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Note

Analysis of labelled fatty acid methyl esters by argentation and reversed-phase two-dimensional thin-layer chromatography

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In a study of the metabolism of fatty acids in marine invertebrates, we needed to analyse individual fatty acids having low radioactivity. Normally, this is done by means of gas–liquid chromatography (GLC) with subsequent scintillation counting of the collected fractions, or by use of special radioactivity detectors[1,2]. Both methods, however, are not without shortcomings. The collection of fractions after GLC results in substantial loss of substance, while gas chromatographic detectors of radioactivity lack sufficient sensitivity, are quite expensive and not too widely used. Besides, it is common knowledge that the polar phases usually used for GLC of fatty acid methyl esters (FAMEs) result in peak overlapping, which complicates analysis. Therefore, labelled FAMEs are often separated according to the number of double bonds, using plates impregnated with silver nitrate[3].

The early sixties witnessed quite a significant development in fatty acid analysis by use of thin-layer chromatography (TLC)[4,5], which was later superseded by GLC. Based on a recently developed high-performance TLC method involving a firmly fixed sorbent layer[6], we now propose a technique for analysing labelled fatty acids that is essentially comprised of two-dimensional TLC on silica gel impreganted with silver nitrate in the first direction, and reversed-phase chromatography in the second direction.

EXPERIMENTAL

Materials

Labelled [1-^{14}C]acetate (50 mCi/mmol), [1-^{14}C]oleate (56 mCi/mmol) and [1-^{14}C]stearate (56 mCi/mmol; Isotope, U.S.S.R.) were used. The FAMEs of sea urchin *Strongylocentrotus intermedius* embryos labelled with [1-^{14}C]acetate at the pluteus stage were used to determine radioactivity. For TLC and GLC, we also used the FAMEs of a lipid extract from rat liver.

TLC

The preparation of silica gel and plates with the sorbent layer firmly fixed with silica sol is described elsewhere[6,7]. Similar results were also obtained using Kieselgel 60 plates (Merck, F.R.G.). The plate (6 × 6 cm) edge was impregnated to a width

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of 1.5 cm with silver nitrate by dipping it in methanol saturated with silver nitrate. Before use, the plates were activated for 10–15 min at 100°C. The separation of FAMEs according to the number of double bonds was performed according to Dudley and Anderson[8]. Following separation, the silver nitrate was washed off with a saturated sodium thiosulphate solution (for 0.5 min) and then with running water (for 3 min). Then the plate was dried in a stream of warm air for 12–15 min. To impregnate the plate with decane, it was dipped in a 5% decane solution in pentane for a short time (3–5 sec) and, following pentane evaporation, developed in the system

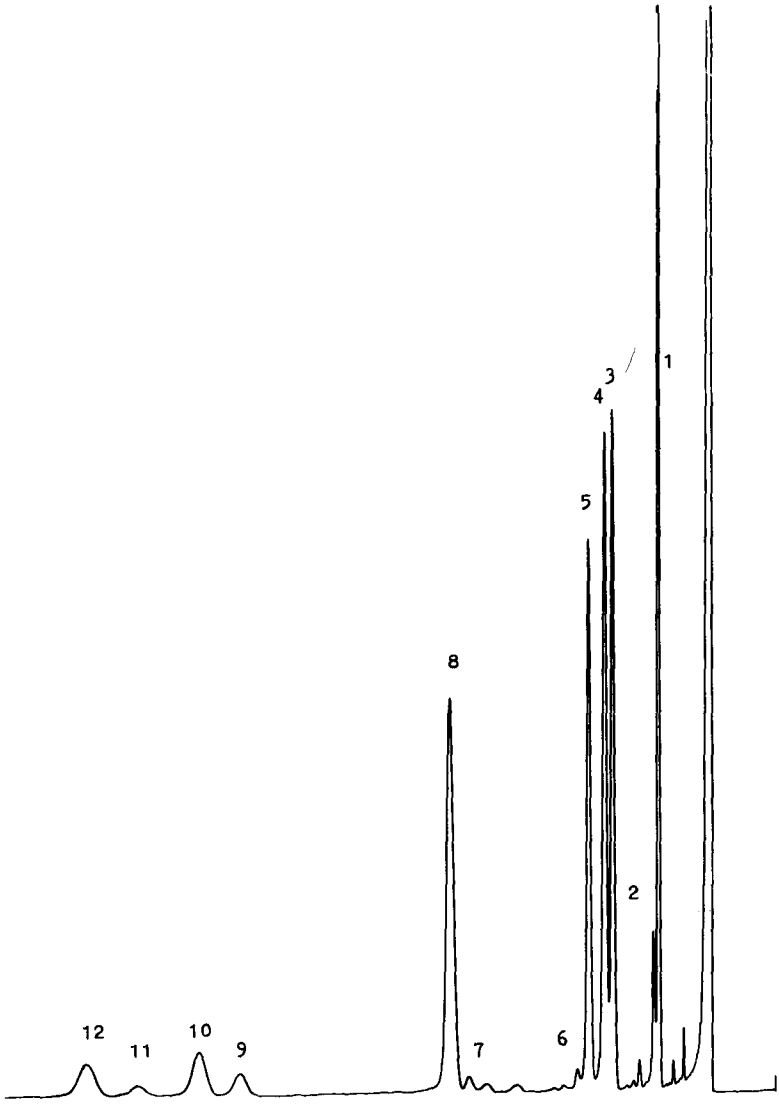

Fig. 1. GLC of FAMEs from rat liver lipids. Chromatographic conditions as indicated in the text. Peaks: 1, 16:0; 2, 16:1; 3, 18:0; 4, 18:1; 5, 18:2; 6, 18:3ω3; 7, 20:3ω6; 8, 20:4ω6; 9, 22;4ω6; 10, 22:5ω6; 11, 22:5ω3; 12, 22:6ω3.

methanol–water (95:5, v/v), 90% saturated with decane. Following separation, the decane was removed by use of a fan 10 min. The FAME-containing zones were detected by use of iodine vapours, collected and their radioactivity determined using a scintillation spectrometer (Intertechnique SL-30, France) accordding to Pyrovolakis *et al.*[9].

GLC

FAMEs were obtained using the Carreau and Dubacq[10] technique and then separated on a glass column (4 m × 3 mm) filled with 3% Carbowax 20M on Chromaton N: separation temperature 215°C; carrier gas (helium) flow-rate 40 ml/min. A Shimadzu 5 GC (Japan) chromatograph was used.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the results of separation of FAMEs from rat liver total lipids according to TLC and GLC. The number of spots on the thin-layer chromatogram corresponds to the number of peaks on the gas–liquid chromatogram. The use of plates with a firmly fixed sorbent layer[6] permits the employment of techniques applied in chromatography on paper impregnated with silica gel[11]. Such plates may be dipped in various aqueous reagents[6] without destroying the layers, and this allows, following TLC on silica gel with silver nitrate, washing with a sodium thiosulphate

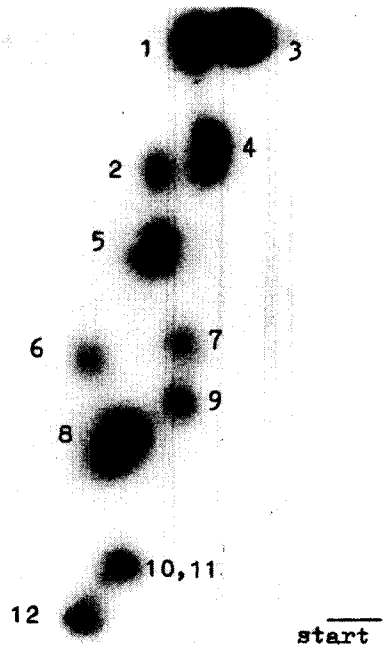

Fig. 2. Two-dimensional TLC of FAMEs from rat liver lipids (100 μg): first direction (vertical), argentation chromatography; second direction (horizontal), reversed-phase chromatography. Conditions as indicated in the text. Detection: 10% sulphuric acid in methanol, followed by heating at 180°C. Designations as in Fig. 1.

TABLE I

EFFECT OF TIME OF WASHING OF THIN-LAYER PLATES ON THE RECOVERY OF LABELLED FAMEs

Washing time (min)		*FAME radioactivity (cpm)*		*Recovery (%)*
Thiosulphate	*Water*	*Spotted on plate*	*Following TLC*	
0.5	3	15 737	15 637	99.4
1.0	5	77 055	70 058	90.9
2.0	10	19 517	16 774	85.9

solution in order to remove the silver nitrate which would interfere with subsequent procedures. Experiments were performed to show that the degree of impregnation with silver nitrate under the given conditions was 8%, and with decane, 10–12%. Decane is readily removable from the plates, and the separated substances may then be studied by various physico-chemical methods.

Experiments with individual labelled FAMEs (16:0, 18:0 and 18:1) and mixtures obtained after introducing [1-^{14}C]acetate into sea urchin embryos showed that over 90% of the radioactivity was recovered (Table I). In chromatography on silica acid-impregnated paper, the substances were revealed by dipping the paper in various reagents and then washed off with water. Though phospholipids and glycolipids are much more polar than FAMEs, their loss appears to be quite insignificant[11].

In 1964, Bergelson *et al.*[4] published a detailed technique for analysing and identifying FAMEs by means of two-dimensional TLC. They first performed reversed-phase chromatography, then removed dodecane by quite a rigid and lengthy procedure, impregnated part of the plate with silver nitrate and then performed a second separation. The detection method used was essentially destructive, and the analysis required as much as 50 μg of each component. Our procedure is characterized by a quite high sensitivity (analysis requires 50–100 μg of FAME complex mixture), is relatively simple and cheap and permits non-destructive detection. At the same time, the resolving power is close to that of GLC on packed columns.

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