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## Note

# Purification of methyl arachidonate using silver resin chromatography

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Silver resin chromatography is a convenient preparative method for purification of saturated, mono-, di- and tri-unsaturated *cis* and *trans* fatty acid methyl esters[1–4]. However, the technique has not been a suitable method for the separation of mixtures of tetra-unsaturated isomers because of poor peak shape and long elution times required for these highly unsaturated compounds. A mixture of acetonitrile–methanol has been reported to reduce the elution time for methyl linolenate and markedly improve peak shape[5]. The results of this development suggested that silver resin chromatography could be applied to the separation of complex mixtures of arachidonate isomers.

The synthesis of radioisotope and stable isotope labeled arachidonate and its isomers for biological investigations and analytical applications requires purification of the reaction products to separate isomers and remove structurally similar intermediates and impurities. In this paper a rapid and effective method is described that uses a low-pressure silver resin chromatographic column and an acetonitrile–methanol elution solvent system to isolate methyl arachidonate. The procedure has been used to isolate pure methyl arachidonate from its geometric isomers and also to remove reaction products formed during synthesis of $^{14}C$-labeled methyl arachidonate.

## EXPERIMENTAL*

Authentic fatty acid ester standards were obtained from Nu-Chek Prep (Elysian, MN, U.S.A.). An unpurified sample of methyl [1-$^{14}C$]arachidonate (55.8 mCi/mmol) was supplied by New England Nuclear Corp. (Boston, MA, U.S.A.). The synthetic procedure for preparing this ester was that of Sprecher[6] and involved Lindlar reduction[7] of tetraynoic $C_{19}$ alcohol prior to addition of the $^{14}C$-label. Over-reduced compounds and *trans* isomers of methyl arachidonate were the impurities (*ca.* 15%).

Geometric isomers of methyl arachidonate were prepared with *p*-toluenesulfinic acid catalysis using a 1:1 molar ratio according to the method of Gibson and Strassburger[8].

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Methyl esters were analyzed by a Perkin-Elmer 3920 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a flame ionization detector. The glass capillary column, 50 m × 0.25 mm I.D., was coated with Silar 10C. The oven was maintained at 180°C. Helium flow-rates were 1.06 ml/min through the column and 175 ml/min at the splitter outlet to give a split ratio of 165.

Silver resin chromatography was performed using a Waters ALC 202 instrument (Waters Assoc., Milford, MA, U.S.A.). The column had been prepared previously[3] and consisted of two 2 ft. × 5/16 in. (61 cm × 7 mm) I.D. stainless-steel tubes connected in series. The packing was XE-284 sulfonic acid resin (Rohm and Haas, 270–325 mesh), which had been fully saturated with $Ag^+$. Eluting solvent consisted of varying amounts (3–15%) of acetonitrile in methanol. In solvent-programmed runs the acetonitrile concentration was increased from 3 to 10% over 30 min along the non-linear curve 10 of a Waters 660 solvent programmer. Isocratic runs were made using 15% acetonitrile in methanol. Flow-rate through the column was 6 ml/min, developing a pressure of 600 p.s.i. A Schoeffel Spectroflow Monitor SF770 variable-wavelength UV detector (Schoeffel, Westford, NJ, U.S.A.) set at 210 nm monitored the column effluent. Radioactive samples were detected with a Berthold HPLC radioactivity monitor LB503 (Beta Analytical, Coraopolis, PA, U.S.A.). The stream splitter was set at 1 sec at 10%. Scintillation cocktail (2.7 g/l 2,5-diphenyloxazole in toluene) was added at 1 ml/min.

Fractions collected from the silver resin column separations were analyzed for per cent *trans* with a Perkin-Elmer 621 grating infrared (IR) spectrophotometer using matched KBr cells[9]. The purity of individual fractions was determined by capillary gas chromatography (GC) and by argentation thin-layer chromatography (TLC) (Brinkman plates, 5 cm × 20 cm × 0.25 mm dipped in 15% alcoholic $AgNO_3$; benzene–light petroleum (b.p. 35–60°C), 80:20) and reversed-phase TLC (Whatman KC18F plates, 5 cm × 20 cm × 200 μm; acetonitrile–acetic acid, 1:1).

## RESULTS AND DISCUSSION

Methods for synthesis of arachidonic acid include coupling of the appropriate unsaturated intermediates followed by selective catalytic reduction of acetylenic precursors[10–13]. In addition to small amounts of geometrical isomers formed by catalytic reductions, other reactants and unwanted products must be removed from the all-*cis* arachidonate. Previously reported isolation procedures include argentation TLC[13], reversed-phase gel partition chromatography (RPPC)[12], partial argentation resin chromatography[4] and reversed-phase high-performance liquid chromatography (HPLC)[14,15]. It has been our experience with argentation TLC that sample size is limited and that with long-chain polyunsaturated acids or esters the product is not always completely pure. Woollard *et al.*[12] used RPPC to isolate "pure" arachidonic acid. This method required five column volumes or about 20 h elution time. The isolated compound was deemed pure after GC analysis on a packed column. Isomeric purity was not determined. As previously reported[16], long-chain polyunsaturated fatty esters were not eluted from a 100% silvered macroreticular resin column with single solvent systems, whereas partial silvering of a sulfonic acid resin gave some separation of a methyl arachidonate standard from shorter chain esters in 3.75 h[4]. Polyunsaturated ester peaks were skewed, and methyl linolenate obviously tailed

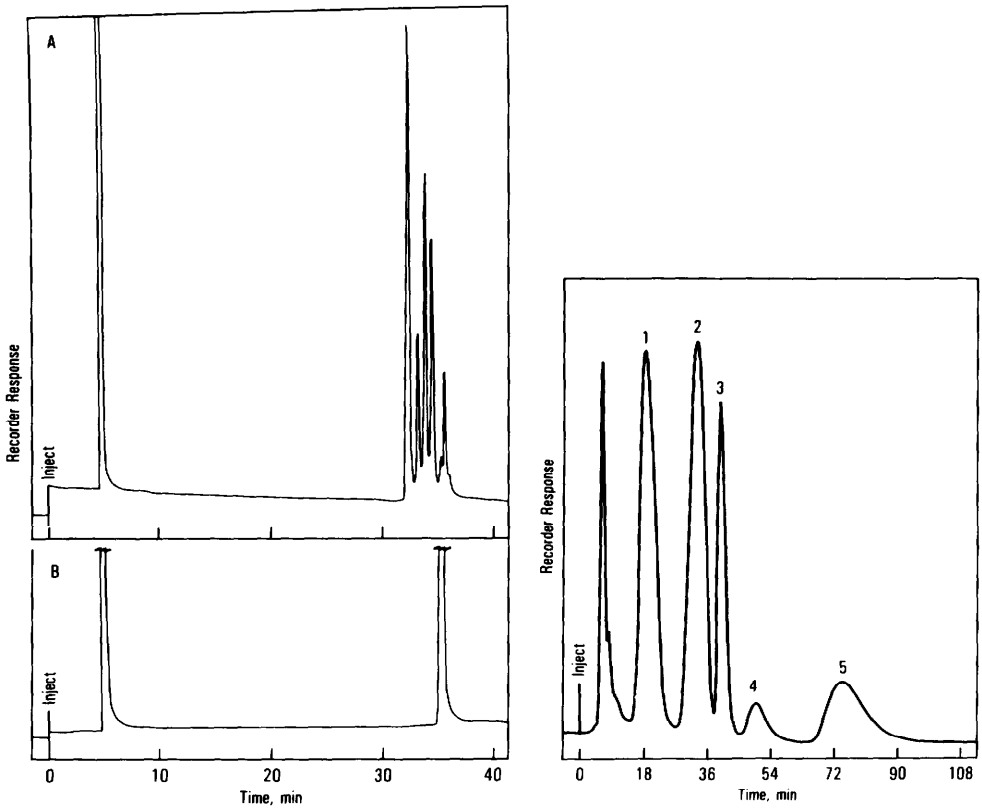
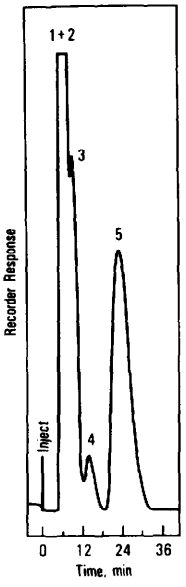



Fig. 1. GC of isomerized methyl arachidonate prior to (A) and after (B) purification by silver resin chromatography. GC was carried out on a 50 m × 0.25 mm I.D. capillary column coated with Silar 10C. Carrier gas was helium at a flow-rate of 1.06 ml/min. Column temperature was maintained at 180°C. Chart speed was 0.5 cm/min.

Fig. 2. Silver resin chromatographic separation of a 10-mg mixture containing isomerized methyl arachidonate. A Schoeffel Spectroflow Monitor SF770 measured absorbance at 210 nm at 0.2 a.u.f.s. The column was 4 ft. × 5/16 in. I.D. stainless-steel packed with XE-284 sulfonic acid resin, which had been fully saturated with $Ag^+$. Solvent gradient from 3 to 10% acetonitrile in methanol in 30 min along non-linear curve 10 (Waters Associates 660 solvent programmer). Pure methyl arachidonate elutes with peak 5. Flow-rate was 6 ml/min. Chart speed was 6 cm/min.

into the methyl arachidonate peak. Reversed-phase HPLC has been shown to separate the 2-naphthacyl esters of tri- and tetra-enoic $C_{20}$ acids[14]. Arachidonic ester would apparently co-elute at 50 min with linoleic ester if both were in the chromatographed mixture. A rapid and simple procedure for isolation of a pure arachidonate fraction after large-scale synthesis remained desirable.

Capillary GC of the isomerized methyl arachidonate used for these silver-resin column separations indicated seven partially resolved components (Fig. 1A). Silver resin chromatography of this mixture using non-linear gradient elution with acetonitrile in methanol (3% to 10% acetonitrile over 30 min along Waters program 10) provided a clean separation of methyl arachidonate from its geometric isomers. With this solvent system, pure arachidonate (Fig. 2, peak 5) was isolated in 96 min and the other isomers were resolved into four separate fractions. These fractions probably



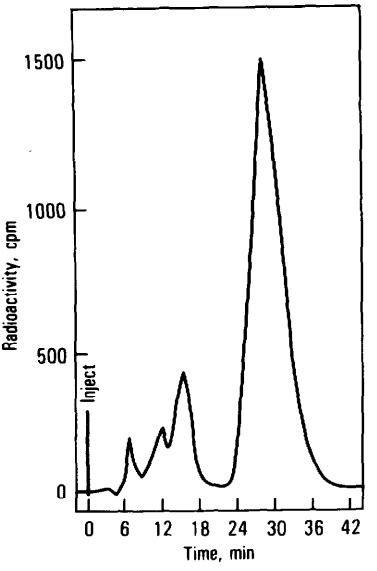




Fig. 3. Silver resin chromatographic separation of a 12-mg sample of isomerized methyl arachidonate. Solvent: acetonitrile–methanol (15:85). Detection, flow-rate, chart speed and numbering of peaks as in Fig. 2.

Fig. 4. Radiochromatogram of methyl [1-$^{14}$C]arachidonate (1 $\mu$Ci, 2.9 $\mu$g) containing *trans* isomers and reaction products. Separations were by silver resin chromatography using acetonitrile–methanol (15:85). Flow-rate and chart speed as in Fig. 2. Isotope detection was with a Berthold HPLC radioactivity monitor LB503.

represent the tetra- (peak 1), tri- (2), di- (3) and mono- (4) *trans* isomers.

Peak 1 showed only a single component on capillary GC and had strong IR absorbance at 10.4 nm. Intermediate peaks (2–4) were composed of multiple components on capillary GC as would be expected, since a number of mono-, di- and tri-*trans* arachidonate isomers are possible. Lack of pure standards for these compounds prevented absolute identification.

The component in fraction 5 was determined to contain only methyl arachidonate based on the single peak found by capillary GC (Fig. 1B), which cochromatographed with a methyl arachidonate standard. This fraction had a single spot by argentation and reversed-phase TLC analyses and IR analyses showed no absorbance for *trans* double bonds confirming complete purification.

Methyl arachidonate can also be fully resolved from its isomers by isocratic elution with acetonitrile–methanol (15:85) in 36 min (Fig. 3) if separation of the *trans* isomers is not desired. Silver resin chromatography with this solvent system has been used to isolate methyl [1-$^{14}$C]arachidonate from reaction co-products (Fig. 4) and to purify a commercial sample of methyl arachidonate (90 mg, 90% pure) to give essentially 100% recovery (80 mg) of pure product in 40 min. Loading capacity of this silver resin column was tested using this same isocratic solvent system with a weighed mixture containing 179 mg methyl arachidonate and 29 mg of geometric isomers and reaction products. Separation of methyl arachidonate from these "impurities" was almost complete even though overloading of the column was indicated by peak tail-

ing. Previous applications of silver resin chromatography to separations of unsaturated fatty esters indicate these separations can readily be scaled up to 10–20 g samples by using proportionately larger diameter columns. A silver resin column can be regenerated periodically by passing 1-hexene through the column to displace any contaminants bound to the $Ag^+$.

Rapid isolation of large amounts of pure long-chain polyunsaturated fatty esters suggests that silver resin chromatography is the method of choice for purification of reaction mixtures containing geometric isomers and over-reduced impurities.

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