Notes

The isolation of the polyunsaturated acids of the fish oils as their methyl esters by preparative scale gas chromatography*

Oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids can be obtained in a pure state by low temperature crystallisation[1], urea adduct formation[2,3], polybromide preparation[4], counter-current distribution[5], adsorption chromatography[6] and partition chromatography[7]. In general, the highly unsaturated acids of the fish oils ($C_{18:4}$; $C_{20:5}$; $C_{22:6}$) cannot be isolated by these techniques. Exceptionally, partition chromatography using a reversed phase, acetonitrile–methanol/heptane, system gives excellent results for $C_{22:6}$, $C_{22:5}$ and $C_{20:5}$ methyl esters[7]. This procedure is objectionable in that it uses large volumes of toxic solvent[8] and may not be suitable for the continuous preparation of large quantities of esters. Analytical gas–liquid chromatography gives excellent separations of the required polyunsaturated esters but this fact does not appear to have been exploited on the preparative scale. In early work with a "home-made" preparative gas chromatograph results were so encouraging that we decided to adapt a commercial preparative instrument for the purpose. A procedure has been developed which makes possible the continuous preparation of highly purified methyl esters of octadecatetraenoic acid ($C_{18:4}$), eicosapentaenoic acid ($C_{20:5}$) and docosahexaenoic acid ($C_{22:6}$).

Experimental

(1) *Starting material*

Cod liver oil which contains all three of the required acids[9] was used. The oil (200 g) was saponified by the cold procedure of DOMART *et al.*[10] The mixed acids had the following analysis:

Iodine value (Wijs): 205.

Ultra-violet spectrum: indicates 0.11 % conjugated diene; 0.07 % conjugated triene; other conjugated isomers absent.

(2) *Preparation of concentrates of the polyenoic esters*

It was considered desirable to work with a simplified concentrate of the polyunsaturated esters since this would facilitate clean separation of these in the preparative gas chromatograph. The concentrate was prepared as follows.

Cod liver oil acids (100 g) were dissolved in methanol (200 ml) and a saturated solution of urea in methanol (3 l) added. The mixture was heated to effect solution and cooled to 1° overnight. After filtration, the crystals were washed with cold methanol (200 ml) and then discarded. The combined filtrate and washings were concentrated on a steam-bath until 2 l of methanol had distilled and the residue cooled again to 1° overnight. The filtration procedure was repeated, the crystals

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discarded as before and the filtrate concentrated on a rotary film evaporator (bath < 40°) until most of the methanol had been removed. Water (1 l) was added to the residue, the mixture acidified with hydrochloric acid, the fatty acids extracted with ether and the solution washed free from acid and urea. The fatty acids when isolated (36.5 g) were found to have an iodine value of 401.0. The acids were refluxed for 1 h under nitrogen in methanol (80 ml) containing concentrated sulphuric acid (2.3 ml) to give the methyl esters (37 g). The esters which were moderately yellow were decolorised by column chromatography on silicic acid (Mallinckrodt). The esters were applied to the column in petroleum ether (b.p. 40–60°). A coloured band eluted quickly from the column. The esters which were very pale yellow were then eluted with petroleum ether–ethyl ether (99:1), a strongly coloured band remaining at the top of the column. The esters had the following analysis.

GLC: $C_{18:4}$ 11 %; $C_{20:5}$ 32.6 %; $C_{22:6}$ 49.7 %.

U.V. spectrum: 1.5 % conjugated diene.

(3) *Preparative gas chromatography*

A Wilkens "Autoprep" equipped with a flame ionisation detector was used. The following operating conditions were used.

Column. 5 % Apiezon L on Chromosorb G (acid washed, dimethyldichlorosilane treated). 5 ft. × 0.25 in., I.D. aluminium spiral column, conditioned for 48 h at 250° before use.

Carrier gas. Nitrogen (O_2 free); 150 ml per min.

Temperatures. Injection port: not heated (on column injection used). Column: 225°. Detector: 250°. Collector: 225°.

Collection. The problem of aerosol formation in the preparative GLC of fatty acid methyl esters has been discussed previously and a method of dealing with this devised[11]. In our experiments with the Autoprep we found that the traps supplied by the manufacturer did not condense the aerosols. Packing the trap with stainless

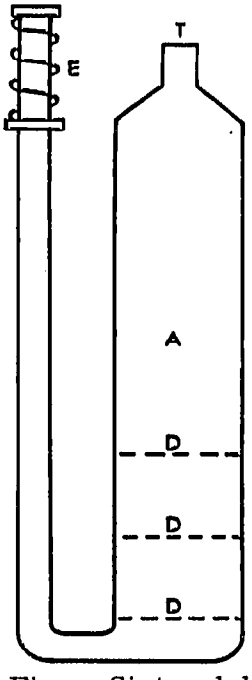

Fig. 1. Sintered disc trap constructed for use with the "Autoprep" gas chromatograph.

steel wool and insertion of an aluminium rod in the central tube to provide a thermal gradient were ineffective. We finally produced a trap, which is a miniature version of the one previously described by us for use with a "home-made" chromatograph[11]. It is shown in Fig. 1. The main chamber (A) is 10 cm long and has an internal diameter of 2 cm. The sintered glass discs (D) (porosity 1) are spaced 1.5 cm apart. The trap is fitted to the turntable of the Autoprep by the spring-loaded glass and silicone rubber seal (E) which can be purchased from Varian Aerograph. The trap is charged with solvent through T, gentle blowing being necessary to obtain a 1 cm layer of solvent above each disc. The contents of the trap can be discharged into a flask in a similar way. On test runs with single fatty acid methyl esters this device was found to give $>$ 85 % recoveries.

In the preparative GLC of the cod liver oil esters the traps contained a solution of butylated hydroxytoluene (BHT) antioxidant in acetone (0.1 %). The traps were partially immersed in a solid carbon dioxide–acetone bath (Dewar flask).

Detector split ratio. It was found in practice that with low split ratios (10:1) the flame of the ionisation detector was extinguished when a trap moved into position over the outlet. This was caused by the high impedance offered to the main flow by the solvent-containing sintered-disc trap. By employing a 50:1 split ratio this difficulty was resolved. This, however, resulted in an erratic recorder response which impaired the reliability of the fraction change mechanism. This was overcome by reducing the recorder sensitivity which resulted in a slightly "stepped" trace but which permitted correct fraction changing. It should also be noted that we found that the Bunsen valves supplied by the manufacturer to seal the outlet end of the traps were not wholly reliable, often causing great variation in recorder response and they were, therefore, not used.

Mode of operation. BHT (0.01 %) was added to the methyl ester concentrate to minimise its oxidation during exposure to the atmosphere in the injection reservoir of the machine. A series of forty 0.05 ml injections were applied automatically with like fractions being bulked.

Further purification of fractions. The three bulked fractions, $C_{18:4}$, $C_{20:5}$ and $C_{22:6}$, were purified by chromatography on silicic acid by an adaptation of the method of LUDDY *et al.*[12] to remove BHT and coloured material. The details of the running of the column and the contents of the fractions are shown in Table I. Thin-layer chromatography was carried out on silicic acid plates using petroleum ether

TABLE I

SILICIC ACID CHROMATOGRAPHY OF ESTERS

Fraction	*Eluting solvent*	*Volume (ml)*	*Contents by TLC*
1	Petroleum ether	100	BHT
2	Petroleum ether	100	Ester (trace)
3	Petroleum ether–ethyl ether (99:1)	100	Ester
4	Petroleum ether–ethyl ether (99:1)	100	Ester
5	Petroleum ether–ethyl ether (99:1)	100	Ester
6	Petroleum ether–ethyl ether (99:1)	100	Ester
7	Petroleum ether–ethyl ether (90:10)	250	Polar material
8	Ethyl ether	100	Polar material

(40–60°)–ethyl ether (99:1) as solvent. Spots were revealed with iodine vapour. The results show that complete separation of unsaturated esters from BHT and polar oxidation products is obtained. Recoveries of the main products, which were colourless, were > 90 %. The GLC analysis of the original mixture and the various fractions on Apiezon L and diethylene glycol succinate (DEGS) analytical columns are shown in Figs. 2 and 3, respectively. There are clearly minor amounts of contaminants in each fraction. Most prominent is the shoulder on the trailing edge of the

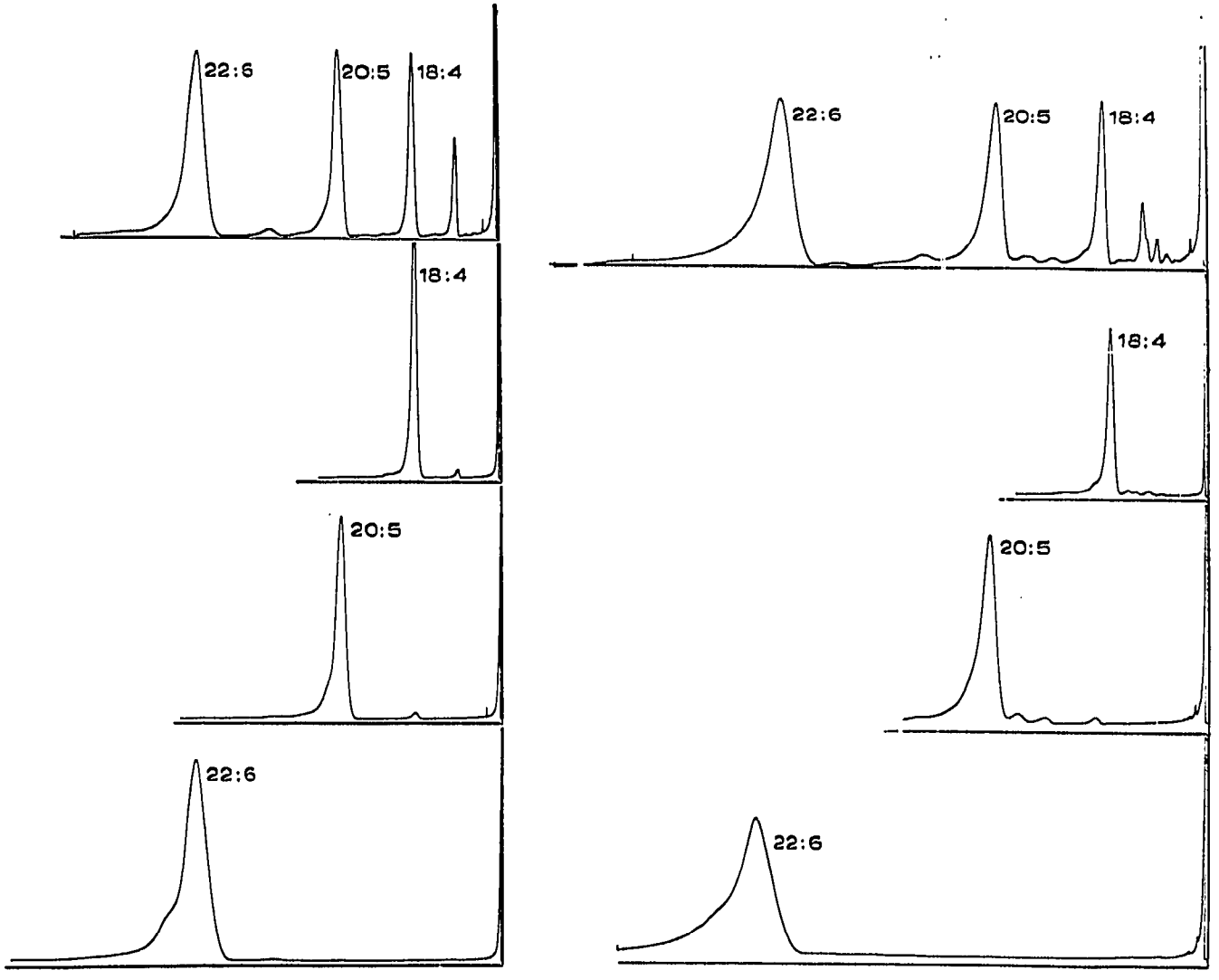

Fig. 2. Gas–liquid chromatograms of the unsaturated ester concentrate and the three purified esters obtained from it. These chromatograms were obtained using a 5 % Apiezon L on Chromosorb G analytical column at 225°.

Fig. 3. Gas–liquid chromatograms of the unsaturated ester concentrate and the three purified esters obtained from it. These chromatograms were obtained using a 20 % DEGS on Chromosorb P analytical column at 190°.

$C_{22:6}$ peak. It is possible that this may be due to one of the thermally produced "artifacts" described by PRIVETT AND NICKELL[7]. The fractions were also chromatographed on a 100 m Apiezon L capillary column with the same result, *i.e.*, the indication of a number of minor constituents. Preparative re-chromatography on a polyester

column did not improve purity. The chemical and spectroscopic analyses of the three esters are shown in Table II and Fig. 4.

(4) Note on iodine value determination

It was noted that the Wijs iodine value method, which gives excellent accuracy and precision with esters such as linoleate and linolenate, produces less satisfactory

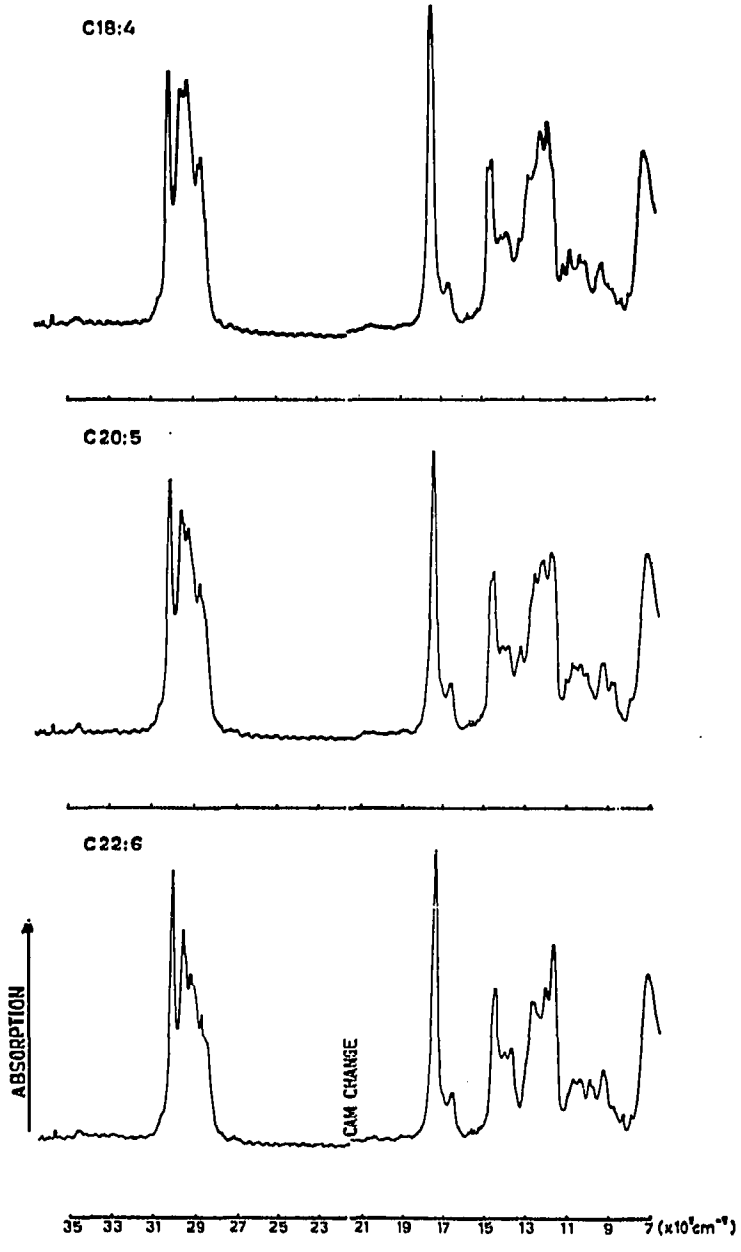

Fig. 4. Infra-red absorption spectra of purified esters obtained by preparative GLC on the "Autoprep". The spectra were run as liquid films between NaCl plates on a Unicam SP 100 spectrophotometer using a prism/grating monochromator.

results with the very highly unsaturated esters. It may be the case that the precipitation of polyhalide which can be seen to occur during the determination may interfere with quantitative addition of halogen to the polyene. In order to avoid this

TABLE II

CHEMICAL AND SPECTROSCOPIC ANALYSIS OF ESTERS

Ester	*Weight (g)*	*Iodine value*		*U.V. spectrum (in cyclohexane)*
		Theoretical	*Found (duplicate)*	
$C_{18:4}$	0.16	349.6	339.8 341.9	1.5 % conjugated triene
$C_{20:5}$	0.46	401.0	393.4 392.2	2 % conjugated triene
$C_{22:6}$	0.61	444.6	445.4 448.8	4.5 % conjugated triene

precipitation it was necessary to keep the sample size down to 10 mg per determination. A difference titration of 4 ml thus obtained can be measured with a piston burette with an accuracy of ± 0.02 ml. This gives an inherent error of ± 2 % which in the case of an iodine value of 400 represents $\pm$ 8 units.

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