https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction*

Graham C. Burdge†, Paul Wright, Amanda E. Jones and Stephen A. Wootton

Institute of Human Nutrition, University of Southampton, Southampton, UK

(Received 2 September 1999 – Revised 31 January 2000 – Accepted 6 March 2000)

Efficient isolation of individual lipid classes is a critical step in the analysis of plasma and lipoprotein fatty acid compositions. Whilst good separations of total lipid extracts are possible by TLC, this method is time consuming and a major rate-limiting step when processing large numbers of specimens. A method for rapid separation of phosphatidylcholine (PC), non-esterified fatty acids (NEFA), cholesterol ester (CE) and triacylglycerol (TAG) from total plasma lipid extracts by solid-phase extraction (SPE) using aminopropyl silica columns has been developed and validated. Following initial separation of polar and neutral lipids, individual classes were isolated by application of solvents with increasing polarity. Recoveries for combined plasma extraction with chloroform–methanol and SPE were (%): PC 74·2 (SD 7·5), NEFA 73·6 (SD 8·3), CE 84·9 (SD 4·9), and TAG 86·8 (SD 4·9), which were significantly greater for TAG and NEFA than by TLC ($P < 0{\cdot}001$). Both GC–flame ionisation detector and GC-MS analysis of fatty acid methyl esters demonstrated that there was no cross-contamination between lipid classes. Measurements of repeatability of fatty acid composition for TAG, PC, CE and NEFA fractions showed similar CV for each fatty acid. The magnitude of the CV appeared to be related inversely to the fractional fatty acid concentration, and was greatest at concentrations of less than 1 g/100 g total fatty acids. There was no evidence of selective elution of individual fatty acid or CE species. In conclusion, this method represents an efficient, rapid alternative to TLC for isolation of these lipid classes from plasma.

Lipids: Solid-phase extraction: Analytical methodology

Determination of the fatty acid compositions of different plasma and lipoprotein lipid classes is an analytical procedure central to a wide range of studies in both human and animal biology. Many such studies require methods for separating individual plasma lipid classes from a large number of specimens. Conventionally, TLC has been used to provide efficient separations of plasma lipids. However, TLC is relatively slow, produces low lipid recoveries and may result in oxidation of polyunsaturated fatty acids due to prolonged exposure to air (for review see Agren *et al.* 1992; Bernhardt *et al.* 1996). Solid-phase extraction (SPE) by column chromatography represents an alternative to TLC for isolation of plasma lipid classes, since up to twenty specimens can be processed simultaneously, compared with typically about five by TLC, with reduced risk of polyunsaturated fatty acid auto-oxidation.

Protocols have been published previously for separation of plasma cholesterol ester (CE) and triacylglycerol (TAG) fractions (Hoving *et al.* 1988), phospholipids (Caesar *et al.* 1988; Kim & Salem 1990), and fatty acid ethyl esters (Bernhardt *et al.* 1996). In addition, separation of plasma CE, TAG, non-esterified fatty acid (NEFA) and phospholipid fractions using a single aminopropyl column has been described (Agren *et al.* 1992). Although recoveries and efficient separations of >99 % were reported for lipid standards, these variables were not reported for the complete extraction procedure from plasma. In addition, an immiscible solvent mixture containing water was used to isolate phospholipids, presumably phosphatidylcholine (PC), and so required post-column extraction prior to methylation which could reduce yields.

We have been unable to reproduce the separation

Abbreviations: CE, cholesterol ester; NEFA, non-esterified fatty acids; PC, phosphatidylcholine; SPE, solid-phase extraction; TAG, triacylglycerol.

* These data have been described previously, in part, in an abstract (Wright P, Burdge GC, Jones AE & Wooton SA (2000) Purification of the major plasma lipid classes by solid-phase extraction on aminopropyl silica cartridges. *Proceedings of the Nutrition Society* **52**, 21A).

† **Corresponding author:** Dr Graham C. Burdge, fax +44 2380 794945, email g.c.burdge@soton.ac.uk

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

efficiency of Agren *et al.* (1992) using aminopropyl silica columns prepared commercially. This may be explained, in part, by physical differences in the absorbent phase which appeared to be a critical factor in the quality of lipid separations (Agren *et al.* 1992). We have developed, therefore, a rapid method for SPE separation of plasma PC, NEFA, CE and TAG using commercial aminopropyl silica columns based upon initial separation of neutral and polar lipids.

Materials and methods

Materials

BondElut aminopropyl silica columns were purchased from Varian (Harbour City, CA, USA). HPLC grade solvents were obtained from Fisher (Loughborough, Leics., UK), and 'Analar' grade glacial acetic acid from Merck Limited (Poole, Dorset, UK). All other chemicals were purchased from Sigma Limited (Poole, Dorset, UK).

Extraction of total plasma lipids

Blood samples were collected using lithium heparin as anticoagulant. Lipid recoveries were determined using plasma specimens (*n* 24) from sequential blood collections from healthy young women (*n* 3). Repeatability of fatty acid composition was assessed using a single blood sample from a healthy male. Total plasma TAG and cholesterol concentrations were within ranges accepted as clinically normal (TAG 0–2·0 mmol/l, cholesterol 2·5–5·2 mmol/l, HDL-cholesterol 1·2–1·5 mmol/l). Plasma and erythrocytes were separated by centrifugation at 1125 ***g***, 15 min at 4°C. Plasma was collected by aspiration, avoiding contamination with leucocytes and platelets. Total plasma lipids were extracted by a modification of the method of Folch *et al.* (1957). Briefly, di-heptadeconoyl PC (60 μg in trifluoroethanol), triheptadecanoin (60 μg in chloroform), cholesteryl behenate (100 μg in chloroform) and heneicosanoate (30 μg in chloroform) were added to plasma (1 ml). Chloroform–methanol (2:1, v/v) containing butyrated hydroxytoluene (50 μg/ml) was added, the preparation mixed briefly and then shaken for 15 min at room temperature. 1 M-NaCl was added (1·0 ml), and organic and aqueous phases separated by centrifugation at 1125 ***g*** for 10 min at 4°C. The aqueous phase was removed and the organic phase collected by aspiration. The interfacial protein disc was homogenised in chloroform–methanol (2:1, v/v) containing butyrated hydroxytoluene (50 μg/ml) and 1 M-NaCl (1·0 ml). Re-extraction of the protein disc increased recovery of NEFA by approximately 40 % (data not shown). The organic phase was separated and collected as before, combined with the initial chloroform layer and dried under N_2 at 40°C.

Solid-phase extraction of triacylglycerol, cholesterol ester, non-esterified fatty acid and phosphatidylcholine

Resolution of individual lipid classes was determined using a mixture of di-pentadecyl PC (100 μg), triheptadecanoin (100 μg), cholesteryl octadecanoate (100 μg) and heneicosanoate (100 μg). Total plasma lipid extracts containing appropriate internal standards were used to determine recovery of individual lipid classes and repeatability of fatty acid composition within a lipid class. The extent of selective elution of individual fatty acid and cholesterol ester species from SPE cartridges was assessed using mixtures of palmitic, stearic, oleic and α-linolenic acids, and cholesteryl palmitate, -stearate, -oleate and -linoleate (100 μg of each component). Total plasma lipid extracts or mixtures of standards were dissolved in chloroform (1·0 ml) and applied to an aminopropyl silica column (Varian; 100 mg packed silica per 1·0 ml cartridge) under gravity. Residual solvent was removed under vacuum. The column was washed with chloroform (2 × 1·0 ml), the void fractions combined and dried under N_2 at 40°C. PC was eluted with chloroform–methanol (3:2, v/v; 1·0 ml) under vacuum (Caesar *et al.* 1988). The column was washed with methanol (1·0 ml) to remove any residual phospholipid, and the NEFA fraction eluted with chloroform–methanol–acetic acid (100:2:2, by vol. 2·0 ml) under vacuum (Agren *et al.* 1992).

TAG and CE were isolated from the void fraction from the first stage. A fresh aminopropyl silica column (Varian) was pre-conditioned with hexane (4 × 1·0 ml). The void fraction was dissolved in hexane (1·0 ml) and applied to the column under gravity and then washed with hexane (2 × 1·0 ml) under vacuum to elute CE. TAG was then eluted with hexane–chloroform–ethylacetate (100:5:5, by vol.) (2 × 1·0 ml) under vacuum. Solvent fractions containing isolated lipids were dried under N_2 at 40°C.

Isolation of plasma lipid classes by TLC

Separation of plasma lipid classes by TLC was carried out essentially as follows. Total lipid extracts were dissolved in chloroform (100 μl) and applied to the origin of a Silica Gel 60 TLC plate (20 cm × 20 cm) (Merck Ltd.). The dried lipid spots were developed with hexane–diethyl ether–glacial acetic acid (70:30:1·8, by vol.). Plates were allowed to dry and then sprayed with fluorescein (2·5 g/l ethanol). Bands corresponding to TAG, CE and NEFA were identified under u.v. illumination and collected by scraping the silica into a glass tube.

Preparation and GC analysis of fatty acid methyl esters

Fatty acid methyl esters were prepared by incubation with acidified methanol. Briefly, lipids isolated by SPE or silica from TLC plates were mixed with toluene (1·0 ml) by vortex mixing. Methanol containing 20 ml H_2SO_4/l (2·0 ml) was added, mixed briefly and incubated at 50°C for 18 h. The reaction mixture was cooled and neutralised with a solution (2·0 ml) containing a mixture of $KHCO_3$ (0·25 M) and K_2CO_3 (0·5 M). Fatty acid methyl esters were isolated by addition of hexane (2·0 ml), separation of organic and aqueous phases by centrifugation at 1125 ***g*** for 10 min at 14°C and collection of the hexane layer. Samples were transferred to GC autosampler vials, dried under N_2 and dissolved in dry hexane. In specimens used to determine lipid recovery, an equal mass of tricosanoic

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

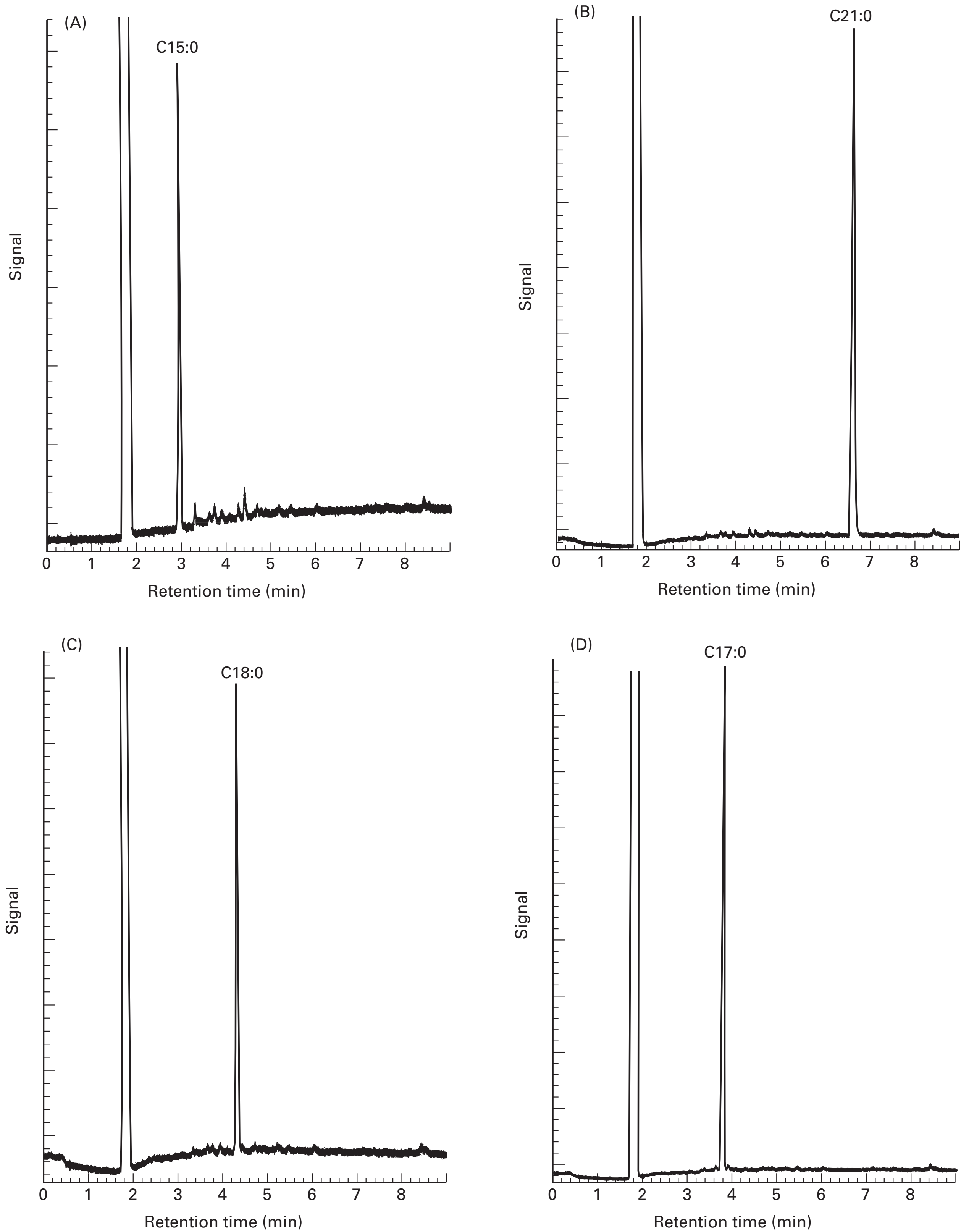

Fig. 1. GC–flame ionisation detector chromatograms of fatty acid methyl esters derived from (A) phosphatidylcholine (pentadecanoic acid), (B) non-esterified fatty acids (heneicosanoic acid), (C) cholesterol ester (stearic acid) and (D) triacylglycerol (heptadecanoic acid) standards separated from a mixture by solid-phase extraction.

acid methyl ester recovery reference standard to the internal recovery standard was added.

Fatty acid methyl esters prepared from lipid fractions isolated by SPE were resolved on a Hewlett Packard 6890 GC equipped with an HP7686 GC autosampler using an Innowax fused silica capillary column (20 m × 100 μm × 0·1 μm) (Hewlett Packard, Stockport, Ches., UK) with flame ionisation detection. Peaks were identified routinely by comparison of retention times with known fatty acid methyl ester standards. Detailed analysis of the fatty acid compositions both of fractions containing lipid standards and of those derived from plasma was carried out by GC–MS on a 6890 GC using an HP5-MS capillary column (30 m × 250 μm × 0·25 μm; Hewlett Packard) connected to an HP5973 mass selective detector (Hewlett Packard). Peaks were identified by comparison of electron impact ionisation spectra with an online reference library (Hewlett Packard).

Statistical analysis

Results are reported as mean values and standard deviations. Statistical analysis was carried out by Student's unpaired t test.

Results and discussion

Efficiency of lipid separation by solid-phase extraction

Analysis by GC of the fatty acid compositions of fractions produced by SPE from mixtures of PC (Fig. 1(A)), NEFA (Fig. 1(B)), CE (Fig. 1(C)) and TAG (Fig. 1(D)) standards showed no detectable co-elution of lipid classes. This was confirmed by GC–MS (data not shown). This indicates that the present SPE method provided efficient resolution of the major fatty acid-containing lipid classes found in plasma. The minor peaks, typically <0·5 % total peak area, seen in some GC–flame ionisation detector chromatograms, for example Fig. 1(A), were identified by GC–MS as long-chain hydrocarbons such as dodecane and phthalates. GC–MS analysis showed that these were derived principally from hexane and methanol used in lipid extraction and fatty acid derivatisation (data not shown). There did not appear, however, to be substantial contamination of eluted lipid classes with compounds derived from the SPE cartridges which Agren *et al.* (1992) reported for commercially-prepared columns.

Comparable resolution of lipid classes was achieved with extracts of total plasma lipids. In these present experiments, recovery standards containing heptadecanoic acid were used for both PC and TAG since when only triheptadecanoin or heptadecanoyl PC were added as internal standards heptadecanoate was not detected in PC or TAG respectively (data not shown). Distinct chromatographic profiles were obtained for PC, NEFA, CE and TAG fractions with no obvious co-elution of lipid classes (Fig. 2) consistent with efficient separation of individual lipid fractions. For example, palmitoleic acid (16:1*n*-7, peak b) was only present in small amounts in PC (typically about 1·2 g/100 g), but represented about 4·8 g/100 g of the TAG fraction. PC also contained both eicosatrienoic acid (20:3*n*-9, peak g) and arachidonic acid (20:4*n*-6, peak h) which were not detected in the TAG fraction (Fig. 2). The fatty acid compositions of plasma PC, NEFA, CE and TAG broadly reflected those published previously (Fosbrooke & Tamir, 1968; Jensen *et al.* 1979; Houwelingen *et al.* 1996), although direct comparisons are likely to be influenced by diet, sex and age of subjects.

Recovery of plasma lipid classes by combined lipid extraction and solid-phase extraction

Recovery of TAG, NEFA, CE and PC were estimated from comparison of the peak areas corresponding to the recovery standards and the tricosanoate methyl ester recovery reference standard. The mean recoveries were (%): PC 74·2 (SD 7·5), CV 10·1 %; NEFA 73·6 (SD 8·3), CV 11·2 %; CE 84·9 (SD 4·9), CV 5·8 %; TAG 86·8 (SD 4·9), CV 5·6 %; n 24 plasma samples. There was no significant difference in the recoveries of PC and NEFA fractions, while recoveries of CE and TAG were significantly greater ($P < 0·0001$). The lower recoveries of PC and NEFA may be due either to incomplete isolation during the initial extraction or partial retention of the polar lipids on the SPE column. Agren *et al.* (1992) reported higher recoveries from SPE separations than those from the present study. In their experiments, however, recoveries were estimated by addition of lipid standards after preparation of a total plasma lipid extract and so do not include losses during initial lipid isolation. These present data represent the combined efficiency of isolation for both lipid extraction and fractionation by SPE. Recoveries from TLC separations (n 10 plasma samples) of TAG (48·1 (SD 11·2) %) and NEFA (32·2 (SD 9·8) %) were significantly lower (TAG 1·8-fold, NEFA 2·3-fold) ($P < 0·0001$) than those obtained by SPE, while recovery of CE (80·9 (SD 5·5) %) was similar for both TLC and SPE. Together these data demonstrate comparable recoveries of PC, NEFA, TAG and CE by SPE, while these lipid classes were eluted differentially from TLC plates. Such variations in recovery from TLC plates between classes may influence ability to detect relatively minor components, in particular polyunsaturated fatty acids, which may be an important consideration in data interpretation. Since recoveries were consistently less than 100 % for both SPE and TLC separations, it is important to include appropriate recovery standards for determination of fatty acid concentrations.

Repeatability of plasma lipid fatty acid composition

Analysis of fatty acid compositions is critically dependent upon the ability to isolate individual lipid classes with minimal modification to fatty acid composition. Comparisons of the repeatability of isolation of four major fatty acids common to TAG, PC, CE and NEFA fractions are summarised in Table 1. The CV was consistently less than 10 % for each fatty acid. The variation in fractional fatty acid content both between and within lipid classes appeared to be greatest for minor fatty acid species. One possible source of such variation may be small differences in the integration of minor peaks on GC chromatograms, for example stearic acid in CE (Table 1), making a greater

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

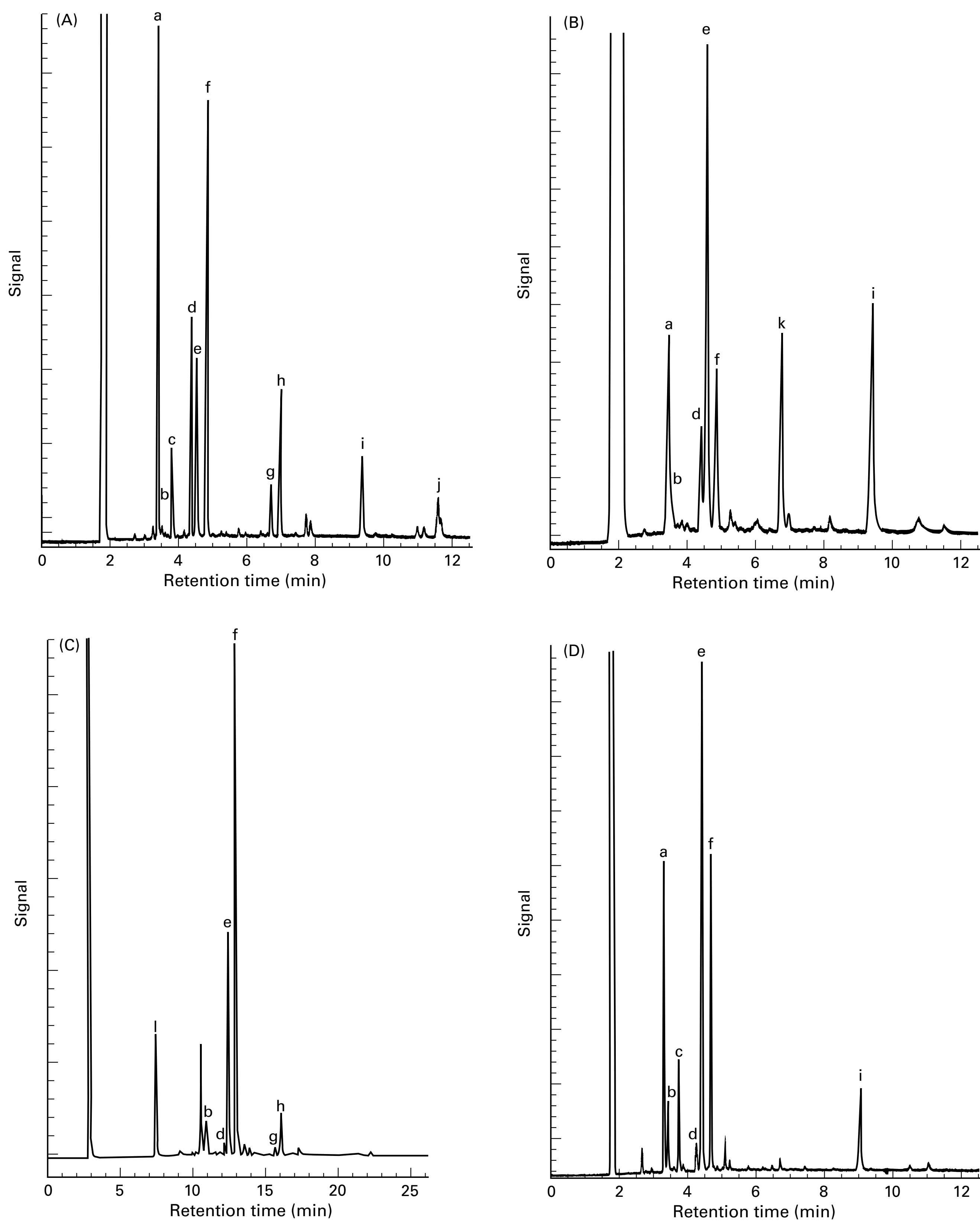

Fig. 2. Representative GC–flame ionisation detector chromatograms of plasma phosphatidylcholine (A), non-esterified fatty acids (B), cholesterol ester (C) and triacylglycerol (D) fractions isolated from total lipid extracts by solid-phase extraction: (a) palmitate; (b) palmitoleate; (c) heptadecanoate; (d) stearate; (e) oleate; (f) linoleate; (g) eicosatrienoate; (h) arachidonate; (i) tricosanoate; (j) docosahexaenoate; (k) henecosanoate, (l) myristate.

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

Table 1. Repeatability of plasma fatty acid composition following separation of lipid classes by solid-phase extraction or TLC†
(Mean fractional concentrations, standard deviations, and coefficients of variation for *n* 10 samples)

	Fractional concentrations of plasma fatty acids (g/100 g)											
	Palmitate			Stearate			Oleate			Linoleate		
	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)
Solid-phase extraction												
PC	40·8	1·0	2·5	13·9	0·6	4·1	18·0	1·0	5·6	27·4	1·4	5·1
NEFA	33·1	0·8	2·5	8·9	0·7	7·8	40·1	1·6	4·0	17·9	1·1	6·4
CE	13·8	0·3	1·9	0·8	0·1	9·2	26·3	0·3	1·0	59·9	0·6	1·0
TAG	33·7	0·9	2·6	5·2	0·4	7·9	44·6	0·7	1·6	16·5	0·5	3·3
TLC												
NEFA	35·4	5·0	14·0	8·8	1·5	16·5	39·5	2·6	6·7	16·2*	1·9	11·8
CE	12·5	1·6	13·0	0·8	0·3	31·2	24·5	2·6	10·8	62·1	4·4	7·0
TAG	31·0	9·2	29·7	3·6	0·6	16·0	46·9	6·3	13·3	18·5	2·5	13·2

PC, phosphatidylcholine; NEFA, non-esterified fatty acid; CE, cholesterol ester; TAG, triacylglycerol.
Mean value was significantly different from that analysed by SPE (*$P < 0.05$).
† For details of procedures see p. 782.

contribution to the total area-under-the-peak compared with similar errors in integrating major fatty acid components.

Comparison of the fractional concentrations of palmitic, stearic, oleic and linoleic acids isolated from aliquots (*n* 10) of the same plasma sample by TLC with SPE showed similar repeatability between these techniques. However, the fractional content of linoleic acid differed significantly ($P < 0.05$) between NEFA prepared by TLC and SPE (Table 1), but not TAG or CE. Furthermore, the CV for linolenate isolated by TLC was greater than for SPE (NEFA 1·8-fold, CE 7·0-fold, TAG 4·0-fold). Such variation could potentially have an important impact upon interpretation of measurements of linoleic acid in plasma. One possible explanation for this increased variability in linoleic acid content between isolation techniques is increased auto-oxidation due to prolonged exposure to air on the TLC plate. PC was resolved poorly by this TLC technique and so was not used in these comparisons.

It is possible that SPE columns may selectively retain individual fatty acid species. This is unlikely to be a major problem with TAG and PC since each molecule typically has a heterogeneous fatty acid composition and so differential isolation of individual molecular species would affect both saturates and unsaturates equally. In separations of NEFA and CE, however, individual species could be eluted differentially and so substantially influence the results of analyses of fatty acid composition. Comparisons of the fractional concentrations of individual fatty acids following SPE with the starting mixtures showed no significant selectivity in elution of fatty acid or cholesterol ester species (Table 2). The influence of SPE upon the relative concentrations of individual fatty acids is unlikely, therefore, to be a major consideration in the interpretation of biological variation in plasma fatty acid content.

The SPE method described here offers a rapid alternative to TLC for separation of plasma lipid classes, with good resolution and increased sample recovery. This method could potentially be applied to other biological specimens, for example adipose tissue, although some modifications to the technique may be required. Although the cost per

Table 2. Effect of solid-phase extraction upon the fractional concentration of individual fatty acid and cholesterol ester species*
(Mean fractional concentrations, standard deviations, and coefficients of variation for *n* 10 samples)

	Fractional concentrations of plasma fatty acids (g/100 g)							
	Fatty acid				Cholesterol ester			
	Mean	SD	CV (%)	*P*	Mean	SD	CV (%)	*P*
Starting mixture								
Palmitic	23·2	0·5	0·5		23·2	0·8	0·7	
Stearic acid	26·4	0·7	0·6		26·0	0·5	0·5	
Oleic acid	27·3	0·6	0·6		26·0	0·3	0·3	
Linoleic acid					24·8	0·3	0·3	
Linolenic acid	23·1	0·2	0·2					
Post solid-phase-extraction								
Palmitic	23·4	0·2	0·1	0·471	23·9	0·9	0·8	0·072
Stearic acid	25·9	0·2	0·1	0·069	25·7	1·0	0·9	0·453
Oleic acid	27·5	0·2	0·2	0·431	25·5	0·8	0·7	0·090
Linoleic acid					24·9	0·9	0·7	0·860
Linolenic acid	23·3	0·2	0·2	0·178				

* For details of procedures see p. 782.
† Values for *P* are comparisons by Students *t* test of fractional concentrations of individual fatty acids before and after solid-phase extraction separation of lipid classes.

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press

specimen in terms of raw materials was slightly greater (1·4-fold) compared with TLC, the greater (3·3-fold) throughput resulted in overall lower labour-cost-h per sample and increased productivity. Furthermore, this method may increase the feasibility of studies in which collection of large numbers of specimens is desirable.

Acknowledgement

This work was supported by a grant from the Ministry of Agriculture, Fisheries and Food, UK.

References

Agren JJ, Julkunen A & Penttila I (1992) Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. *Journal of Lipid Research* **33**, 1871–1876.

Bernhardt TG, Cannistraro PA, Bird DA, Doyle KM & Laposata M (1996) Purification of fatty acid ethyl esters by solid-phase extraction and high performance liquid chromatography. *Journal of Chromatography B* **675**, 189–196.

Caesar PA, Wilson SJ, Normand ICS & Postle AD (1988) A comparison of the specificity of phosphatidylcholine synthesis by human fetal lung maintained in either organ or organotypic culture. *Biochemical Journal* **253**, 451–457.

Folch J, Lees M & Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.

Fosbrooke AS & Tamir I (1968) A modified method for the preparation of methyl esters of a mixture of medium-chain and long-chain fatty acids. Application to the determination of serum triglyceride and non-esterified fatty acid composition and concentration by gas-liquid chromatography. *Clinica Chimica Acta* **20**, 517–522.

Houwelingen ACv, Foreman-van, Drongelen MMHP, Nicolini U, Nicolaides KH, Al MDM, Kester ADM & Hornstra G (1996) Essential fatty acid status of fetal plasma phospholipids: similar to postnatal values obtained at comparable gestational ages. *Early Human Development* **46**, 141–152.

Hoving EB, Jansen G, Volmer M, Van Doormaal JJ & Muskiet FAJ (1998) Profiling of plasma cholesterol ester and triglyceride fatty acids as their methyl esters by capillary gas chromatography, preceded by a rapid aminopropyl-silica column chromatographic separation of lipid classes. *Journal of Chromatography* **434**, 395–409.

Jensen RG, Clark RM, Gerrior SA, Fey MB & Gotto AM (1979) Cholesterol ester and triacylglycerol fatty acids in type V hyperlipidaemia. *Lipids* **14**, 691–694.

Kim HY & Salem N (1990) Separation of lipid classes by solid phase extraction. *Journal of Lipid Research* **31**, 2285–2289.

© Nutrition Society 2000

https://doi.org/10.1017/S0007114500002154 Published online by Cambridge University Press