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Purification of fatty acid ethyl esters by solid-phase extraction and high-performance liquid chromatography

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Abstract

We have developed a two-step method to purify fatty acid ethyl esters (FAEE) using solid-phase extraction (SPE), with a recovery of 70±3% (mean±S.E.M.) as assessed using ethyl oleate as a recovery marker from a standard lipid mixture in hexane. The first step of the SPE procedure involves application of a lipid mixture to an aminopropyl-silica column with simultaneous elution of FAEE and cholesteryl esters from the column with hexane. Gas chromatographic analysis of FAEE without interference from cholesteryl esters may be performed using the eluate from the aminopropyl-silica column, thus eliminating the need for an octadecylsilyl (ODS) column in this case. The FAEE can then be separated from the cholesteryl esters, if necessary, by chromatography on an ODS column and elution with isopropanol–water (5:1, v/v). Both the aminopropyl-silica and ODS columns were found to be effective for up to four uses. To permit isolation of specific FAEE species following isolation of total FAEE by the two-step SPE method, we have also developed a purification scheme for individual FAEE by high-performance liquid chromatography (HPLC). Thus, this simple method allows for reproducible isolation of total FAEE by SPE and isolation of individual FAEE species by HPLC.

Keywords: Solid-phase extraction; Fatty acid ethyl esters

1. Introduction

Fatty acid ethyl esters (FAEE) are esterification products of ethanol and fatty acids [1]. It has been shown that FAEE and the enzyme responsible for their synthesis (FAEE synthase) are present predominantly in the organs most often damaged by ethanol abuse, notably pancreas and liver [2]. This has led to the speculation that FAEE, lipids more hydrophobic than triglycerides, are mediators of ethanol-induced organ damage [2–7]. Following ethanol ingestion by human volunteers, FAEE have also been found in serum lipoproteins [8]. It has been recently demonstrated that FAEE within low density lipoprotein (LDL) have a toxic effect on intact cells [9]. In this study, LDL were reconstituted with FAEE and delivered to a hepatoblastoma cell line (HepG2). The FAEE were shown to inhibit protein synthesis and decrease the rate of cell proliferation.

Studies involving FAEE have proceeded at a relatively slow pace in comparison to studies on the

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enzyme, FAEE synthase, responsible for their synthesis. A significant hindrance to studies involving FAEE is the practice of isolating FAEE by liquid–liquid extraction and thin-layer chromatography (TLC) prior to FAEE species identification and quantitation. The isolation of FAEE by these conventional methods has been shown to often result in low yields. The small amounts of this very hydrophobic lipid present in human plasma following ethanol ingestion are not uncommonly lost from the sample during extraction. As with fatty acids, FAEE moieties which contain two or more double bonds can be oxidized within minutes on a dried TLC plate and are thereby lost prior to quantitation. To enhance recovery of the relatively small amounts of FAEE in biological samples, we developed a consistently effective solid-phase extraction (SPE) method, without the need for TLC, for isolation of FAEE. In addition to the isolation of total FAEE by solid phase extraction, in this report we also describe a method for isolation of individual FAEE species using HPLC.

2. Experimental

2.1. Materials

Aminopropyl-silica SPE columns (500 mg) and a Vac-Elut vacuum manifold were purchased from Varian (Harbor City, CA, USA). Octadecylsilyl (ODS) columns (500 mg) were obtained from Whatman (Clifton, NJ, USA) and silica gel 60 plates were from EM Science (Gibbstown, NJ, USA). Ethyl oleate, cholesteryl oleate, oleic acid, triolein, and cholesterol were purchased from Nu Check Prep (Elysian, MN, USA). Phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL, USA). Cholesteryl [1-^{14}C]oleate (55 mCi/mmol), [1,2,6,7-^{3}H]cholesteryl oleate (82.9 Ci/mmol), [1-^{14}C]oleic acid (53.8 mCi/mmol), [carboxy-^{14}C]triolein (110 mCi/mmol), and [1-^{14}C]dipalmitoyl phosphatidylcholine (115 mCi/mmol) were obtained from DuPont-New England Nuclear (Boston, MA, USA) and [1a,2a(*n*)-^{3}H]cholesterol (40–60 mCi/mmol) was from Amersham (Arlington Heights, IL, USA).

Ethyl [^{14}C]oleate was synthesized by dissolving approximately 39 nmol of the [carboxy-^{14}C]triolein (110 mCi/mmol) in 0.6 ml dichloromethane and incubating with 1 ml of 0.5 *M* KOH in ethanol at room temperature for 45 min [10]. The reaction was terminated by the addition of 1 ml of 6 *M* HCl and the mixture extracted with 2 ml of dichloromethane. The extract was then spotted on a silica gel 60 plate and the plate developed with petroleum ether–diethyl ether (75:5, v/v) to purify the ethyl [^{14}C]oleate. Elution of FAEE from the silica gel scrapings was performed with acetone in an atmosphere of nitrogen.

2.2. Aminopropyl-silica column chromatography

Lipid samples used as standards were prepared by the addition of 25 000 dpm each of ethyl [^{14}C]oleate, cholesteryl [^{14}C]oleate, [^{14}C]dipalmitoyl phosphatidylcholine, [^{14}C]oleic acid, [^{14}C]triolein, and [^{3}H]cholesterol. The radioactive lipids were mixed with corresponding nonradiolabeled lipids to achieve final amounts of 3.1 μg of ethyl oleate, 975 μg of cholesteryl oleate, 120 μg of oleic acid, 1 μg of triolein, 525 μg of cholesterol, and 80 μg of dipalmitoyl phosphatidylcholine. These amounts are similar to the amount of each lipid extracted in 1 ml of serum. The lipid mixtures were evaporated under nitrogen and resuspended in 200 μl of hexane.

The SPE procedure employed was a method modified from that described by Kaluzny et al. [11]. Briefly, the aminopropyl-silica columns were placed on a Vac-Elut vacuum apparatus set at 10 kPa. The columns were conditioned with 4 ml of hexane. Immediately after the solvent reservoir became empty, the sample (200 μl) was applied followed by 4 ml of hexane for column elution. The hexane eluate was then evaporated under nitrogen to a volume of approximately 200 μl which was spotted onto a silica gel 60 plate for TLC to identify the lipids in the eluate. Nonradiolabeled standards were spotted in a separate lane. The plate was developed in a solvent system consisting of petroleum ether–diethyl ether (75:5, v/v). Fractions were then scraped from the plate and the radioactivity determined by liquid scintillation counting.

In experiments to determine the reusability of the aminopropyl-silica columns, four identical lipid mix-

tures were prepared. Each mixture contained the amount of ethyl oleate, cholesteryl oleate, oleic acid, triolein, cholesterol, and dipalmitoyl phosphatidylcholine noted above. In one of the four lipid mixtures, 25 000 dpm of each lipid class was present. The other three mixtures contained no radiolabeled lipids. The four mixtures were applied to an aminopropyl-silica column in one of two different sequences, washing the column between addition of each mixture with 6 ml of methanol, 6 ml of chloroform–isopropanol (2:1, v/v), 6 ml of chloroform, and 6 ml of hexane and then allowing the column to dry. The first sequence was designed to assess carry-over from one sample to another after repeated use of the column. In these studies, the radioactive mixture was added third and the final 4 ml of hexane eluate of the fourth mixture was collected for determination of cross-contamination from the third sample. The hexane eluate was concentrated by evaporation under nitrogen, spotted onto a silica gel 60 plate for TLC, and radioactivity (representing cross-contamination from the third mixture) quantitated as described above. The second sequence was designed to assess the chromatography of the column after repeated use. In these studies, the radioactive mixture was added last and the final 4 ml of hexane eluate analyzed as described above to assess the ability of the column to separate cholesteryl esters and FAEE from the other lipid classes after repeated use.

2.3. Octadecylsilyl column chromatography

Lipid mixtures were prepared by the addition of 25 000 dpm each of ethyl [^{14}C]oleate and [1,2,6,7-^{3}H]cholesteryl oleate mixed with 3.1 μg and 975 μg of nonradiolabeled ethyl oleate and cholesteryl oleate, respectively, to achieve relatively physiologic concentrations. The lipid mixture was evaporated to dryness under nitrogen and resuspended in 300 μl of isopropanol–water (5:1, v/v). This sample was applied to an ODS column connected at the top to a 5-ml syringe used as a loading reservoir. The column was preconditioned sequentially with 5 ml of methanol and 5 ml of water. Elution of lipids was performed with isopropanol-water (5:1, v/v) and the radioactivity quantitated by liquid scintillation counting. If gas chromatography is to be used for FAEE analysis, only the aminopropyl-silica column procedure is necessary and the ODS column procedure may be omitted. This is possible due to the large difference in boiling point between FAEE and cholesteryl esters. The ODS column protocol permits the separation of FAEE from cholesteryl esters if needed.

To determine the reusability of the ODS columns, the same procedure was employed as the one used to determine the reusability of the aminopropyl-silica columns. However, in the ODS column experiments, the lipid mixtures contained only ethyl oleate and cholesteryl oleate, rather than the mixture of five lipids, and only 5 ml of hexane was used to wash the column between sample applications.

2.4. Solid-phase extraction of FAEE from serum

Sera from patients with detectable blood ethanol levels (averaging 54 m*M*, 0.25 g%) were divided into 1-ml aliquots. Extraction of the lipids from each 1-ml aliquot of serum was initiated by the addition of 2 ml of acetone to the serum followed by 5 ml of hexane. After vortex-mixing for 1 min, the samples were centrifuged at 175 *g* for 5 min and the hexane–acetone layer transferred to a separate tube. In separate experiments involving the addition of an ethyl [^{14}C] oleate standard to serum, the recovery of FAEE in the hexane–acetone layer was determined to be 96.3$\pm$1.2% (n=5, mean$\pm$S.E.M.). The hexane extract was then evaporated to dryness under nitrogen, resuspended in 200 μl of hexane and applied to an aminopropyl-silica column as described above. The hexane eluate was then evaporated under nitrogen and resuspended in a small aliquot of hexane for GC analysis.

2.5. Gas chromatography–mass spectrometry

GC analysis was performed on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5970 mass spectrometer with a WCOT Supelcowax capillary column (30 m$\times$0.25 mm I.D., 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). The oven temperature was increased at a rate of 10°C/min from 150°C to 250°C and remained at 250°C for 6 min while the injector and mass spectrometer were maintained at 260°C and 280°C,

respectively. Total ion chromatograms were obtained using an ionization energy of 70 eV. Calibration curves were developed using FAEE standards that related the amount and response of each individual FAEE to that of ethyl heptadecanoate (ethyl 17:0). Quantitation of FAEE from serum samples was achieved using the aforementioned calibration curves and peak areas, and 500 pmol of ethyl heptadecanoate was included as an internal quantification standard after the addition of acetone.

2.6. Comparison of SPE and TLC in the recovery of individual FAEE from serum

To eight 1-ml aliquots of serum, 500 pmol each of ethyl palmitate, ethyl stearate, ethyl oleate, ethyl linoleate, and ethyl arachidonate were added. A lipid extraction of each aliquot into acetone was then performed as described above, except that the addition of 500 pmol of the ethyl heptadecanoate internal quantification standard was omitted. Four of the lipid extracts were processed using an aminopropyl-silica column only, and the other four extracts were processed using the TLC system described above. In order to quantitate the amount of each individual FAEE species remaining in the processed samples, an external standard of 500 pmol of ethyl heptadecanoate was then added to each of the 4-ml hexane eluates from the aminopropyl-silica column and the acetone elutions from the silica gel scrapings. All samples were then evaporated to dryness under nitrogen and resuspended in a small volume of hexane for GC–MS analysis. The recoveries of each individual FAEE for the two methods were compared.

2.7. High-performance liquid chromatography

For separation of individual FAEE species by HPLC, lipid samples were prepared by dissolving 4 μg of ethyl arachidonate, 16 μg of ethyl linoleate, 80 μg of ethyl oleate, 320 μg of ethyl stearate, 600 μg of ethyl heptadecanoate, 160 μg ethyl palmitoleate, and 300 μg of ethyl palmitate in 100 μl of acetonitrile–isopropanol–water (28:28.8:43.2, v/v). Half of the sample was then injected into a Rainin HPLC mounted with a Spherisorb C_6 250×4.6 mm I.D. column, with sorbent size of 5 mm (Alltech, Deerfield, IL, USA). A Knauer variable wavelength detector (Rainin Instruments, Woburn, MA, USA) was set at 205 nm to detect FAEE. For the first 62 min, the mobile phase was acetonitrile–isopropanol–water (28:28.8:43.2, v/v) with a flow-rate of 0.85 ml/min. From 62 to 110 min, a linear gradient was employed to reach a final solvent mixture of acetonitrile–isopropanol–water (52:19.2:28.8, v/v) at a flow-rate of 1.25 ml/min.

3. Results and discussion

In the first set of experiments, an aminopropyl-silica solid phase column was used to isolate FAEE. In these studies, 25 000 dpm of each of six different lipids (ethyl [^{14}C]oleate, cholesteryl [^{14}C]oleate, [^{14}C]oleic acid, [^{14}C]triolein, [^{14}C]dipalmitoyl phosphatidylcholine and [^{3}H]cholesterol) were dissolved in hexane. The mixture was applied to the aminopropyl-silica column, after which lipids were eluted with hexane. Fractions were collected and the lipid moieties evaluated for purity by TLC. Fig. 1 shows the lipids isolated by TLC from the hexane eluate. As shown in Fig. 1, hexane eluted FAEE and cholesteryl esters (CE) from the aminopropyl-silica column. Phospholipids (PL), free fatty acids (FFA), triglycerides (TG), and free cholesterol were not eluted by the hexane.

To separate FAEE from cholesteryl esters, an experiment was performed in which ethyl [^{14}C]oleate and [1,2,6,7-^{3}H]cholesteryl oleate (each 25 000 dpm) were dissolved in isopropanol–water (5:1, v/v) and then applied to an ODS column. The lipids were then eluted with isopropanol–water (5:1, v/v) in 1-ml fractions. The radioactivity in each fraction was determined. The FAEE were eluted in this mixture while the cholesteryl esters were retained in the column (Fig. 2).

To determine the recovery of FAEE using SPE, ethyl [^{14}C]oleate (25 000 dpm) was mixed with unlabeled cholesteryl oleate, triolein, oleic acid, cholesterol, and phosphatidylcholine in hexane. These were applied to an aminopropyl-silica column followed by elution with hexane and collection of 1-ml fractions. The fractions were dried and resuspended in isopropanol–water (5:1, v/v). The

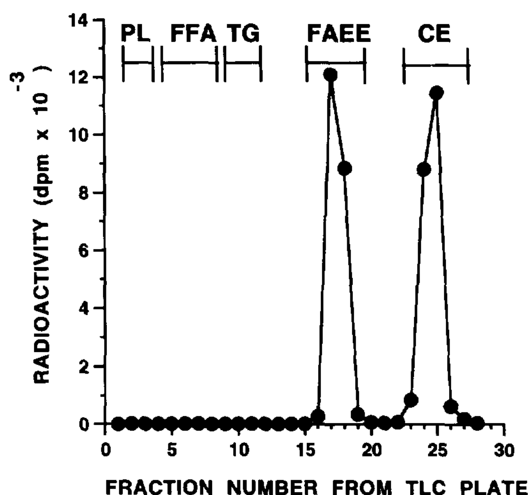

Fig. 1. Elution from the aminopropyl-silica column. A lipid mixture containing 25 000 dpm each of ethyl [^{14}C]oleate (FAEE), cholesteryl [^{14}C]oleate (CE), [^{14}C]dipalmitoyl phosphatidylcholine (PL), [^{14}C]oleic acid (FFA), [^{14}C]triolein (TG), and [^{3}H]cholesterol with corresponding nonradiolabeled lipids was dissolved in hexane and applied to a conditioned aminopropyl-silica column. Elution was performed with 4 ml of hexane. The hexane eluate was dried under nitrogen to a volume of less than 50 μl and applied to a silica gel 60 plate for TLC. After developing the plate, fractions were scraped and the radioactivity determined by scintillation counting. The extents of migration of PL, FFA, TG, FAEE and CE from the point of origin (fraction 2) are designated. Values represent the mean of three experiments.

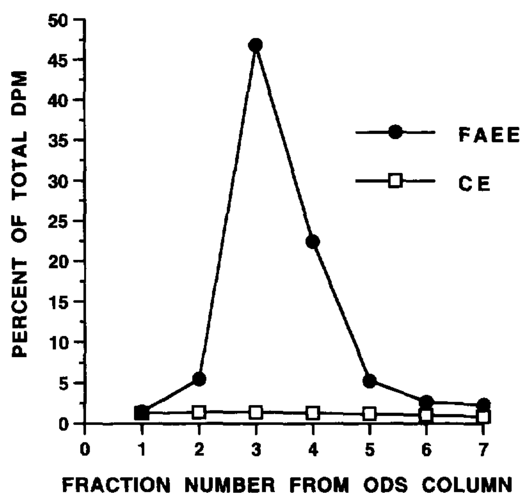

Fig. 2. Elution from the ODS column. A lipid mixture containing 25 000 dpm each of ethyl [^{14}C]oleate (FAEE), [1,2,6,7-^{3}H]cholesteryl oleate (CE), 3.1 μg ethyl oleate and 975 μg cholesteryl oleate was dissolved in isopropanol–water (5:1, v/v) and applied to a conditioned ODS column. Elution was performed with isopropanol–water (5:1, v/v) collecting 1-ml fractions. The radioactivity in each fraction was determined by scintillation counting. Values represent the mean of three experiments.

hexane eluate contained FAEE and cholesteryl esters. These were applied to an ODS column to separate the two lipids. The FAEE were eluted with isopropanol–water (5:1, v/v) and then extracted from this mixture into hexane. The hexane fractions were counted for radioactivity. It was found that 70±3% (range of 60–78%) of the ethyl oleate marker was recovered from a standard mixture of lipids in hexane (n=9, mean±S.E.M.).

Fig. 3 shows a total ion chromatogram from the gas chromatograph–mass spectrometer of FAEE following elution from the aminopropyl-silica column. In this study, the FAEE in 1 ml of serum containing a relatively high blood ethanol concentration (37.0 mM, 0.17 g%) were extracted into hexane. The extract was applied to an aminopropyl-silica column and then the hexane eluate was dried under nitrogen and resuspended in a small volume for injection into the gas chromatograph–mass spectrometer. The total ion chromatogram shows definitive peaks confirmed to be ethyl esters by comparison of the electron impact spectra of the individual peaks with spectra of authentic standards. The chromatogram in Fig. 3 shows ethyl palmitate (E 16:0, 1.11 nmol), ethyl heptadecanoate (internal quantification standard, E 17:0, 0.50 nmol), ethyl stearate (E 18:0, 0.47 nmol), ethyl oleate (E 18:1, 0.70 nmol), ethyl linoleate (E 18:2, 0.20 nmol), and ethyl arachidonate (E 20:4, 0.05 nmol). Control studies were performed in which twenty serum samples containing no detectable ethanol were extracted and processed for FAEE analysis by GC–MS. No FAEE were detected in any of these samples.

A number of experiments using standard lipid mixtures were performed to assess the reusability of both the aminopropyl-silica column and the ODS column. The amount of each lipid present in the standard mixtures closely approximated the amount that would be extracted from 1 ml of serum. It is extremely unlikely that there was a matrix effect from the serum sample which would differentiate it from a standard lipid mixture on an aminopropyl-silica column. Serum samples are processed by

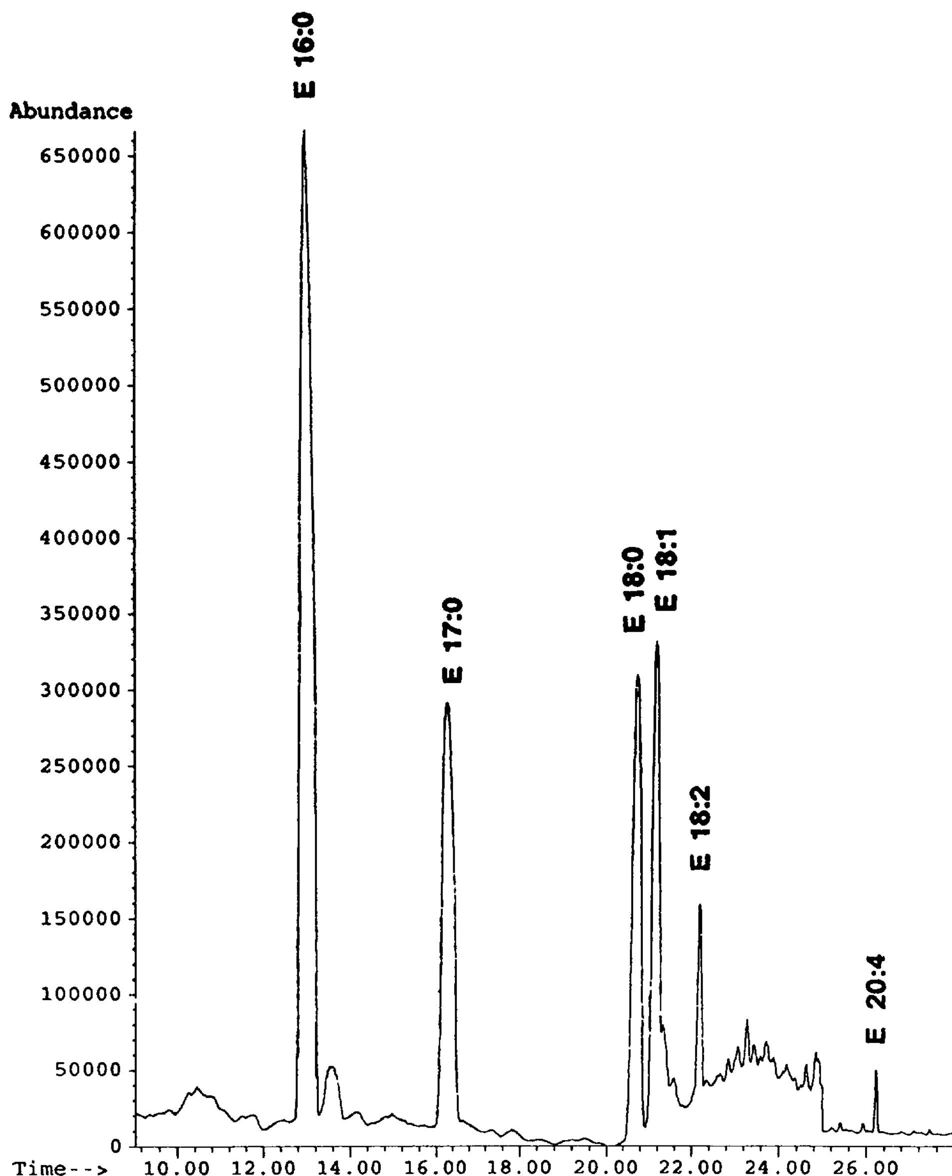

Fig. 3. Analysis of FAEE from human plasma. Lipids from sera of patients with markedly elevated blood ethanol levels were extracted into hexane and applied to an aminopropyl-silica column. Lipids eluted from the column were dried under nitrogen to a small volume and an aliquot injected into a gas chromatograph-mass spectrometer. This chromatogram is representative of four similar preparations. The peaks identified as FAEE are labeled as E 16:0 for ethyl palmitate, E 17:0 for ethyl heptadecanoate, E 18:0 for ethyl stearate, E 18:1 for ethyl oleate, E 18:2 for ethyl linoleate, and E 20:4 for ethyl arachidonate.

extraction with acetone and hexane and drying to completion, with resuspension of extracted lipids in hexane. Therefore, the serum sample and the standard lipid mixture are both in hexane at the time of application to the aminopropyl-silica column. In these experiments it was demonstrated that both columns could be used at least four times without cross-contamination from one sample to another, as

shown by the lack of radioactivity found in the fourth use of the column when the radioactive lipid mixture was added in the third sample. It was also demonstrated that the aminopropyl-silica columns were able to separate cholesteryl esters and FAEE from the other lipid classes after three prior uses of the column. The same was shown for ODS columns, yielding elution profiles similar to those shown in Fig. 2.

Experiments were performed to compare the recovery of individual FAEE species (ethyl 16:0, ethyl 18:0, ethyl 18:1, ethyl 18:2, and ethyl 20:4) added to serum using either aminopropyl-silica column elution or TLC to process the acetone–hexane lipid extracts. Recoveries were assessed using the GC–MS procedure described above with ethyl heptadecanoate added as an external standard just prior to injection. The data in Table 1 indicate that the recoveries were approximately 20% higher and more reproducible for samples processed by aminopropyl-silica column elution than those processed by TLC. The S.E.M. value for TLC was approximately twice that for the aminopropyl-silica column.

A cost analysis was performed to determine the cost of analyzing samples by TLC vs. SPE using aminopropyl-silica and ODS columns. If both the aminopropyl-silica and ODS columns are used four times before disposal, the total cost for the method involving SPE is $1.64 per sample. If only the aminopropyl-silica column is required, the cost of analysis is $1.16 per sample. The cost of analyzing a sample by the TLC protocol described above, with eight samples on a single 20×20 cm TLC plate, is $0.60 per sample. The principal advantage of SPE is a reproducibly higher recovery. There is also an advantage for SPE in the time saved in sample processing. The processing of eight samples by TLC for GC analysis, including all steps, required approximately 3 h, and the processing of eight samples using only the aminopropyl-silica column required less than 1 h. If the ODS column is required for further separation of FAEE from cholesteryl esters, an additional hour of processing is required.

Table 1
Recovery of FAEE from serum for both aminopropyl-silica column and TLC protocols

FAEE	% Recovery	
	Aminopropyl-silica column[a]	TLC[b]
Ethyl 16:0	87.3±0.9	61.4±2.1
Ethyl 18:0	83.7±1.0	62.5±2.5
Ethyl 18:1	85.1±2.0	66.8±2.7
Ethyl 18:2	88.1±1.2	62.0±1.5
Ethyl 20:4	72.9±0.9	62.8±4.4

[a] Mean±S.E.M. for $n=4$.
[b] Mean±S.E.M. for $n=3$.

An HPLC program was developed to separate the individual FAEE species. Ethyl palmitoleate, ethyl arachidonate, ethyl linoleate, ethyl palmitate, ethyl oleate, ethyl heptadecanoate, and ethyl stearate dissolved in 100 μl of acetonitrile–isopropanol–water (28:28.8:43.2, v/v/v) were injected into an HPLC with a Spherisorb C_6 column. A linear gradient of increasing acetonitrile was used to separate the FAEE. The retention times for ethyl palmitoleate, ethyl arachidonate, ethyl linoleate, ethyl palmitate, ethyl oleate, ethyl heptadecanoate, and ethyl stearate were 51.0, 54.8, 59.1, 74.4, 78.0, 85.4, and 95.3 min, respectively. It is important to note that at 205 nm, the sensitivity of this method is low. Approximately 0.5 μmol is required for detection of saturated FAEE. With this in mind, either large serum volumes must be used for FAEE detection, or fractions must be collected based on the retention times. The combination of SPE with HPLC permits recovery of individual FAEE species with a high yield and excellent reproducibility.

4. Conclusions

A two-column SPE method was used to purify FAEE with a reproducibly high recovery. The first step of the SPE procedure involved the use of an aminopropyl-silica column with elution of FAEE and cholesteryl esters, after which GC–MS analysis of FAEE may be performed. The second SPE step involved the separation of the FAEE from the cholesteryl esters using an ODS column. This method can be used to isolate small quantities of FAEE from lipid extracts of human plasma. Individual FAEE species can subsequently be isolated by HPLC. This combination of methods allows for isolation of total FAEE by two SPE steps and isolation of individual FAEE species by HPLC.

Note added in proof

We have recently observed that the $n-3$ ethyl esters of eicosapentaenoate (20:5) and docosahexaenoate (22:6) elute from the aminopropyl-silica column only if the column is preconditioned with 4 ml of dichloromethane before the 4 ml of hexane used in preconditioning. In addition, the elution of the $n-3$ FAEE from the column requires 4 ml of dichloromethane following the 4-ml elution with hexane. The hexane and dichloromethane eluates can be combined to obtain a mixture of FAEE including the long-chain $n-3$ FAEE.

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