, I., and B. Shapiro. *J. Clin. Pathol.* **6:** 158, 1953.
10. Lowry, O. H., N. R. Roberts, K. Y. Leiner, M.-L. Wu, and A. L. Farr. *J. Biol. Chem.* **207:** 1, 1954.
11. Horrocks, L. A., D. G. Cornwell, and J. B. Brown. *J. Lipid Res.* **2:** 92, 1961.
12. Privett, O. S., M. L. Blank, and J. A. Schmit. *J. Food Sci.* **27:** 463, 1962.