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THE RAPID SEPARATION OF FATTY ACIDS FROM FOSSIL LIPIDS BY IMPREGNATED ADSORBENT THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The separation of fatty acids from complex fossil lipid mixtures is described. The technique developed uses thin-layer plates coated with silica gel containing potassium hydroxide; the method is compared with a standard column technique. It is also shown that glycerides and fatty acid methyl esters are not saponified under the conditions of analysis.

INTRODUCTION

The use of thin-layer chromatography in examining biological lipids is well established. The technique has been extended by the use of impregnated adsorbents, for example, silica gel containing silver nitrate for the separation of unsaturated compounds[1,2], borate for hydroxy compounds[2], oxalic acid for some phospholipids[3], urea for fatty acids[4], ferrous sulphate for porphyrin esters[5], and aromatic polynitro compounds for aromatic hydrocarbons[6].

The isolation and identification of bound and free fatty acids from the lipid fraction of a variety of geological sources including soil, petroleum, peat, lignite, mud, and both recent and ancient sediments has been well documented. We have previously discussed these occurrences[7] and PARKER[8] has recently reviewed the subject.

Perhaps the simplest and most rapid method of separating free fatty acids from complex mixtures of lipids is that devised by MCCARTHY AND DUTHIE[9] who used a column packing of silica gel containing potassium hydroxide. Neutral lipids were removed from the column with diethyl ether and the free acids were subsequently removed with a diethyl ether–formic acid mixture.

The many advantages associated with thin-layer chromatography suggested it as a method for the separation of free fatty acids from lipid extracts of sediments and this communication describes separations which have been made with plates coated with silica gel containing 2–3 % of potassium hydroxide. The fatty acids are not desorbed from the silica gel, but rather they are methylated with boron trifluoride–methanol directly by a method similar to that described by METCALFE *et al.*[10]. The methyl esters are subsequently examined by gas–liquid chromatography.

MATERIALS

All solvents were of Analar grade and were distilled prior to use. For the rapid evaluation of solvent systems, microscope slides 7.5 × 2.5 cm and 7.5 × 5.0 cm were coated with a slurry prepared from one part of silica gel (Kieselgel G, Merck) plus two parts of a 3 % solution of potassium hydroxide in distilled water. Analytical and preparative plates (20 × 20 cm) were coated with the same mixture to give layers 0.25 mm and 0.5 mm thick respectively. Air dried plates were activated at 110–120° for 1–2 h; deactivated plates were prepared by exposing the activated plates to the atmosphere for *ca.* 24 h. Plates were developed in jars or tanks lined with filter paper and equilibrated with the developing solvent. Plates were visualised by spraying with a 0.2 % ethanolic solution of 2′,7′-dichlorofluorescein. For the reference chromatographic column, silica gel impregnated with potassium hydroxide was made according to the procedure of McCARTHY AND DUTHIE[9] without treating the silica gel to remove fines; Whatman's column Chromedia SG-31, 100–200 mesh was used. Gas chromatographic conditions are given in the figure legend. *n*-Fatty acids (B.D.H., specially pure) were esterified with BF_3–MeOH and standard mixtures of normal and branched esters (Applied Science Laboratories BC Mix L, containing C_{14}–C_{17} esters and BC Mix 4 containing C_{27}–C_{31} esters) were used.

METHODS AND RESULTS

Preliminary experiments to find a suitable solvent system were made on microscope slides using a 10% solution of stearic acid in crude mineral oil as sample. Inspection of the slides in U.V. light showed that when using acetone, ethanol, chloroform, ether or ethyl acetate as developer only the last three solvents moved the brightly fluorescent oil to the solvent front. Analyses of some carboxylic acids and hydroxy compounds on analytical (20 × 20 cm) plates showed (Table I) that for some polyhydroxy compounds chloroform and ether gave R_F values which were too low, and unsatisfactory for the required separations; better results were obtained using a mixture of ether–methanol (95:5). Comparable separations were obtained when the impregnated plates were 'deactivated' by exposure to the atmosphere for *ca.* 24 h; this is shown in Table I where the R_F values are given in parentheses.

The separation and recovery of stearic acid (14.1 mg) from crude mineral oil (127 mg) was achieved by applying the above mixture to a 0.5 mm preparative plate and developing with ether–methanol (95:5). The origin was removed as a 1 cm band, transferred to a small flask and boiled under reflux with BF_3–MeOH (3 ml) for 5 min. The reaction mixture was extracted three times with 30 ml of petroleum ether–ether (95:5), evaporated and filtered through a column of Florisil (2.5 × 0.5 cm). The ester (14.4 mg, 98 % recovery) was recovered by evaporation; its I.R. spectrum was identical to that of methyl stearate and gas–liquid chromatography afforded only one peak which was enhanced by co-injection with methyl stearate. In another experiment stearic acid (10.9 mg) was added to a rock extract (102 mg) prepared in the following manner. Finely powdered shale (100 g) from the Carboniferous Coal Measure deposits of Northumberland was extracted in a Soxhlet apparatus with benzene–methanol–chloroform (1:1:1) to give a dark gum, which was boiled with 5 % sodium hydroxide solution. Extraction of this alkaline solution with petroleum ether provided the

TABLE I

R_F VALUES OF SOME CARBOXYLIC ACIDS AND HYDROXY COMPOUNDS ON SILICA GEL PLATES IMPREGNATED WITH POTASSIUM HYDROXIDE

Figures in parentheses are for plates which were deactivated by exposure to the atmosphere for *ca.* 24 h.

Developer	*Tri-palmitin*	*Dipalmitin*	*Mono-palmitin*	*Stearic acid*	*Methyl stearate*	*Phenol*	*Resorcinol*	*Pyrogallol*	*Phthalic acid*	*Trimesic acid*	*Abietic acid*
Chloroform	0.79	0.36	0.04	0.04	0.65	0.26 (0.27)	0.04 (0.03)	0 (0)	0 (0)	0 (0)	0 (0.04)
Ether	0.92	0.71	0.10	0.02	0.78	0.59 (0.62)	0.49 (0.50)	0.15 (0.18)	0 (0)	0 (0)	0.04 (0.05)
Ether–methanol (95:5)	0.93 (0.83)	0.86 (0.71)	0.40 (0.33)	0.04 (0)	0.87 (0.76)	0.70 (0.78)	0.60 (0.67)	0.29 (0.26)	0 (0)	0 (0)	0.08 (0.07)

acid free material used for admixture with stearic acid. Chromatography of this mixture on a potassium hydroxide–silica gel plate, followed by methylation and filtration through Florisil as described above, afforded the pure ester (9.9 mg, 92 % recovery). The purity of the ester was established by co-injection gas–liquid chromatography and by recording its I.R. spectrum, as described above.

Fatty acids isolated from Green River Shale (Eocene, ca. 60 × 10⁶ years)

Pieces of Green River Shale with surfaces cleaned ultrasonically in a mixture of toluene–methanol (1:1), were broken into small lumps and powdered in a disc mill. The powder (118 g) was boiled under reflux for 24 h with methanol (400 ml) containing water (20 ml) and potassium hydroxide (40 g), cooled, filtered and washed with methanol. Washings and filtrate were combined, diluted with an equal volume of water and extracted with ether (200 ml, × 3). The aqueous methanolic solution was acidified with hydrochloric acid to pH 2 and extracted with petroleum ether–ether (90:10, 200 ml, × 3) to give a dark gum (336 mg). The I.R. spectrum (thin film) of this extract showed that it contained free fatty acids since there was a broad absorption band at *ca.* 2500–3400 cm^{-1} and there were also bands at 2920, 2850, 1709, 1462, 1413, 1378, 1368, 1285, 1225, 945, 726 and 717 cm^{-1}. This gum was dissolved in ether and two aliquots, each containing 84 mg, were used for the separation of the fatty acids by (a) the KOH plate method described above, and (b) the column method of McCARTHY AND DUTHIE.

(*a*) *Plate method.* The above extract (84 mg) in about 1 ml ether was loaded on a preparative plate containing 3 % KOH and developed with ether–methanol (97:3). A 1 cm band at the origin was removed and methylated directly with BF_3–MeOH (4 ml) as described earlier. Water (20 ml) was added and the crude esters (34.4 mg) were extracted. Analysis of the crude esters by analytical thin-layer chromatography on normal silica gel plates (developer petroleum ether–ether, 90:10) showed an extended band with an R_F value of 0.5–0.7 (n-C_{14} ester = 0.53, C_{27}–C_{31} Applied Science standard mixed esters, BC4 = 0.64) and a number of spots with lower R_F values. The range of R_F values obtained by thin-layer chromatographic separation of saturated fatty acid methyl esters having widely different chain lengths indicates that care is needed to recover all such esters from the plates. The spots with R_F values < 0.5 are due in part to branched and unbranched α,ω-dicarboxylic acid esters[11,12]. The pure monocarboxylic acid esters (7.8 mg) were obtained by preparative thin-layer chromatography of the crude ester fraction (30 mg, developer as above); the I.R. spectrum of this fraction had bands at 2915, 2845, 1740, 1193 and 1165 cm^{-1} which are appropriate for fatty acid methyl esters. A gas-liquid chromatogram of this mixture is shown in Fig. 1A.

(*b*) *McCarthy and Duthie method.* Silica gel (10 g, Whatman SG-31) was treated with isopropanol–KOH according to the method of McCARTHY AND DUTHIE[9], the slurry was charged to a glass column (12 × 1.5 cm) and washed with ether (150 ml). The shale extract (84 mg) in about 1 ml of ether was placed on top of the column and the column was eluted with ether (200 ml). The acids were then desorbed by eluting with ether–formic acid (97:3, 150 ml) and recovered by evaporation of this mixture. We found that the removal of formic acid on a rotary evaporator (water pump pressure and water bath at 60–70°) was rather difficult. The residue was boiled with BF_3–MeOH (4 ml) to give a crude ester fraction (28.2 mg). Analytical thin-layer chro-

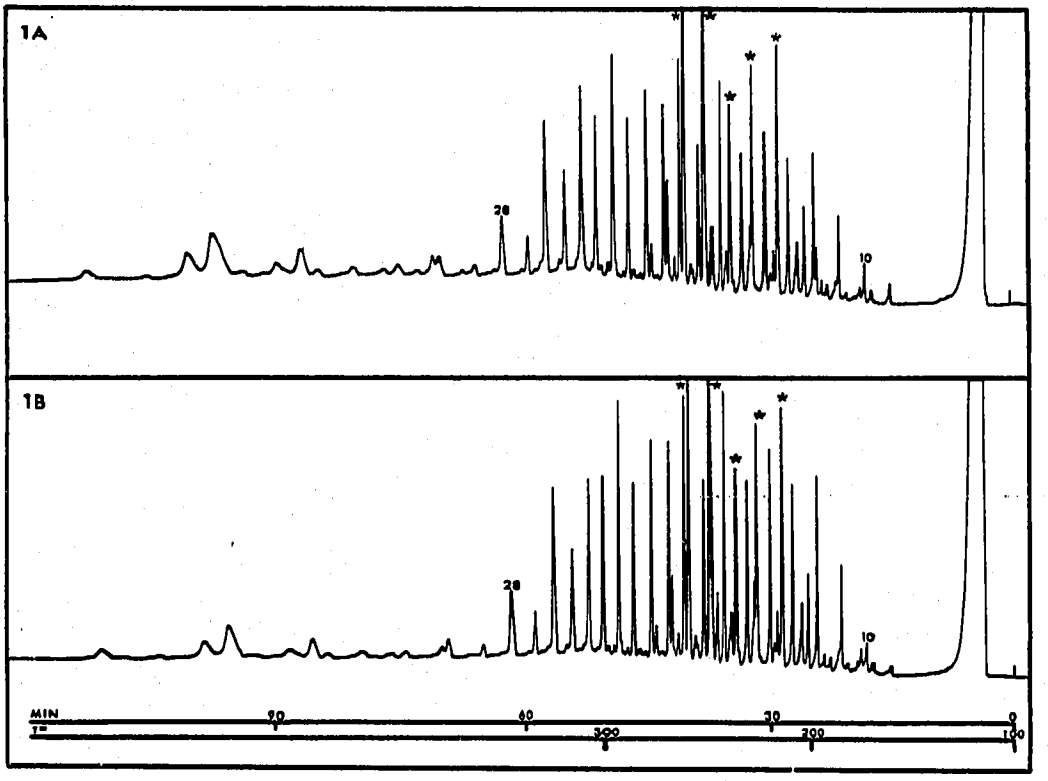

Fig. 1. Fatty acid methyl esters from Green River Shale. Gas chromatographic conditions: column 21 ft. × 0.040 in. (I.D.) containing 3 % OV-1 on 100–120 mesh Gas-Chrom Q; nitrogen 5 ml/min; temperature programmed from 100–300° at 4°/min; injector temperature 310°; detector temperature 330°; attenuation 16 × 10; chart 12 in./h. This column exhibited about 10,300 plates, measured for methyl stearate. A, Esters obtained by impregnated adsorbent TLC. B, Esters obtained by method of McCarthy and Duthie.

matography against standards indicated the presence of esters with lower R_F values than the monocarboxylic esters, which were obtained as a pure fraction (7.9 mg) by preparative thin-layer chromatography. The I.R. spectrum of this fraction had absorption bands, typical of fatty acid esters, at 2915, 2848, 1743, 1194 and 1166 cm^{-1}. A gas–liquid chromatogram of this mixture is shown in Fig. 1B. Both traces are remarkably similar with only small variations in peak heights; there is also a small extra peak in Fig. 1A with an equivalent chain length of about 11.90. The series of evenly spaced peaks ranging from about C_{10}–C_{30} are due to normal fatty acid esters. Those peaks marked with an asterisk represent some of the isoprenoid acids present in Green River Shale[11,13]. The two largest peaks on the chromatograms are due to pristanic (2,6,10,14-tetramethylpentadecanoic) and phytanic (3,7,11,15-tetramethylhexadecanoic) acid methyl esters respectively. The normal esters ranging from C_{12}–C_{19} and the isoprenoid esters marked with an asterisk were identified by mass spectrometry[13–15] using a combined gas chromatograph–mass spectrometer.

We have also determined that fatty acid esters and glycerides may be chromatographed without hydrolysis on potassium hydroxide treated plates. Tripalmitin, purified by preparative thin-layer chromatography, to remove traces of palmitic acid, was charged to a plate coated with silica gel containing 3% potassium hydroxide. After development with ether–methanol (97:3), a 1 cm band of adsorbent at the origin

was removed and boiled under reflux with BF_3–MeOH; the mixture was then worked up as previously described. Gas–liquid chromatography of the concentrated eluate from the small Florisil column gave no peaks, indicating that there had been no hydrolysis of the tripalmitin on the plate. A similar result was obtained when monopalmitin was used instead of tripalmitin. Finally, a mixture containing n-C_{12} and n-C_{16} fatty acids plus n-C_{14} and n-C_{18} fatty acid esters was separated on a potassium hydroxide treated plate. Removal of the band at the origin, methylation, and gas–liquid chromatography of the recovered chromatographic eluate gave only peaks due to the n-C_{12} and n-C_{16} methyl esters (determined by co-injection), but no peaks in the positions expected of n-C_{14} and n-C_{18} esters, again confirming that no hydrolysis had taken place on the treated silica gel.

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