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Short Communication

High-resolution separation of polyunsaturated fatty acids by argentation thin-layer chromatography

Robert Wilson and John R. Sargent

Department of Biological and Molecular Sciences, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland (UK)

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ABSTRACT

A simple procedure is described for separating methyl esters of polyunsaturated fatty acids (PUFAs) by argentation chromatography using silver nitrate-impregnated thin-layer chromatography plates. Esters are resolved into discrete classes containing 2, 3, 4, 5 and 6 double bonds and within each of these groupings esters are separated according to chain length. All commonly encountered (n − 6)PUFAs from C_{18} to C_{22} are resolved from each other, as are all commonly encountered (n − 3)PUFAs from C_{18} to C_{22}. The technique is particularly useful for metabolic studies of the chain elongation and further desaturation of PUFAs by separately incubating isolated cells with [1-^{14}C]18:2(n − 6) and [1-^{14}C]18:3(n − 3). R_F values for individual PUFAs are very reproducible and recoveries of individual [^{14}C]PUFAs for radioassay exceed 90%.

INTRODUCTION

Fatty acid methyl esters (FAMEs) can be efficiently separated and quantitated by gas chromatography (GC) [1] and high-performance liquid chromatography (HPLC) [2], and both GC and reversed-phase HPLC have been applied to study lipid metabolism using radio-labelled compounds [3–13]. Radio-labelled FAMEs separated by either packed column GC or reversed-phase HPLC can be either counted directly with commercial radiodetectors, or fractions collected for further analysis and/or liquid scintillation radioassay [3–13]. However, not all components may be well separated by packed column GC or HPLC and further analysis using other techniques is often required [3,4,7,8,12].

Argentation thin-layer chromatography (TLC) has been used to separate FAMEs according to their degree of unsaturation and to study the metabolism of polyunsaturated fatty acids (PUFAs) using radioisotopes [14–18]. However, not all PUFAs are well separated by existing silver nitrate techniques [14–21] and, in particular, separation of PUFA methyl esters with four or more double bond is difficult [18–21]. Furthermore, separation of FAMEs with the same degree of unsaturation but with different chain lengths is seldom possible [19]. We report here a simple method for separating PUFA methyl esters according to both degree of unsaturation and chain length. The application of the method in studies of chain elongation and further desaturation of C_{18} PUFAs is illustrated.

Correspondence to: Dr. Robert Wilson, Department of Biological and Molecular Sciences, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK.

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MATERIALS AND METHODS

Materials

Butylated hydroxytoluene (BHT) and standard FAMEs (at least 99% pure) were obtained from Sigma, Poole, UK. Silver nitrate was obtained from Johnson Matthey, Royston, UK. [1-^{14}C]18:2(n − 6) (54.7 mCi/mmol), [1-^{14}C]18:3(n − 3) (53.9 mCi/mmol) and radioactive ink were purchased from Amersham International, Aylesbury, UK. Ecoscint A scintillation fluid was supplied by National Diagnostics, Manville, NJ, USA. Konica A2 X-ray film was supplied by MAS Stirling, Crief, UK. Silica gel 60 TLC plates (Merck 5721, 20 cm × 20 cm × 0.25 mm) were purchased from BDH, Poole, UK. Aminopropyl columns were purchased from Jones Chromatography, Hengord, UK. 10% CP-Wax 51 coated on Chromosorb 100–120 mesh was obtained from Chrompack, Middelburg, Netherlands. All solvents were of HPLC grade and were purchased from Rathburn Chemicals, Walkerburn, UK.

Impregnation of silica gel plates with silver nitrate

Silica gel 60 TLC plates (20 cm × 20 cm × 0.25 mm) were sprayed uniformly with 20 ml acetonitrile containing 2 g silver nitrate until the plates were saturated. Spraying TLC plates gives a coating of silver nitrate as uniform as dipping [17,19]. The plates were air-dried in subdued light, heated at 110°C for 30 min to achieve activation [19] and used within 1 h.

Preparation of fatty acid methyl esters

FAMEs were prepared from mixtures of either (n − 3) or (n − 6) PUFAs by transmethylation in methanol containing 1% sulphuric acid for 16 h under nitrogen at 50°C [20]. The composition of the mixtures is given in the legend to Fig. 1. In other experiments, human skin fibroblasts cultured in 10 ml medium, were incubated with either 1 μM (0.05 μCi/ml) [1-^{14}C]18:2(n − 6) or 1 μM (0.05 μCi/ml) [1-^{14}C]18:3(n − 3) for 24 h to generate metabolic intermediates as described previously [4–6]. Total lipid was isolated from human skin fibroblasts [22] and FAMEs prepared as above [20]. FAMEs were purified on 500 mg aminopropyl (NH_2) columns (Bond-Elut) [23]. Using a syringe attached to the column by an adapter, 7 column volumes hexane–acetic acid (98:2, v/v) followed by 7 column volumes hexane were slowly pushed through the column. FAMEs were then applied to the column in hexane and eluted from the column using 7 column volumes hexane. The solvent was evaporated under a stream of nitrogen. FAMEs were redissolved in hexane containing 0.01% BHT at 50 mg/ml. Cholesterol and PUFA degradation products were retained on the column and are eluted using 7 column volumes hexane–acetic acid (98:2, v/v) followed by 7 column volumes hexane.

Separation of (n − 3) and (n − 6) mixtures by argentation TLC

FAME mixtures were applied to the impregnated TLC plates as a narrow band over 1 cm using a Hamilton micro-syringe at 0.5–1.0 mg/cm. Approximately 100 μg of each component were present in the mixtures using this loading. The plates were developed with toluene–acetonitrile (97:3, v/v) to 1 cm from the top in a standard TLC chamber lined with tissue to saturate the atmosphere with solvent. The plates were dried, lightly sprayed with 3% (w/v) copper acetate–8% (v/v) orthophosphoric acid in water and charred at 180°C for 20 min to visualise FAMEs.

Quantification of [^{14}C] FAMEs by argentation TLC

[^{14}C]FAMEs dissolved in hexane were applied to plates as a narrow band over 2–5 cm at 50 000 dpm/cm and plates developed using toluene–acetonitrile (97:3, v/v) to 1 cm from the top. The plates were marked with radioactive ink, including solvent front and origin, and subjected to autoradiography for 4 days using Konica A2 X-ray film. Areas of silica corresponding to radioactive FAMEs were located, scraped from the plates into vials and 5 ml Ecoscint A added. Radioactivity was determined using a Packard Tricarb 2000CA liquid scintillation analyser [6,17]. In other experiments FAMEs were located, scraped from the plates and eluted using 5 ml ice-cold chloroform–methanol (2:1) containing 0.01% BHT; 1.25 ml 0.88% KCl were added and the solutions mixed [23]. After separation, the chloroform layer was removed and the solvent removed under nitrogen. FAMEs were redissolved in hexane containing 0.01% BHT and washed with 20% NaCl to precipitate any remaining silver. The hexane layer was removed and an aliquot was radioassayed after adding 5 ml Ecoscint A and the remainder used for further analyses by GC or TLC.

Identification of [^{14}C] FAMEs by GC

[^{14}C] FAMEs eluted from thin-layer plates as above were added to authentic non-radioactive standards and subjected to GC on a Pye 104 gas chromatograph using a glass column (2 m × 4 mm I.D.) packed with 10% CP Wax 51 coated on Chromosorb (100–120 mesh) with nitrogen as carrier gas at a flow-rate of 30 ml/min and a programmed temperature from 220 to 250°C [6]. Separated components were identified by reference to standards and quantified using flame ionisation detection (FID) and a recording integrator [6]. The instrument was also equipped with a stream splitter situated before FID that allowed 90% of the column effluent to be collected outside the chromatograph. Fractions of column effluent corresponding to individual FAMEs were trapped on Gilson pipetteman safety filters wetted in Ecoscint A scintillation fluid [6]. Radioactivity on the filters was determined by liquid scintillation counting as described above. Location of radioactivity with authentic standards confirmed the identity of [^{14}C] FAMEs [6].

RESULTS

Representative lanes of TLC plates developed in toluene–acetonitrile (97:3, v/v) and charred are shown in Fig. 1. Separate mixtures of (n − 6) and (n − 3) FAMEs are resolved according to the degree of unsaturation with dienes being clearly separated from trienes that were in turn separated from tetraenes. Pentaenes and hexaenes were also well separated and in addition hexaenes had migrated clear of the origin. FAMEs were also resolved according to chain length with the same degree of unsaturation, this being most evident with PUFAs containing two or three double bonds. Thus, 18:2(n − 6), 20:2(n − 6) and 22:2(n − 6) were all resolved as were 18:3(n − 3), 20:3(n − 3) and 22:3(n − 3).

Although the R_F values for (n − 3) and (n − 6) PUFAs with identical degree of unsaturation and chain lengths are slightly different, they cannot be satisfactorily resolved when both are present in the same mixture (Fig. 1, see trienes). Thus, the mixture (n − 6) PUFAs + (n − 3) PUFAs is incompletely resolved. As shown in Fig. 2, however, this limitation can be simply overcome in metabolic studies by separate incubations with [1-^{14}C]18:2(n − 6) and [1-^{14}C]18:3(n − 3) and subsequent simultaneous analyses of the products from the two incubations. In the experiment shown the conversions of 18:2(n − 6) to 18:3(n − 6), 20:2(n − 6), 20:3(n − 6) and 20:4(n − 6), and of 18:3(n − 3) to 18:4(n − 3), 20:3(n − 3), 20:4(n − 3), 20:5(n − 3) and 22:5(n −

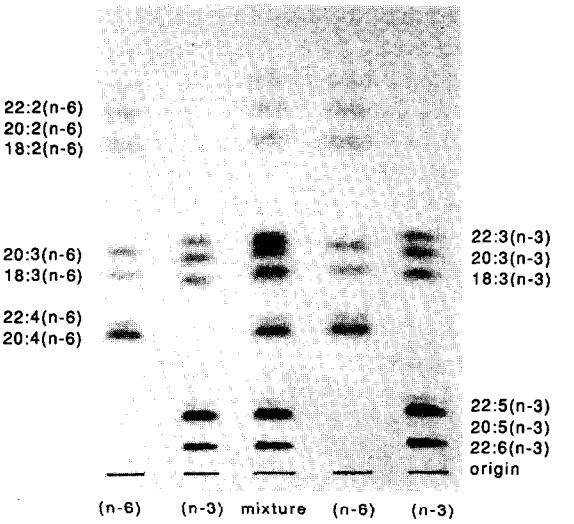

Fig. 1. Methyl esters prepared from the mixtures of (n − 6) and (n − 3) PUFAs were separated by argentation TLC as described in the Materials and Methods section. The separated esters were visualised by charring. Mixture compositions were, (n − 6) PUFAs: 18:2(n − 6), 20:2(n − 6), 22:2(n − 6), 18:3(n − 6), 20:3(n − 6), 20:4(n − 6) and 22:4(n − 6); (n − 3) PUFAs: 18:3(n − 3), 20:3(n − 3), 22:3(n − 3), 20:5(n − 3), 22:5(n − 3) and 22:6(n − 3). Mixture: (n − 6) PUFAs + (n − 3) PUFAs.

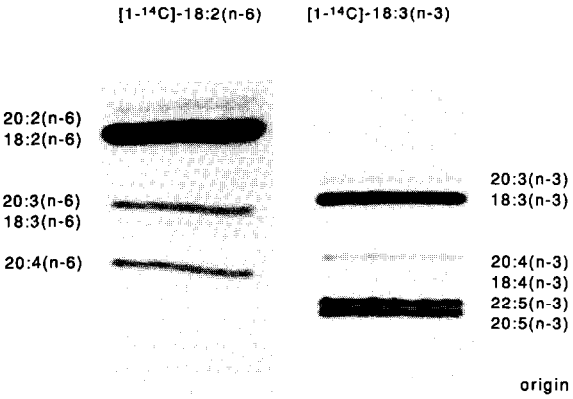

Fig. 2. FAMEs derived from the metabolism of [1-^{14}C]18:2(n − 6) and [1-^{14}C]18:3(n − 3) by cultured skin fibroblasts were separated by argenation TLC and visualised by autoradiography as described in the Materials and Methods section.

3) in cultured skin fibroblasts are clearly seen. Note that conversion to 22:6(n − 3) did not occur under the culture conditions employed.

A linear relationship exists between carbon number, degree of unsaturation and R_F values of individual methyl esters (Fig. 3), so that for each class of FAMEs containing 2, 3, 4 or 5 double bonds, an elution profile graph is created when R_F values are plotted against carbon number. As noted above, (n − 3) and (n − 6) PUFAs with identical degree of unsaturation and carbon number cannot be resolved and thus the graph for trienes and tetraenes in Fig. 3 represents both groups of PUFAs. 24:5 (n − 3) was identified from autoradiograms as a product of metabolism of [1-^{14}C]18:3(n − 3) in skin fibroblasts. This PUFA was characterised using a number of techniques, including catalytic hydrogenation, reversed-phase TLC and capillary GC.

The technique is highly reproducible with only 3–6% variation in R_F values being recorded over 5 separate analyses of the same mixture (data not shown). Recoveries of individual radioactive methyl esters, *e.g.* from experiments as in Fig. 2, were 90–92% as determined by elution of individual zones from the plates, radioassaying aliquots of the eluates, and re-chromatographing and radioassaying.

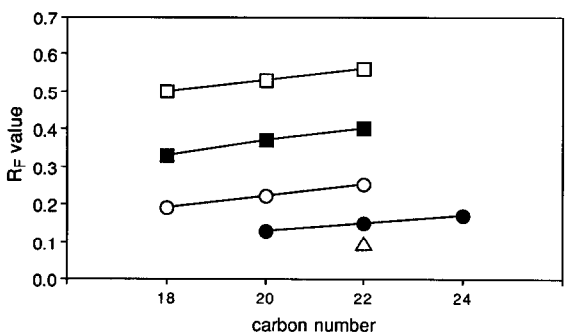

Fig. 3. R_F values were plotted against carbon number for dienes (□), trienes (■), tetraenes (○), pentaenes (●) and hexaenes (△). Methyl esters of (n − 3) PUFAs and (n − 6) PUFAs with identical degree of unsaturation and carbon numbers have slightly different R_F values but cannot be resolved. Thus the graphs for trienes and tetraenes represent both groups of PUFAs.

DISCUSSION

Development of plates with benzene–ethyl acetate (90:10, v/v) has been used previously to separate methyl esters of PUFAs with up to four double bonds with compounds of different chain lengths and the same degree of unsaturation being resolved [19]. However, complete separation of components with four or more double bonds has usually required multiple development. Separation of saturates, monoenes, dienes, trienes, tetraenes and pentaenes is achieved by a triple development technique in toluene–acetone (95:5, v/v) at −20°C [18], whereas pentaenes and hexaenes are also resolved when plates are developed twice in hexane–diethylether–acetic acid (94:4:2, v/v/v) [21]. Although these solvents resolved FAMEs into a number of classes, no separation according to chain length within a given class was noted [18,20,21].

We have examined several solvent systems for separating PUFA methyl esters and found toluene–acetonitrile (97:3, v/v) to be the most efficient. Increased resolution between pentaenes and hexaenes could be achieved using toluene–acetonitrile (95:5, v/v) but this led to poorer separation of some components in the mixture according to chain length. However, the use of this solvent may be advantageous in studies using longer-chain PUFAs. The present solvent system resolved FAMEs not only according to the degree of unsaturation but also due to chain length with the same degree of unsaturation. The technique is very reproducible and the relationship described in Fig. 3 is analogous to the homologous series of FAMEs in isothermal GC or isocratic HPLC [1,2]. In these techniques FAMEs can be identified by reference to equivalent chain length plots that can be created from retention time data and by reference to known standards [1,2]. Similarly, in the argentation TLC technique described in this paper, FAMEs can be identified from the elution profile graphs created from R_F data.

We applied this system to study the products of metabolism of [1-^{14}C]18:2(n − 6) and [1-^{14}C]18:3 (n − 3) in cultured skin fibroblasts. Other studies of PUFA metabolism using radio-labelled fatty acids have used GC or HPLC equipped with radiodetectors and computer data acquisition for analysis [3–5,10–13]. These techniques have various drawbacks, not least being the expense of the equipment

required and a need for high specific radioactivities in the analytes when flow detectors are used [1,2,20]. The technique here can be applied using low levels of radioactivity, it is inexpensive and it can be operated reliably and routinely by relatively non-skilled operators. It offers an excellent alternative and complementary method to GC or HPLC for studying the metabolism of PUFA using radiolabelled substrates.

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