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USE OF REVERSED-PHASE GEL PARTITION CHROMATOGRAPHY FOR THE PURIFICATION OF CHEMICALLY SYNTHESISED [5,6,8,9,11,12,14,15(*n*)] OCTADEUTERIUM- AND OCTATRITIUM-LABELLED ARACHIDONIC ACID

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SUMMARY

The development of a method is described for the preparation and purification of [5,6,8,9,11,12,14,15(*n*)-^{2}H]arachidonic acid ($^{2}H_8$-AA). The $^{2}H_8$-AA was chemically synthesised by the selective reduction of 5,8,11,14-eicosatetraynoic acid (ETYA) with deuterium gas.

Using reversed-phase partition chromatography on a Lipidex 5000 column support, it was shown that:

(1) The reaction products could readily be separated from each other to yield $^{2}H_8$-AA of greater than 98% mass purity by gas chromatography.

(2) Closely related C20 *cis*-ethylenic fatty acids differing only in the degree of unsaturation are efficiently separated. The resolution increases exponentially on saturation of double bonds.

(3) Commercially available [5,6,8,9,11,12,14,15(*n*)]octatritium-labelled arachidonic acid ($^{3}H_8$-AA) was readily purified. Both [$^{3}H_8$]- and [1-^{14}C]arachidonic acid (^{14}C-AA) co-chromatographed with $^{2}H_8$-AA.

(4) The mass spectra of the methyl ester and trimethylsilyl ester of the purified $^{2}H_8$-AA showed molecular ions at *m/e* 326 and 384, respectively.

INTRODUCTION

In recent years mass spectrometric (MS) techniques have been successfully applied to the structural analysis and measurement of naturally occurring compounds.

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The use of deuterium-labelled analogues as reference compounds and internal standards in the combined gas–liquid chromatography (GLC)–MS measurement of biologically active compounds and their metabolites has proven to be a powerful technique for the estimation of low levels of such compounds in tissue samples[1]. This has certainly been the case with the prostaglandin family and their related lipids[2].

In many instances, it is desired to extract and measure picomole quantities of prostaglandins from tissues containing large amounts of potentially interfering and closely related lipids. Many of these compounds yield ions with corresponding *m/e* values during electron impact fragmentation. Structural isomers also exist in the prostaglandin (PG) family, *e.g.* PGE_2 and PGD_2, and 15-keto$PGF_{2\alpha}$ which would yield the same M + 1 ion during chemical ionisation mass spectrometry. In some cases, therefore, lengthy extraction procedures are necessary to ensure the specificity of the assay.

Addition of a stable isotope at the earliest possible stage in the extraction procedure allows compensation for losses of endogenous compound during extraction assuming that both isotopes act identically in these procedures. This also allows calculation of the concentration of endogenous compound from constructed standard lines with the authentic compound[3]. However, the use of stable isotopes as carrier compounds during GLC–MS has been questioned[4].

In this paper we present the development of a method for the production and purification of octadeuterium-labelled arachidonic acid ($^2H_8$-AA). This isotope not only provides a means for GLC–MS determination of arachidonic acid (AA) (ref. 5), but it can also be used as a substrate for the biosynthetic production of deuterium-labelled metabolites[6] of AA. Of special interest to the authors are the highly biologically active prostanoid products of the cyclooxygenase pathway and their stable metabolites, in particular thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$ (refs. 7, 8).

Reversed-phase gel partition chromatography (RPPC) was considered to be an ideal method for the isolation of chemically synthesised $^2H_8$-AA from the crude reaction mixture, because of the relatively mild conditions of pH and temperature (ambient) that are afforded by this liquid chromatography system. Secondly, while the chromatographic characteristics of methyl esters, particularly of the PGs have been investigated[9] using straight-phase partition chromatography, the reversed-phase system allows purification of the free acids. These are more suitable as internal standards for GLC–MS analysis of PGs, as they can be added at the earliest possible stage in the extraction procedure.

A disadvantage in using reversed-phase chromatography for the purification of highly unsaturated fatty acids is their differential chemical stability in aqueous media.

EXPERIMENTAL

Materials

Arachidonic acid, N,N-bis(trimethylsilyl)trifluoroacetamide and dimethyl dichlorosilane were purchased from Sigma (St. Louis, Mo., U.S.A.).

Deuterium gas (98% D_2 atom pure) and quinoline were purchased from BDH (Poole, Great Britain).

Lindlar catalyst for selective reduction was obtained from Fluorochem (Derbyshire, Great Britain).

Lipidex 5000 gel was obtained from Packard (Downers Grove, Ill., U.S.A.) and the thin-layer chromatography plates were purchased from Anderman & Co. (East Molesey, Great Britain).

The diazomethane was generated from Diazald purchased from Aldrich (Gillingham, Great Britain).

[1-^{14}C]Arachidonic acid (55 mCi/mmol) and [5,6,8,9,11,12,14,15(*n*)-^{3}H]arachidonic acid (135 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Great Britain).

The 5,8,11,14-eicosatetraynoic acid (ETYA) was kindly supplied by Dr. Phillpott, Roche Products, Welwyn, Great Britain.

Authentic [5,6,8,9,11,12,14,15-^{2}H]arachidonic acid was obtained from Dr. R. L. Jones, Department of Pharmacology, Edinburgh University, Edinburgh, Great Britain.

All solvents were of analytical grade, or the best available, and were redistilled before use. Water was glass distilled prior to deionisation before use.

Storage of fatty acids. Fatty acid solutions of 100–1000 μg/ml methanol were stored at −20°.

Chemical reduction of 5,8,11,14-eicosatetraynoic acid with deuterium gas

Samples of approximately 100 mg of ETYA were each dissolved in 10 ml of methanol. Lindlar catalyst (50 mg) and quinoline (50 μl) were added and the reaction mixture shaken vigorously under 1 atm of deuterium gas at room temperature in a hydrogenerator previously flushed several times with deuterium gas. The rate of reaction was determined by measuring the rate of uptake of deuterium gas and the reaction was terminated when the deuterium uptake reduced towards zero. (In all cases more than the theoretical uptake occurred.) The reaction products were then filtered through Whatman No. 1 filter paper and collected in a 100-ml Quickfit round bottomed flask. A further 20 ml of methanol (two 10-ml aliquots) were used to wash the catalyst and reaction vessel. These washes were then filtered and pooled with the original filtrate. The solvent was then evaporated under reduced pressure at 40°, leaving a brown oily mixture. This crude mixture was subjected, without prior purification, to RPPC.

Chromatographic techniques

Reversed-phase gel partition chromatography. A glass column (300 × 15 mm I.D.) which had been silanized by treatment with 5% dimethyl dichlorosilane in heptane, was plugged at the base with glass wool and packed with 100 ml of Lipidex 5000 gel, suspended and equilibrated in the eluting solvent system to be used for chromatographic separation. A constant pressure-head of solvent was maintained above the surface of the gel, giving a flow-rate of approximately 20–25 ml/h.

The column eluent passed through narrow-bore PTFE tubing and was collected in 5-ml fractions (1/20 bed volumes) by an LKB 7000 Ultrorac fraction collector as described previously[10].

Compounds to be chromatographed on the Lipidex 5000 column were first taken to dryness under reduced pressure at 40° prior to redissolving in 2 ml of the same solvent system to be used for chromatography. By varying the ratio of polar to non-polar solvents in the eluting system, a suitable solvent mixture was determined and

used subsequently. This consisted of methanol–water–1,2-dichloroethane–acetic acid (700:150:150:1 by volume). When more than one authentic standard was to be chromatographed through the column, the mixture was made prior to evaporation and resuspension in the eluting solvent. Amounts of the free acids (2–10 mg or 0.5 μCi of the radiolabel) constituted the samples to be eluted. When the crude ETYA reduction mixture containing the $^2H_8$-AA was eluted from the column, no more than 50 mg amounts of ETYA were loaded at any one time. The column was allowed to regenerate overnight prior to further use, with excess solvent.

Where colloidal Lindlar catalyst appeared to contaminate the surface of the Lipidex 5000 column support, this was removed as soon as practically possible and replaced with fresh Lipidex 5000 gel.

Thin-layer chromatography. Thin-layer chromatography (TLC) was carried out using neutral silica gel on glass plates (200 and 100 $\times$ 0.25 mm). The F6 solvent system used was that of Anderson[11] containing ethyl acetate–acetone–acetic acid (90:10:1 by volume). Marker fatty acids, ETYA and AA, were spotted at the side of the test samples and chromatography was carried out in the dark at ambient temperature in glass tanks lined with filter paper. Radiolabelled samples were applied to the plate 3 cm from the lower edge with an SGE 100-μl glass syringe in a streak 3 mm wide, 4 cm long, and developed to a distance of 15 cm. Scrapes (0.5 cm: origin $\pm$ 0.5 cm) were then removed, into 10 ml of scintillation fluid containing naphthalene–2,5-diphenyloxazole (PPO) (112.5:10.5, w/w) in toluene–2-ethoxyethanol–methanol (1500:900:432 by volume), and counted for either ^{14}C or ^3H, using an LKB 1012 liquid scintillation counter. A correction for quenching using the automatic external standard channels ratio method and a quench calibration curve construction for the scintillation mixture was used to obtain disintegrations per minute (dpm) where necessary.

The purity of the radiolabelled compounds was determined using the F6 solvent system, which gives good separation of AA from more polar impurities. Tritiated arachidonic acid ($^3H_8$-AA) was also subjected to TLC, using the solvent system light petroleum (b.p. 60–80°)–diethyl ether–acetic acid (80:50:1). The $^3H_8$-AA was shown to contain several impurities and was therefore purified by RPPC on Lipidex 5000 column support, using the solvent system described above. Several of the radiochemical impurities separated by RPPC were found to co-chromatograph on TLC with $^3H_8$-AA. The purity of AA and ETYA was also checked using TLC. Aliquots of 10–20 μg, spotted at the origin, were developed to 15 cm. The fatty acids were then visualised on the plate by exposing it to iodine vapour.

Treatment of Lipidex 5000 column effluent

Radioactive samples. Aliquots of 100–250 μl of solvent were removed from each alternate fraction and added to 10 ml of scintillation fluid. The radioactivity was measured as described. Where eluting compounds were required for further analysis, the relevant fractions were pooled and taken to dryness under reduced pressure at 40° and stored at −20°.

Unlabelled samples. Aliquots (200–750 μl) were removed and were vacuum desiccated at ambient temperature until dry.

The methyl esters of the fatty acids were produced by reacting the residues with freshly distilled diazomethane in ethereal methanolic solution (9:1, v/v) for the

shortest contact time feasible before removing the solvent under a stream of nitrogen at 40°. The resulting residues were resuspended in 25 μl of methanol.

Alternatively, the trimethylsilyl esters were prepared by treating the vacuum desiccated column residues with 50 μl of N,N-bis(trimethylsilyl)trifluoroacetamide at room temperature for 30 min.

As a quick guide to the position and identity of the mass eluting from the column, 10–20-μl amounts from alternate fractions were spotted on TLC plates and either visualised directly by exposure to iodine vapour, or visualised after development in F6 solvent system to monitor the separation of quinoline from the fatty acids.

Methyl esters of the reaction products (after reduction of ETYA with deuterium gas) were produced from aliquots of the mixture before chromatography on Lipidex 5000 gel and of the pooled purified eluent, containing the $^{2}H_{8}$-AA.

Gas–liquid chromatography with flame ionisation detection

GLC of pure samples was carried out on a Pye 104 series gas chromatograph with a flame ionisation detector (FID), using columns (2.1–3.0 m × 4 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100–120 mesh, Pye Unicam), operating at oven temperatures between 235 and 275° and nitrogen carrier gas flow-rate of 35–40 ml/min.

GLC–FID of the methylated deuterium reduction products before and after RPPC was carried out on a Hewlett-Packard chromatograph using a column (1.2 m × 2 mm I.D.) of stationary phase 10% diethylene glycol succinate (DEGS-PS) on 80–100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.). The oven temperature was programmed between 150 and 250° at 8°/min. Helium was used as the carrier gas at a flow-rate of 45–50 ml/min.

Combined gas–liquid chromatography–mass spectrometry

Electron impact mass spectra were obtained using an AEI MS-30 double focusing mass spectrometer coupled via a membrane separator to a Pye 104 series gas chromatograph equipped with a 3% OV-1 column (1.5 m × 2 mm I.D.). Helium was used as the carrier gas at a flow-rate of 20 ml/min. The mass spectrometer was linked to a DS50 data system and mass spectra were recorded at 3 sec per decade and at 70 eV.

RESULTS AND DISCUSSION

To investigate the capability of RPPC on Lipidex 5000 to purify the $^{2}H_{8}$-AA from closely related reduction products likely to be present in the reaction mixture after deuteration of ETYA, a number of C20 fatty acids differing only in extent of unsaturation were chromatographed on this system.

The acetylenic starting material ETYA was shown to separate efficiently from [1-^{14}C]arachidonic acid (^{14}C-AA) used as a marker for $^{2}H_{8}$-AA, and also from the fully reduced entity, arachidic acid during RPPC (Fig. 1).

It was also demonstrated that ^{14}C-AA and also $^{3}H_{8}$-AA acted as true markers during chromatography of $^{2}H_{8}$-AA. The chromatography was carried out in two consecutive elutions on the Lipidex 5000 column (Fig. 2a and b). $^{2}H_{8}$-AA was loaded on-column with either ^{14}C-AA or $^{3}H_{8}$-AA, and ETYA was added as an internal stan-

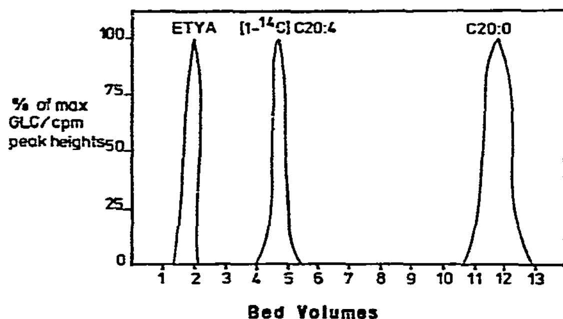


Fig. 1. RPPC on Lipidex 5000 gel of ETYA, ^{14}C-AA and arachidic acid. Results expressed as percentage of maximum peak height as measured by GLC-FID of the TMS esters and cpm from fractions eluting from the column. One bed volume is equivalent to 20 fractions.

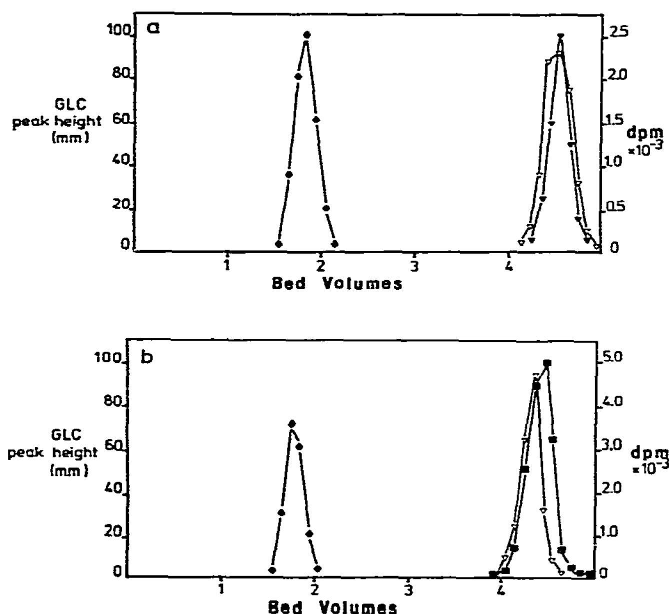
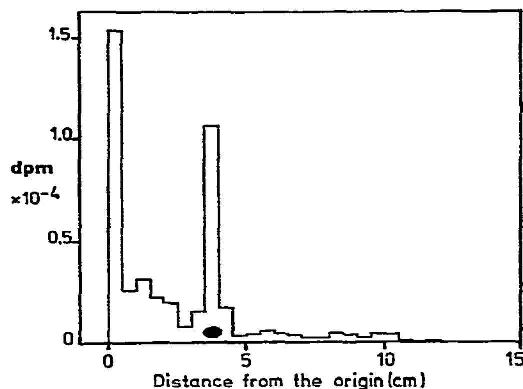


Fig. 2 (a and b). RPPC on Lipidex 5000 gel of $^{2}H_{8}$-AA with both ^{14}C-AA and $^{3}H_{8}$-AA as markers. ETYA was chromatographed as an internal standard. Results expressed as dpm of radiolabel and peak heights (mm) recorded after GLC–FID of TMS esters of eluting fatty acids. ◆, ETYA; ▼, ^{14}C-AA; ■, $^{3}H_{8}$-AA; ▽, $^{2}H_{8}$-AA.

dard. The retention volumes of each compound showed a good degree of reproducibility.

Advantages in using RPPC instead of TLC for purification of chemically impure arachidonic acid were revealed when commercially obtained $^{3}H_{8}$-AA was purified by RPPC. TLC of an aliquot of the $^{3}H_{8}$-AA indicated major radiochemical impurities more polar than AA (Fig. 3). After RPPC of the $^{3}H_{8}$-AA solution (Fig. 4) the fractions containing more polar radioactive impurities were divided arbitrarily in three approximately equal pools. On TLC with F6 solvent system, approximately 30% of the impurity co-chromatographed with AA (Fig. 5a–c). Re-chromatography of 5%



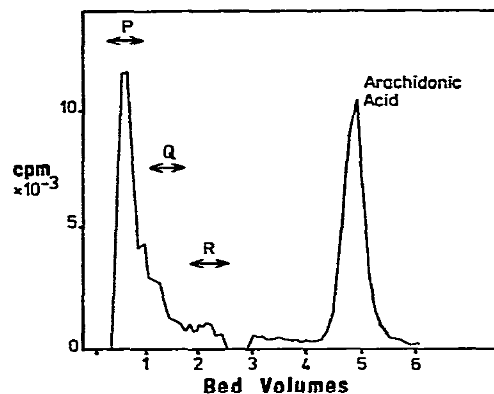
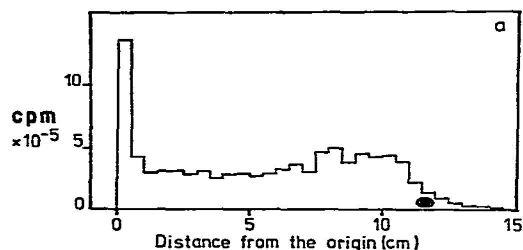
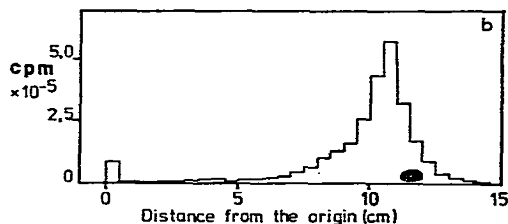


Fig. 3. TLC of $^{3}H_{8}$-AA containing radiochemical impurities. Solvent system: light petroleum (b.p. 60–80°)–diethyl ether–acetic acid (80:50:1 by volume).

Fig. 4. RPPC on Lipidex 5000 gel of $^{3}H_{8}$-AA containing radiochemical impurities. Fractions containing radioactivity eluting from the column were pooled as follows: pool P: fractions 7–21; pool Q: fractions 22–35; pool R: fractions 36–51.





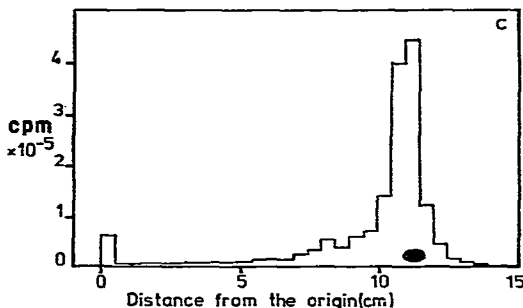


Fig. 5. TLC of the residues from pool P (a), Q (b), and R (c) after evaporation of solvent. Solvent system: ethyl acetate–acetone–acetic acid (90:10:1 by volume).

of the purified $^{3}H_{8}$-AA showed that impurities were not produced on RPPC (Fig. 6).

A second group of C20 *cis*-ethylenic unsaturated fatty acids differing in the number and positions of their double bonds was investigated. Fatty acids differing in the positions of the double bonds present in the molecule were chromatographed separately with arachidic acid added as an internal standard (Table I). Baseline separation was demonstrable for all the fatty acids tested differing in degree of un-

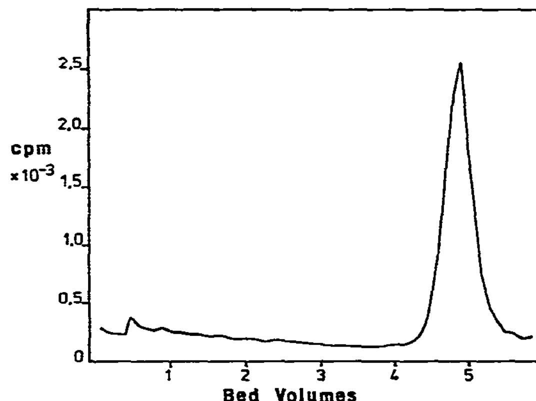


Fig. 6. RPPC on Lipidex 5000 gel of 5% of $^{3}H_{8}$-AA purified by this system. Results expressed as cpm eluting from the column.

TABLE I

RETENTION VOLUMES OF C20 *cis*-ETHYLENIC UNSATURATED FATTY ACIDS ON RPPC ON LIPIDEX 5000 GEL

One bed volume is equivalent to 20 fractions.

C20 fatty acid	*No. of double bonds*	*Retention volume (fraction No.)*
Arachidic	0	261
11-Eicosaenoic	1	179
11,14-Eicosadienoic	2	143
8,11,14-Eicosatrienoic	3	109
Arachidic	0	237
11,14,17-Eicosatrienoic	3	105
5,8,11,14,17-Eicosapentaenoic	5	73

saturation, while C20:3 W6 and W9 co-chromatographed (Fig. 7a and b). The mean retention volume of arachidic acid was calculated from three separate experiments and from this the relative retention volume of each fatty acid was deduced from the equation:

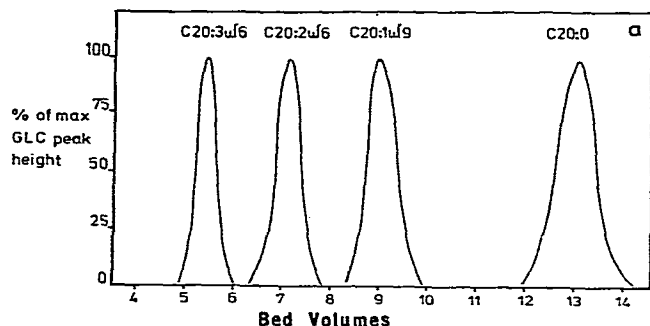
$$RV_{relative} = RV_{measured} \times \frac{RV_{C20:0}}{RV_{C20:0\ (mean)}}$$

where RV = retention volume.

From this it was found that the retention volume varied logarithmically with the number of double bonds present in the molecule (Fig. 8). Therefore, the resolution of these fatty acids increases exponentially with the degree of saturation of the double bonds and the chromatographic system is more efficient in separating reduction products less polar than arachidonic acid than those of greater polarity.

Purification and analysis of $^{2}H_{8}$-AA

After reductive deuteration of ETYA, the reaction products were methylated with diazomethane prior to RPPC on Lipidex 5000. The mass eluting as AA after



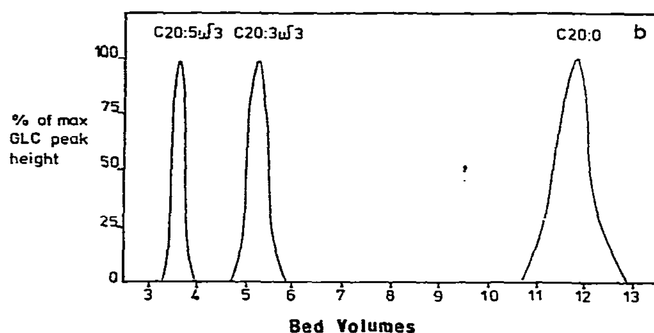


Fig. 7. RPPC on Lipidex 5000 gel of C20 *cis*-ethylenic unsaturated fatty acids: (a) 8,11,14-eicosatrienoic acid (C20:3 W6); 11,14-eicosadienoic acid (C20:2 W6); 11-eicosaenoic acid (C20:1 W9); arachidic acid (C20:0); and (b) 5,8,11,14,17-eicosapentaenoic acid (C20:5 W3); 11,14,17-eicosatrienoic acid (C20:3 W3); arachidic acid (C20:0). Arachidic acid was chromatographed as an internal standard. Results are expressed as percentage of maximum peak height as measured by GLC–FID of the TMS esters of the fatty acids from fractions eluting from the column.

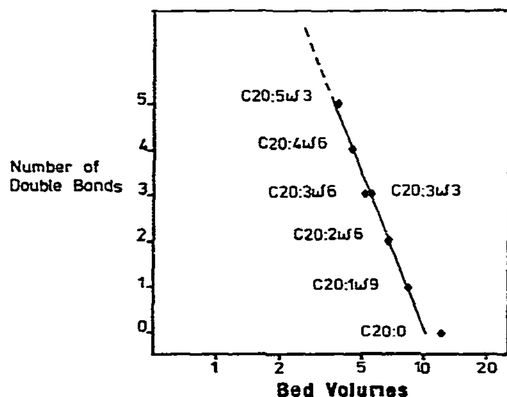


Fig. 8. Graph relating the number of double bonds in C20 fatty acids to the logarithm of the retention volume on RPPC. The nomenclature is as in Fig. 7.

RPPC was also methylated and both methylated residues subjected to GLC–FID as described above (Fig. 9). Whereas, prior to RPPC, quinoline, ETYA and the reduced products were identified, after RPPC, all interfering peaks were removed leaving a single peak which co-chromatographed with authentic AA. TLC of the eluting fractions showed that quinoline added to poison the catalyst during reduction eluted early during RPPC (about 1 bed volume).

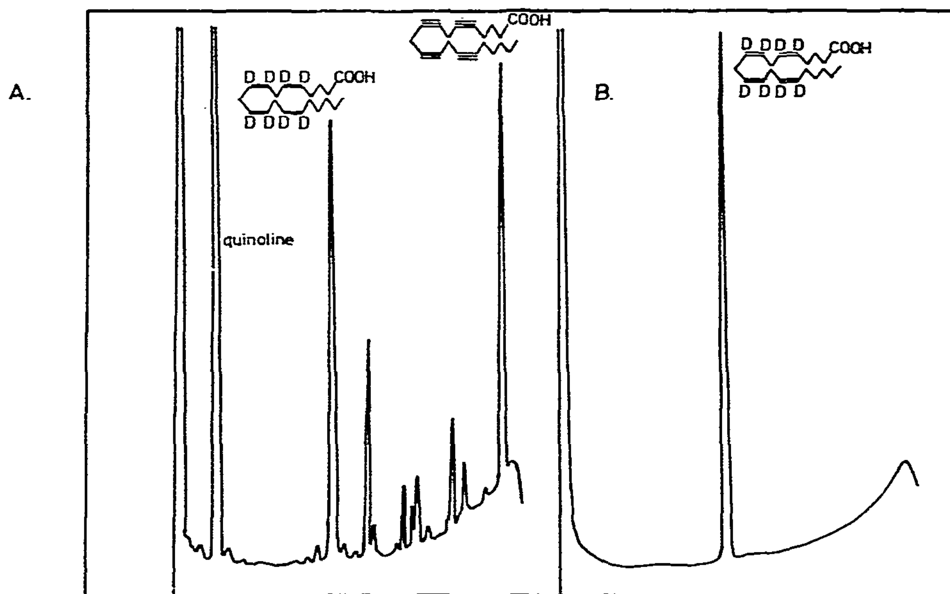


Fig. 9. Temperature-programmed GLC–FID of the reduction products (methyl esters) prior to (A), and after purification of $^2H_8$-AA by RPPC on Lipidex 5000 gel (B). GLC was carried out using 1.2 m × 2 mm I.D. column packed with 10% DEGS on Supelcoport. Carrier gas was helium at a flow-rate 50 ml/min. Chart speed was 15 in./h. Column temperature was programmed between 150° and 250° at 8°/min. Structures of the free acids are shown.

GLC–MS of the reduced purified product

Electron impact mass spectra of the methyl ester and the trimethylsilyl ester (TMS ester) of the purified reduced compound were obtained. The fragmentation patterns are identical to those of the authentic $^2H_8$-AA and show molecular ions at nominal *m/e* values of 326 (methyl ester) and 384 (TMS ester) (Fig. 10a and b). Similar fragmentation patterns were obtained for the AA methyl and TMS esters which yielded molecular ions at the nominal *m/e* values of 318 (methyl ester) and 376 (TMS ester) (Fig. 11).

No significant contribution of AA fragments were detected in the spectra of the $^2H_8$-AA derivatives.

CONCLUSION

Following the synthesis of $^2H_8$-AA by the reduction of ETYA with deuterium gas, chromatography of the reaction mixture on Lipidex 5000, RPPC provided a quick, simple and reproducible method for its purification, as subsequently determined by GLC–MS.

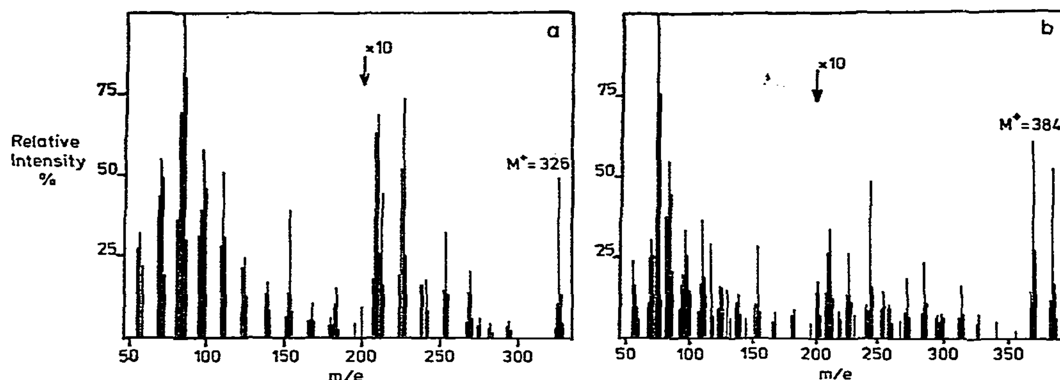


Fig. 10. Mass spectra of (a) the methyl ester and (b) the TMS ester of the purified $^2H_8$-AA.

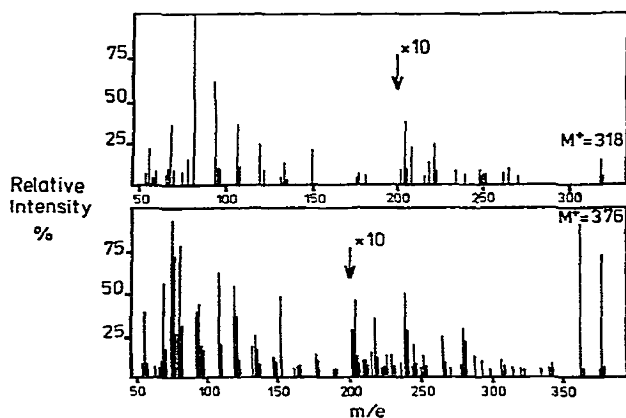


Fig. 11. Mass spectra of the authentic AA methyl ester (top) and TMS ester (bottom).

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