notes on methodology

Gas–liquid chromatography of ethyl ester artifacts formed during the preparation of fatty acid methyl esters

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Summary Ethanol is typically used as a stabilizer in chloroform. Failure to remove this ethanol from the chloroform used in the extraction of lipids leads subsequently to the formation of ethyl ester artifacts during the preparation of methyl esters by a commonly employed transesterification procedure. Depending on the conditions and phases used during gas–liquid chromatography, the ethyl esters may be resolved from the corresponding methyl esters. The resulting chromatograms contain extraneous peaks and may be incorrectly identified.

Supplementary key word retention times

A simple and rapid method for the extraction of triacylglycerols from fatty materials rich in these compounds, such as adipose tissue, is to grind or homogenize the material with chloroform in the presence of anhydrous sodium sulfate to remove water. The chloroform solution containing extracted lipid is then filtered. The rapid transesterification method of Glass and Christopherson (1) may then be applied directly to the filtered chloroform extract to prepare fatty acid methyl esters for gas–liquid chromatography (GLC). This method involves the use of sodium methoxide in methanol to catalyze the formation of the methyl esters. However, it must be remembered that ethanol is normally added to stabilize chloroform and that, unfortunately, some manufacturers do not specify this on the product label. Under the conditions of transesterification, sodium methoxide catalyzes transethylation as well as transmethylation in mixtures containing chloroform–methanol–ethanol. In the original Glass and Christopherson procedure (1) the volume of chloroform used is small, so if the ethanol has not been removed the possible formation of ethyl esters may not be noticed. However, when chloroform is used to extract triacylglycerols the ratio of ethanol to methanol in the transesterification step may be much greater, so significant amounts of ethyl esters are produced. The problem can be readily overcome by washing the chloroform with water just before use. When the chloroform is used in the Bligh and Dyer procedure (2) for extracting lipid, any ethanol present is removed in the aqueous phase during the course of the extraction, thus eliminating the problem.

An aspect of particular interest concerns the behaviour of the ethyl and methyl esters during GLC on different stationary phases and at temperatures that allow good resolution of the methyl esters. It

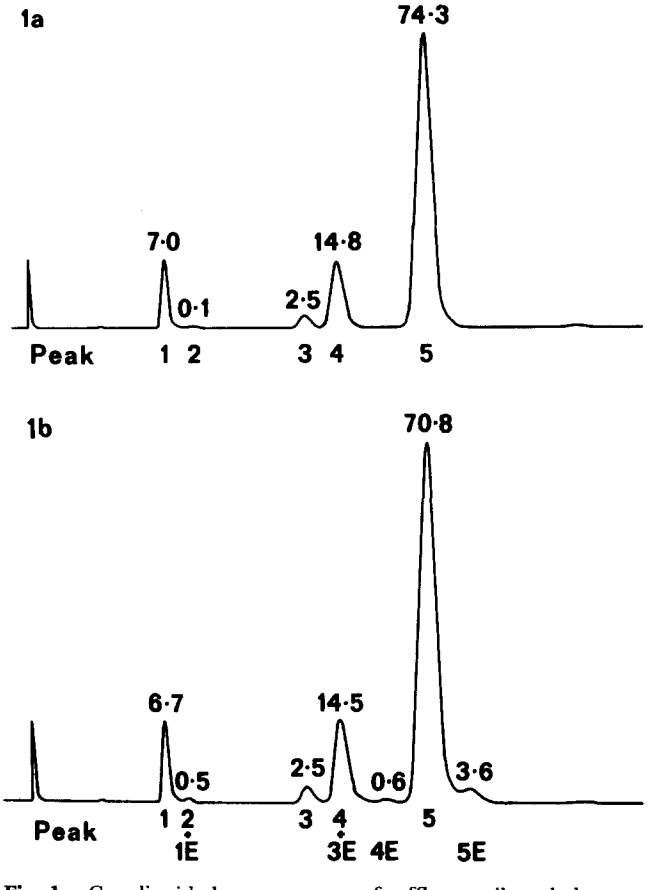

Fig. 1. Gas–liquid chromatograms of safflower oil methyl esters prepared in the presence of chloroform that had been either washed with water to remove traces of ethanol (Fig. 1a), or not washed with water (Fig. 1b). In each case peaks 1, 2, 3, 4, and 5 are, respectively, 16:0, 16:1, 18:0, 18:1 and 18:2 (methyl esters), while peaks 1E, 2E, 3E, 4E, and 5E are ethyl esters corresponding to 1, 2, 3, 4, and 5. The amount of each peak (wt percent as determined by an Infotronics CRS 208 Integrator) is shown above the peak. Phase 10% SP-222-PS at 155°C.

Abbreviation: GLC, gas–liquid chromatography

TABLE 1. Effect of column temperature on retention times of methyl and ethyl esters of some fatty acids during GLC on SP-222-PS

Fatty Acid Ester	Retention Time Relative to Methyl Stearate				
	160°C	170°C	180°C	190°C	200°C
16:0 Me[a]	0.51	0.53	0.55	0.58	0.60
Et	0.58	0.59	0.60	0.62	0.61
18:0 Me	1.00	1.00	1.00	1.00	1.00
Et	1.18	1.16	1.10	1.09	1.04
18:1 Me	1.13	1.14	1.16	1.16	1.17
Et	1.28	1.25	1.26	1.24	1.20
18:2 Me	1.44	1.45	1.49	1.49	1.52
Et	1.57	1.56	1.57	1.54	1.52

[a] 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid. Me signifies methyl ester, Et signifies ethyl ester.

was not possible to separate ethyl esters from the corresponding methyl esters on a column of 17% DEGS on Chromosorb W (Hewlett Packard 5700A) even when different temperatures (173°C and 190°C) were used for the analysis. However, it was possible to separate corresponding fatty acid ethyl and methyl esters when the analysis was carried out on 10% SP-222-PS or 10% SP-2340 on 100/120 Chromosorb W.AW (Supelco, Bellefonte Pa.), (Packard Model 7401 with glass U-tube columns 180 mm long × 2 mm ID). In this laboratory, columns containing SP-222-PS were operated at temperatures between 165°C and 175°C, since the optimum operating temperature varied slightly between different batches of SP-222-PS. At these temperatures ethyl esters were readily resolved from methyl esters. The effect was clearly demonstrated by reducing the column temperature to about 155°C (**Fig. 1**). When the temperature at which the analysis was carried out was increased to 200°C the separation of corresponding ethyl and methyl esters became increasingly difficult (**Table 1**).

During the quantitative analysis of fatty acids by GLC, the presence of ethyl esters in the methyl esters can affect the accuracy of the results. The peaks due to ethyl esters may be incorrectly attributed to unusual fatty acids, or they may coincide with genuine methyl esters. For example, ethyl stearate and methyl oleate are coincident at 170°C (Table 1), so the analysis would underestimate the level of stearic acid and overestimate the level of oleic acid.

TABLE 2. Fatty acids of beef adipose tissue lipids extracted either with water-washed chloroform or with non-washed chloroform[a]

Fatty Acids beyond C16:0	Percent Total Fatty Acids	
	Washed Chloroform	Non-washed Chloroform[b]
16:0	25.2	24.6^{-}
16:1 *trans*/16:0 ethyl	0.8	1.6^{+}
16:1 *cis*	2.6	2.6
17:0	1.9	1.9
17:1/16:2	0.7	0.7
18:0	24.3	23.4^{-}
18:1/18:0 ethyl	33.6	$31.8^{+,-}$
18:1 ethyl		2.2^{+}
18:2	3.2	2.7
20:0	0.2	0.2
18:3/20:1	1.1	1.2

[a] As determined by GLC of the fatty acid methyl esters on 10% SP 2340 at 185°C, using an Infotronics CRS 208 integrator to determine peak areas.

[b] A minus sign indicates that the value was low because the corresponding ethyl ester appeared in the following peak. A plus sign indicates that the value was high because of the contribution of the ethyl ester from the preceding peak.

The problem caused by the presence of ethyl esters becomes more acute when cyanosilicone phases such as SP 2340 are used. These phases are capable of resolving *cis*- and *trans*- isomers of fatty acid methyl esters, and peaks due to ethyl esters may be mistaken for *trans*- unsaturated methyl esters. (**Table 2**).

For the reasons outlined above, users of the recently-developed highly polar phases for GLC should ensure that ethanol is removed from chloroform used in the extraction of lipid and the preparation of methyl esters.

REFERENCES

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