

A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil

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Abstract

A low expense process is developed for recovering esterified eicosapentaenoic acid (EPA) from microalgae and fish oil. Over 70% of the EPA content in the esterified crude extract of microalgae were recovered at purities exceeding 90%. The recovery scheme utilizes either wet or freeze-dried algal biomass. The process consists of only three main steps: 1) simultaneous extraction and transesterification of the algal biomass; 2) argentated silica gel column chromatography of the crude extract; and 3) removal of pigments by a second column chromatographic step. Argentated silica gel chromatography recovered about 70% of the EPA ester present in the crude fatty ester mixture of fish oil, but at a reduced purity (~83% pure) compared to the microalgal derived EPA. The optimal loading of the fatty ester mixture on the chromatographic support was about 3% (w/w) but loadings up to 4% did not affect the resolution significantly. The process was scaled up by a factor of nearly 320 by increasing the diameter of the chromatography columns. The elution velocity remained constant. Compared to the green alga *Monodus subterraneus*, the diatom *Phaeodactylum tricorutum* had important advantages as a potential commercial producer of EPA. For a microalgal EPA process to be competitive with fish oil derived EPA, *P. tricorutum* biomass (2.5% w/w EPA) needs to be obtained at less than \$4/kg. If the EPA content in the alga are increased to 3.5%, the biomass may command a somewhat higher price. The quality of microalgal EPA compares favorably with that of the fish oil product. Compared to free fatty acid, EPA ester is more stable in storage. Shelf-life is extended by storing in hexane. The silver contamination in the final purified EPA was negligibly small (<210 ppb). © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Argentated silica gel column chromatography; Eicosapentaenoic acid; Fish oil; Microalgae; *Monodus subterraneus*; *Phaeodactylum tricorutum*; Polyunsaturated fatty acids

1. Introduction

Eicosapentaenoic acid (EPA, 5,8,11,14,17-*cis*-eicosapentaenoic acid) is an n-3 C₂₀-polyunsaturated fatty acid that is metabolically active. EPA and its derivatives have proved beneficial in prevention and treatment of certain medical conditions including coronary heart disease, blood platelet aggregation, abnormal cholesterol levels, and several carcinomas [1]. EPA is effective also in arresting and minimizing tumor growth [2]. At least one patent protected drug (1,3-propanediol ester of EPA) derived by coupling EPA to other molecules is in advanced clinical trials [2]. This product is being developed by Scotia Pharmaceuticals Ltd.

EPA is currently sourced from fish oil. No other sources are commercially available. Fish oil fluctuates in price and quality. In addition, there are important concerns regarding contamination of fish oil with pesticides and heavy metals. Thus, an alternative economic and consistent source of EPA is needed. Microalgae are one potential source. Annual EPA demand is about 125 tones in Japan and world-wide demand is much greater. With emerging evidence of clinical effectiveness of this compound, demand is expected to increase. Because of dwindling supplies, fish oil may not be able to satisfy the future demand of EPA. This, too, suggests the need for a robust alternative source. Current market price of EPA ethyl ester (95% pure) in bulk quantities is about \$650/kg and any new source would need to compete with that price.

This work reports on a process for large scale recovery of highly pure (>95% purity) EPA ester from microalgal biomass. The process is developed for the two microalgae

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Nomenclature

A_i	area under the chromatographic peak of component i (mm^2)
A_s	area under the chromatographic peak of the internal standard (mm^2)
ASTM	American Society for Testing and Materials
A_{xxx}	absorbance at xxx nm (–)
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
C_i	concentration of the fatty acid methyl ester i (g/l)
C_s	concentration of the fatty acid methyl ester standard (g/l)
D	dilution factor for the lipid sample (–)
DHA	docosahexaenoic acid
D_L	diameter of the larger column (m)
D_S	diameter of the smaller column (m)
EPA	eicosapentaenoic acid
f	fractional contribution of downstream processing to total cost of production (–)
L	fractional loading of the total fatty ester on the stationary phase (% w/w)
M_A	dry weight of algal biomass needed to produce 1 kg EPA (kg)
M_L	amount of ester loaded on larger scale column (kg)
M_S	amount of ester loaded on small scale column (kg)
n	number of purification cycles before regeneration (–)
P_{ox}	peroxide value (mEq O_2 /kg EPA)
PUFA	polyunsaturated fatty acids
PUFE	polyunsaturated fatty esters
T	total cost of producing EPA from fish oil (\$/kg)
T_m	total cost of producing EPA from microalgae biomass (\$/kg)
T_R	cost of the fish oil or algal biomass for 1 kg of EPA (\$)
t	time (d)
V_t	total volume of liquid in the cuvette (ml)
V_s	volume of the sample added (ml)
x	percent recoverable EPA in the biomass (% w/w)
y	solvent recovery costs as a fraction of those of the fish oil process (–)
z	mass fraction of EPA (as ester) in the algal biomass (–)

ters from the algal biomass; 2) a silver ion column chromatography step; and 3) a chlorophyll removal step. Optimal processing conditions, the scale up of recovery, and the relative economics of producing microalgal EPA are discussed. The quality and stability characteristics of EPA from microalgae are reported.

In the past, several process schemes have been advanced to purify polyunsaturated fatty acids from complex mixtures. To attain high purities, these schemes invariably employed too many processing operations that reduced overall recovery and magnified costs [3,4]. In several cases, the methods developed could not be scaled up or had other problems. In one study, a complicated procedure involving a two-step winterization, saponification, and urea fractionation of sardine oil successfully produced a fraction containing 90% mixture of EPA and DHA [5], but failed to resolve those two compounds. Among other possibilities, polyunsaturated fatty esters (PUFE) may be selectively extracted from a water immiscible organic phase into an aqueous solution of silver nitrate [6]. However, this approach is suitable largely for separating the PUFEs from saturated ones; it does not allow purification of a single compound such as EPA from a complex mixture such as esterified fish oil. Similarly, $n-3$ PUFEs may be selectively obtained by adsorption of the esterified oil on aminopropyl bonded silica columns and selective elution of saturated and monounsaturated fatty acid esters with hexane [7]. The polyunsaturated fatty acid esters are subsequently eluted with dichloromethane. Again, this method does not resolve highly pure EPA from the other polyunsaturated esters. Another variant of column chromatography envisions using aluminum oxide stationary phase and supercritical or liquid carbon dioxide as the mobile phase for fractionation of unsaturated fatty acids [8], but few clear details have been published. Fish oil and whole triglyceride extracts of other marine organisms may of course be fractionated directly on silica gel [9] and Ag-impregnated silica gel, but initial fractionation of whole triglycerides is not efficient for simultaneously obtaining a high purity and good recovery of most of the EPA in an oil.

A more recent novel approach employed kinetic resolution to separate EPA from fish oil [10]. Kinetic resolution is based on differences in selectivity and rates of lipase catalyzed esterification of different fatty acids in a mixture. Whereas this approach has allowed high recovery of EPA (up to 75%) from the oil, the purity of the product did not exceed 18% [10]. When free fatty acids were used as the starting material rather than the triglycerides, EPA recovery by kinetic resolution improved to 93% but purity declined to less than 8% [10]. Obviously, kinetic resolution as a method of purification has limited capabilities. In addition, kinetic resolution using lipases under anhydrous conditions is difficult to economically implement in practice and the process is comparatively slow. Other fatty acid recovery schemes, mostly useful only in the laboratory, have been reviewed elsewhere [4,11].

Phaeodactylum tricornutum and *Monodus subterraneus* and it is proved also with fish oil. The process has three main steps: 1) combined extraction-transesterification of fatty est-

2. Materials and methods

2.1. Simultaneous extraction and transesterification of microalgal fatty acids

The fatty esters were extracted either from freeze-dried microalgal biomass or from centrifugally harvested biomass paste that had been stored in a freezer. The biomass paste had a moisture content of 82% by weight. The biomass belonged to either the diatom *P. tricornutum* or the green alga *M. subterraneus*. The wet and dry biomass were processed differently as explained next. The biomass had been produced by outdoor continuous culture in tubular photobioreactors [12]. The culture medium was sea water, additionally supplemented with nutrient salts as detailed elsewhere [13]. The culture temperature was 20°C.

2.1.1. Freeze-dried biomass

Dry biomass (either 100 g *P. tricornutum* or 70 g *M. subterraneus*) was added to a mixture of methanol (500 ml), acetyl chloride (25 ml), and hexane (500 ml). The resulting slurry was placed in a stainless steel pressure vessel (2 l, 0.16 m id, 0.15 m depth; built in house) and held in an ultrasonic bath for 10 min. The pressure vessel was then transferred to a boiling water bath and held there for 30 min from the time the pressure reached its maximum value of 3.5 atm. Then the pressure reactor was cooled to ambient in a cold water bath. The biomass slurry was filtered with suction (Büchner funnel). The reactor was washed with 500 ml hexane into the Büchner funnel. The liquid phase was allowed to separate in a separator funnel (15 min). The top hexane layer was recovered and concentrated to 100 ml by evaporation under argon (rotary evaporator). Various amounts of this concentrated extract were applied to chromatography columns as needed.

2.1.2. Biomass paste

Biomass paste (500 g, 82% moisture by wt) of *P. tricornutum* was added to a mixture of methanol (1 l) and acetyl chloride (50 ml). The resulting slurry was placed in a pressure vessel and held in an ultrasonic bath for 10 min. The pressure vessel was then transferred to a boiling water bath and held there for 120 min from the time the pressure reached its maximum value of 2.5 atm. Then the pressure reactor was cooled to ambient in a cold water bath. Hexane (1 liter) was added to the biomass slurry. The slurry was agitated (~10 min) and left overnight at 4°C. The next day, the hexane layer was removed using a peristaltic pump and without disturbing the settled solids. A 500 ml portion of fresh hexane was carefully added to the top of the settled solids and after 15 min this layer of hexane was also withdrawn. The two portions of the hexane extract were mixed, filtered, and evaporated under argon (rotary evaporator) at 30–35°C to obtain the crude fatty ester mixture. Various amounts of this extract were applied to chromatography columns as needed.

2.2. Transesterification of fish oil

Cod liver oil (9.1 g) was mixed with absolute ethanol (500 ml), acetyl chloride (25 ml), and hexane (1 l). The resulting solution was transferred to a pressure vessel that was placed in a boiling water bath (30 min). Then the pressure container was cooled to ambient in a cold water bath. The recovered solution was evaporated under argon (rotary evaporator) and hexane (100 ml) was added. Various amounts of this concentrate were applied to chromatography columns as needed. The cod liver oil used had been purchased from Acofarma (Barcelona, Spain). The oil had the following properties: a density of 925 kg/m³, a refractive index of 1.479, an acidity value of 0.16, an iodine value (Hanus) of 150.3, and a non-saponifiable content of 1.46% (w/w).

2.3. Column chromatography

2.3.1. Fractionation of fatty esters

Argentated silica gel column chromatography was used for fractionation of fatty esters. The Ag-silica gel was prepared as follows: 200 g of silica gel (0.06–0.2 mm, 70–230 mesh ASTM; mean pore diameter of 6 nm, specific surface area of 500 m²/g) for column chromatography (Scharlau, Barcelona, Spain) was slurried in ethanol (400 ml, 10 min). A solution of silver nitrate (20 g) in 70% (v/v) ethanol (~70 ml) was added. Agitation was continued for a further 10 min. Ethanol was evaporated in a rotary evaporator under vacuum at ~60°C. The silver impregnated silica gel was activated by overnight heating at 120°C. This material was cooled and kept in the dark in a desiccator until needed. In an alternative method of preparing the stationary phase, a small volume of an aqueous solution of silver nitrate is added to silica gel which is then dried at 110 to 120°C [14].

Chromatography columns were packed by one of two methods. Either a slurry of Ag-silica gel (220 g) in hexane (400 ml) was poured into a column that had been half filled with hexane, or dry support material was sprinkled into a similarly filled column. In the latter case, the sides of the column were gently tapped during pouring of solids to aid packing. A slight flow of hexane was allowed to occur during packing. The packed height of glass chromatography columns was generally 0.08 m, although in preliminary experiments the height was varied from 7 to 9 cm in a 0.5 cm diameter column. Depending on the scale of operation, the column diameter was 0.5, 2.5, or 9 cm. The hexane level was lowered until it was 1 cm above that of the stationary phase. A filter paper disc was placed on top of silica gel and the column was ready for use. The exit of a chromatography column was plugged either with glass wool or a sintered glass disc to retain solids.

About 9 g of the crude methylated extract obtained in the simultaneous extraction and transesterification step were dissolved in hexane (25 ml). Portions of this solution were applied to chromatography columns at specified loadings of

extract-to-stationary phase. Unless otherwise noted, a column was eluted with the following sequence of solvents: 1) hexane with 0.5% (v/v) acetone; 2) hexane with 1% (v/v) acetone; and 3) hexane with 5% (v/v) acetone. The volume of each solvent fraction used varied with the size of the column, as noted later.

2.3.2. Removal of pigments

The final EPA fraction obtained from the Ag-silica gel chromatography of microalgal fatty esters had a slight green color because of chlorophylls. The chlorophylls were removed in a second chromatography step. Thus, the final EPA fraction (7.5 l) was concentrated to about 15 ml in a rotary evaporator under vacuum (30–35°C) and applied to a non-argentated silica gel chromatography column (8 cm tall, 2.5 cm diameter). The column had been prepared using the earlier specified silica gel (18 g) slurried in hexane (300 ml). The column was eluted with hexane : acetone (98.5 : 1.5 v/v). All material eluting ahead of the green chlorophyll band was collected. The entire applied amount of EPA was recovered. This EPA fraction was pale yellow.

2.4. Solvent recovery and reuse

The solvent in the various fractions of the column chromatographic step was recovered and reused. Solvent recovery was by vacuum distillation at 30 to 35°C under argon. The amount of acetone in the recovered solvent was determined by measuring its absorbance at 250 nm and using the equation

$$\text{Percent acetone (v/v)} = 1.2091 \cdot A_{250} - 0.0885. \quad (1)$$

In Eq. (1), A_{250} is the absorbance at 250 nm. Once the amount of residual acetone had been established, calculated amounts of either fresh hexane or acetone were added to the recovered solvent to obtain a fresh elution mixture containing the requisite quantity of acetone. Eq. (1) was determined by measuring the absorbance of standard mixtures of acetone and hexane. The equation was linear for up to 5% (v/v) acetone in hexane. The correlation coefficient for Eq. (1) was 0.998.

Hexane, methanol, ethanol, and acetone were synthesis grade. Trichloromethane was analytical grade. All solvents were purchased from Panreac Quimica S.A. (Barcelona, Spain) unless otherwise noted.

2.5. Analyses

The crude extracts and the fractions obtained by column chromatography were analyzed by gas chromatography to establish recovery and purity of EPA as described next.

2.5.1. Gas chromatography

A HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with an autoinjector

(model HP 6890) and a flame ionization detector was used. The separation was done on a Supelco Omega wax 250 (30 m × 0.25 mm) fused silica (0.25 μm) capillary column. The oven time-temperature profile was as follows: 205°C (10 min), 6°C per min to 240°C, 240°C (9 min), giving a total heating time of about 25 min. The internal standard was nonadecanoic acid methyl ester (19 : 0 methyl ester). The amounts of various fatty acid methyl esters present were calculated using the equation

$$C_i = C_s \left(\frac{A_i}{A_s} \right), \quad (2)$$

where C_i is the amount of any methyl ester i , C_s is the amount of the standard, A_i is the area under the chromatographic peak of component i , and A_s is the area under the nonadecanoic acid methyl ester standard peak. The internal standard had been prepared by dissolving 25 mg of 19 : 0 methyl ester in hexane (1 ml).

2.5.2. Silver contamination of product

The amount of silver leached into the eluent was quantified in a set of experiments to assess the extent of potential silver contamination of the purified fatty esters. Thus, a freshly prepared argentated silica gel column (0.5 cm diameter, 8 cm height), not loaded with any ester, was eluted with hexane containing various percentages of acetone as follows: 0.5% (40 ml) 1% (20 ml), 5% (80 ml), and 10% (20 ml). The compositions and volumes of mobile phase were identical to those used in other identical columns that were loaded with the PUFEE mixture. The pure solvent fractions were collected and analyzed for silver by using a high performance quadrupole ICP mass spectrometer (VG PlasmaQuad 3, Brighton, England). The instrument was calibrated with standards containing 0, 1, 4, 7, and 10 ppb silver in 2% (v/v) nitric acid. Indium (2 ppb) was added as an internal standard. About 2 g of each sample (hexane with various proportions of acetone) was evaporated and reconstituted with 2% (v/v) nitric acid (10 ml) and an internal standard (In, 2 ppb) was added. If initial analysis revealed silver in the ppm range, the sample was further diluted to reduce the concentration to the ppb level. The instrument was operated under standard plasma conditions, using a conventional glass concentric nebulizer.

2.5.3. Peroxide value

Lipid hydroperoxides were determined using the spectrophotometric method described by Løvaas [15]. The peroxide value (P_{ox} in milliequivalents per kg lipid) was calculated using the equation

$$P_{ox} = \frac{A_{360} V_s}{18.3 D V_t}, \quad (3)$$

where A_{360} is the absorbance at 360 nm after 15 min, D is the dilution factor for the lipid sample in butanol, V_t is the total volume of liquid in the cuvette, and V_s is the volume

of the sample added [15]. In Eq. (3), 18.3 is the mM extinction coefficient for the I_3^- ion at 360 nm. The EPA ethyl ester samples for peroxide value measurements were stored individually in sealed vials held at various conditions for up to 38 days. The storage conditions were ambient temperature or refrigeration (4°C), with and without hexane, and with normal air or argon atmosphere in the vials. Two identically held vials were analyzed at appointed times and discarded.

3. Results and discussion

3.1. Simultaneous extraction and transesterification of fatty esters

A single step simultaneous extraction-transesterification of algal biomass was used to reduce costs. It was unclear whether transesterification occurred first within the cells, or if the lipids first came out of the cells as triglycerides. Combined extraction-transesterification was effective in quantitatively recovering the fatty acids from dry biomass of both algae. This one step process was successful also for the moist *P. tricorutum*, but not for *M. subterraneus*. The one step operation eliminated several intermediate processing steps that would be otherwise needed [16]. Because drying is prohibitively expensive for commercial use, a mechanical cell disruption step [17] may be necessary to pretreat the moist *M. subterraneus* prior to extraction-transesterification.

3.2. Fractionation

3.2.1. Fatty ester loading

For a given separation performance, establishing the maximum permissible loading of the mixed fatty esters on the stationary phase is essential. The effect of the fractional loading L of the total fatty ester on the stationary phase is illustrated in Fig. 1. The EPA purity attainable could be related to the fractional loading by the polynomial

$$\text{Purity (\%)} = 91.61 - 2.053L + 4.494L^2 - 0.9286L^3. \quad (4)$$

Fig. 1 is for the purification of crude ester extract of freeze-dried *P. tricorutum*. As shown in Fig. 1, the optimal loading of the ester extract on the stationary phase is about 3% (w/w); however, a somewhat higher loading of 4% was generally used in subsequent experiments. A higher loading is necessary for better economics and a loading of up to 4% reduces purity only slightly relative to the lower optimal loading. Note that the standard deviation of the first three data points in Fig. 1 is less than or equal to the width of the data point symbols.

Whether the fatty ester mixture loading can be increased by increasing the loading of silver ion on silica gel is not clear. In the past, in fractionating fish oil triglycerides on

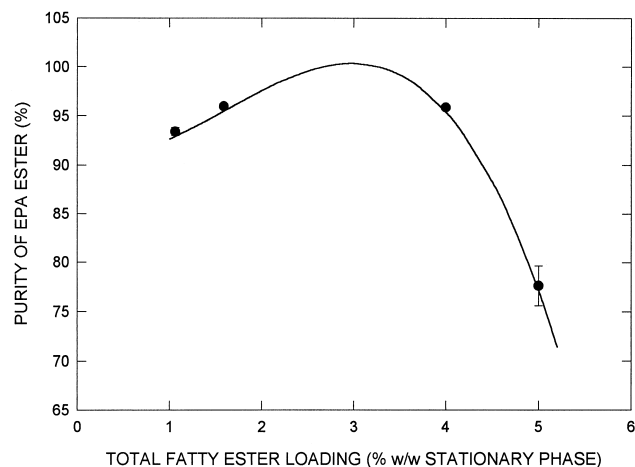


Fig. 1. Effect on the EPA ester purity of the amount of the crude fatty ester mixture loaded per unit mass of the stationary phase.

non-argentated silica gel columns, Hayashi and Kishimura [9] employed a whole oil loading of 2.5% w/w on the stationary phase. A somewhat lower loading of 2.3% w/w of perilla oil was used by Ryu et al. [18] on Ag-silica gel columns. In view of the data in Fig. 1, a loading of 4% cannot be exceeded for columns containing 10% (w/w) silver nitrate on silica gel. The data in Fig. 1 were obtained in a 0.5 cm diameter column that was 8 cm tall. Column heights below 7 cm reduced resolution, hence, 8 cm was the minimum acceptable height for attaining the requisite purification (>90%) at a given flow of the eluent through the column.

3.2.2. Scale up and comparative performance

The same column packing and sequence of eluents were used at all scales. The scale up method aimed to attain identical purification performance at the various scales even though the scale factors were up to 25. Thus, to attain an identical number and height of theoretical plates at the various scales, the residence times and the hydrodynamics in the columns had to be identical. Therefore, the columns had identical packing heights (0.08 m) irrespective of scale, only the diameters varied. The superficial velocity of the eluent remained unchanged on scale up because the pressure drop across the columns did not change. The mass loading of the fatty ester mixture per unit cross sectional area of the columns at various scales remained unchanged. Thus, the amount (kg) of fatty ester loaded on to a column of a given scale was calculated as

$$M_L = M_S \left(\frac{D_L}{D_S} \right)^2, \quad (5)$$

where M is the amount of ester loaded, D is the diameter of the column, and the subscripts L and S denote large and small scales, respectively. The optimal loading at the small scale M_S was established empirically as discussed in the previous section. Scale up according to Eq. (5) is equivalent

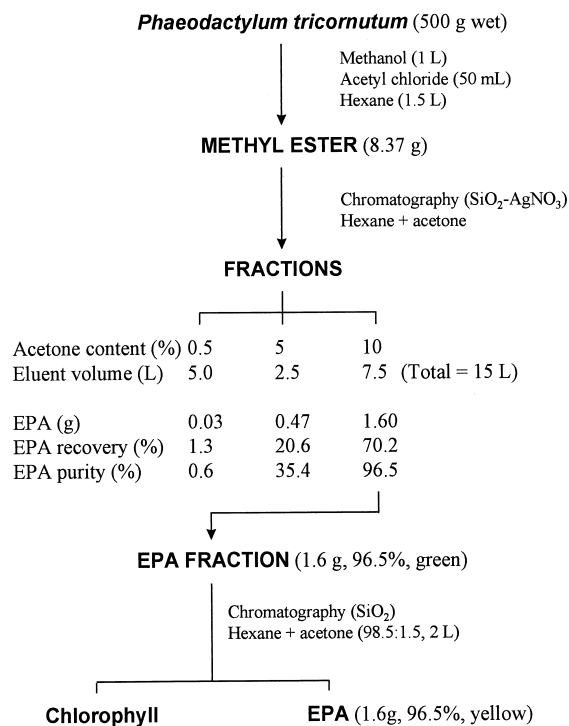


Fig. 2. EPA recovery from *P. tricoratum* paste. The percent EPA recovery numbers for the various solvent fractions are based on the EPA ester content in the crude total fatty ester extract.

to holding constant the loading of the mixture per unit volume or mass of the stationary phase in columns of identical heights.

The gas chromatographic purities of the EPA ester fractions obtained at the analytical, semipreparative, and the preparative scales (corresponding nominal volumes of the stationary phase were roughly 1.6, 39.3, and 509 ml) were 95.9%, 92.9%, and 93.5%, respectively, for fractionation of total fatty ester extract of freeze-dried *P. tricoratum*. These differences in purities are statistically significant, as the maximum standard deviation in purity data was less than $\pm 0.15\%$. The differences are attributed to variations in the quality of packing of the stationary phase. Whereas the nominal height of the packing was always 8 cm, depending on small variations in packing methodology, the same amount of silica gel could provide packed heights that differed by ± 1 cm, or about 12%. The two smaller bore columns were easier to pack consistently than the wider one. The scale ratio in going from the smallest to the largest column was 1 : 25 : 324, based on the nominal volume of the stationary phase. In all cases, the packed height of chromatographic columns remained the same (0.08 m), only the diameter increased from 0.5 to 9 cm.

The sequence of operations in purification of EPA from *P. tricoratum*, *M. subterraneus*, and fish oil are summarized in Figs. 2, 3, and 4, respectively. The figures also note the EPA recovery and purity at various stages. In all cases, the total fatty ester loading with respect to the amount of the

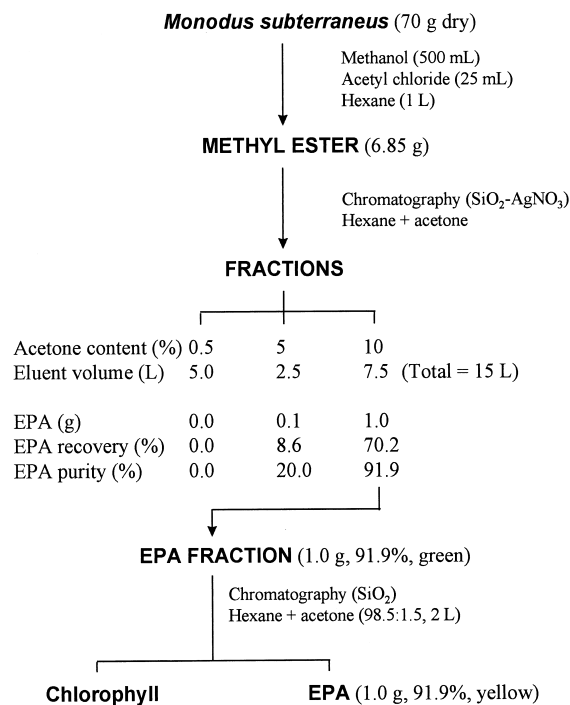


Fig. 3. EPA recovery from *M. subterraneus* freeze-dried biomass. The percent EPA recovery numbers for the various solvent fractions are based on the EPA ester content in the crude total fatty ester extract.

stationary phase was about 4% (w/w). The total EPA contained in the various eluent fractions was 92.1, 78.8, and 81.2% of the EPA that was originally present in the starting total fatty extract in Figs. 2, 3, and 4. The effectiveness of EPA recovery from the crude extracts was in the order *P. tricoratum* > fish oil > *M. subterraneus*. These differences are associated with differences in the fatty ester profiles of the crude extracts (see Fig. 5). The relevant profiles are compared in Fig. 5. The fatty esters that occur in all

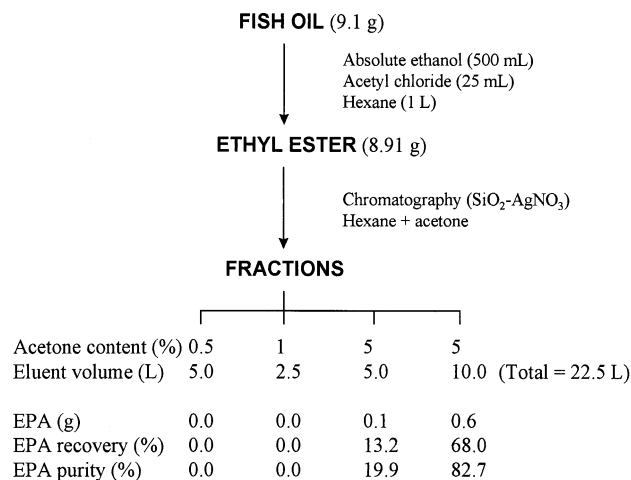


Fig. 4. EPA recovery from fish oil. The percent EPA recovery numbers for the various solvent fractions are based on the EPA ester content in the crude total fatty ester extract.

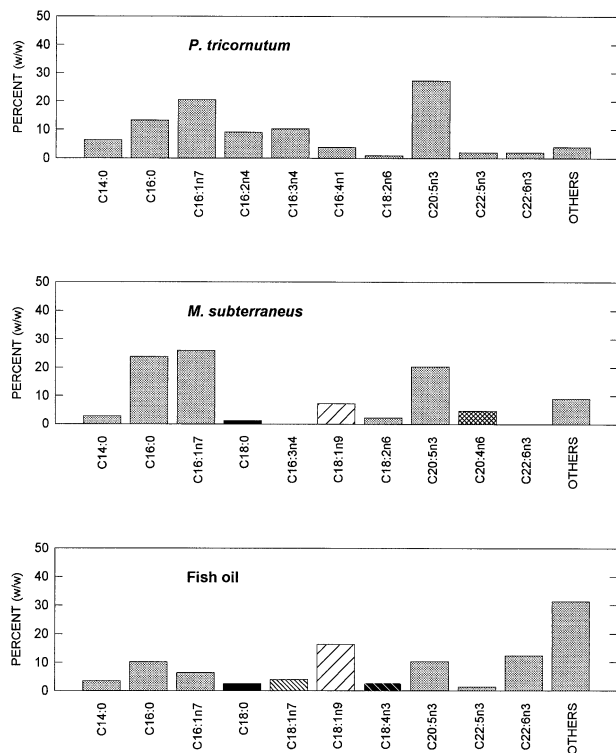


Fig. 5. Comparison of the methyl fatty ester profiles of *P. tricorutum*, *M. subterraneus*, and fish oil (ethyl ester). The individual components are expressed as percent (w/w) of the total fatty esters in a given extract.

three extracts are shown with a gray shading; the bars corresponding to esters that are detected only in fish oil or *M. subterraneus* are shown with different shading patterns.

Note that the 500 g of moist *P. tricorutum* in Fig. 2 was equivalent to 90 g of dry biomass. Thus, comparing the two algae on an identical basis, *P. tricorutum* had 1.8% (w/w) recoverable EPA and *M. subterraneus* had a somewhat lower 1.4%. The recoverable EPA content in fish oil were 6.6% by weight. The actual total EPA content in the various raw materials were of course greater: 2.5% (as methyl ester) in *P. tricorutum* biomass, 2% (as methyl ester) in *M. subterraneus* biomass, and 10% (as ethyl ester) in fish oil.

In silver-silica gel fractionation of fatty acid esters, saturated esters elute first, followed by unsaturated ones. The number, position, and geometric configuration of double bonds determine the order of elution of unsaturated fatty esters. The resolving power of Ag-silica gel is attributed to reversible charge-transfer complexation of Ag^+ with carbon-carbon double bonds. The extent and the strength of complexation control the mobility of a solute and so does the polarity of the mobile phase. The loading of the silver ion on the gel also affects the retention time of a solute because the loading determines the surface density of Ag^+ ions on the solid matrix and this in turn influences the frequency of complexation during elution. The areal density also determines whether a molecule of polyunsaturated fatty ester will simultaneously complex with silver at multiple

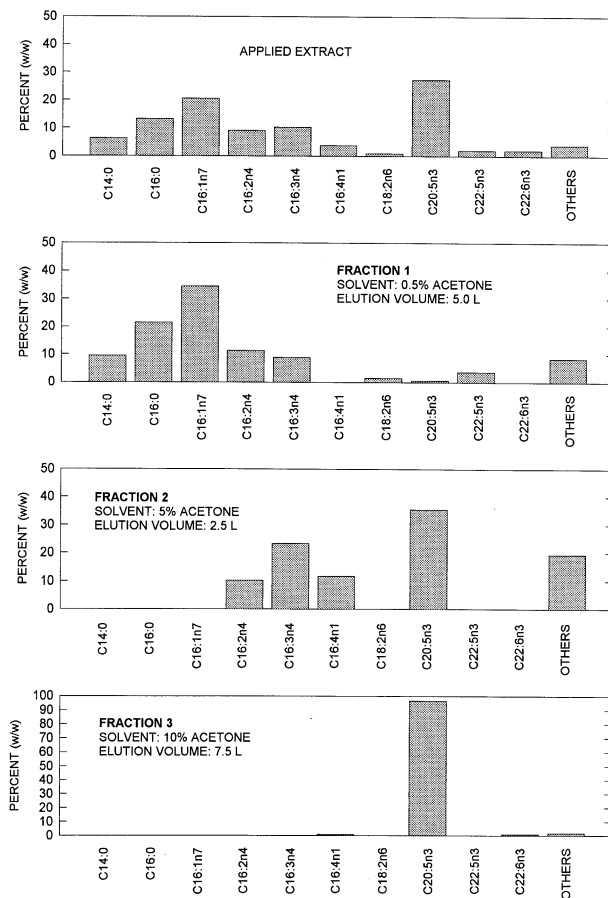


Fig. 6. The fatty ester profiles of the three solvent fractions obtained at the silver chromatography stage during EPA ester recovery from *P. tricorutum* paste. The recovery scheme is given in Fig. 2. The profile of the crude extract applied to the column is also shown.

sites. Simultaneous multipoint complexation does not seem plausible during fractionation of cod liver oil fatty esters when the silver loading on the solid phase does not exceed 1.875×10^{-7} kg Ag^+ per m^2 of surface [14]. In our case, the estimated areal loading of Ag^+ was 1.27×10^{-7} kg per m^2 of surface; therefore, a molecule of an unsaturated ester apparently interacted with only one silver ion at any given instance.

The fatty ester profiles of the various solvent fractions obtained at the silver chromatography stage of Fig. 2 are shown in Fig. 6. The figure also shows the fatty ester profile of the crude extract applied to the column. Because the saturated fatty esters (C14:0, C16:0) and the monounsaturates (C16:1n7) associate least strongly with the stationary phase, these esters are eluted quantitatively with the first solvent fraction (i.e. the one containing 0.5% acetone in hexane). Most of the other unidentified esters in this solvent fraction (Fig. 6) are also likely to be unsaturated and mono-unsaturated types that are of no interest in this work. The highly unsaturated EPA (C20:5n3) is barely eluted in this first fraction; however, most of the equally unsaturated C22:5n3 is removed. This is certainly because C22:5n3 is

more hydrophobic relative to EPA and, therefore, has a higher affinity toward the relatively apolar eluent. This case is identical to that of the observed higher distribution ratio (i.e. ratio of concentrations in the aqueous and organic phases) of C18 : 4n3 compared to C20 : 4n3 when the compounds were agitated in a biphasic mixture of a silver nitrate containing aqueous phase and n-hexane [6]. A higher distribution ratio is indicative of a greater affinity of a compound toward the polar silver ion solid support.

The most unsaturated identified component, methyl ester of docosahexaenoic acid (C22 : 6n3), complexes strongly with the adsorbed silver ions and fails to elute even with 5% acetone in hexane (Fig. 6). The two C16 esters with 2 and 3 carbon-carbon double bonds each (i.e. C16 : 2n4 and C16 : 3n4) commence eluting with 0.5% acetone and they also appear in the 5% acetone fraction (Fig. 6). This suggests that those two fatty esters commence elution most likely in the tail end of the 0.5% acetone fraction. Therefore, a slight increase in the volume of the 0.5% acetone solution before changing over to 5% acetone could potentially remove the C16 : 2n4 and C16 : 3n4 in the first eluent fraction, leaving the 5% acetone fraction much cleaner than it is now (Fig. 6).

Of course, the elution of C16 : 4n1 (Fig. 6) is delayed to the 5% acetone fraction because of that ester's greater unsaturation relative to the other C16 esters present. The other unidentified esters in the 5% acetone fraction are most likely to be the polyunsaturated esters with 20 or fewer carbon atoms and between 2 and 5 carbon-carbon double bonds. The trace of C16 : 4n1 in 10% acetone fraction suggests that this ester commenced elution near the tail end of the 5% acetone eluent. Clearly, it would be impossible to obtain a 5% acetone fraction having EPA (C20 : 5n3) totally free of C16 : 4n1. The final fraction eluted with 10% acetone in hexane is virtually pure EPA methyl ester. The slight trace of C16 : 4n1 (Fig. 6) can be potentially eliminated if the elution volume of the 5% acetone is increased slightly. The slight contamination of EPA with C22 : 6n3 in the final acetone fraction can be eliminated by discarding the tail volume of this fraction during collection. Because the various acetone fractions were collected as single portions, the order of elution of the different components within a given fraction is not known.

The behavior of the chromatographic fractionation of the *M. subterraneus* fatty ester extract was generally consistent with the earlier noted observations for purification of the *P. tricornutum* extract. Thus, for the separation scheme of Fig. 3, the corresponding fatty ester profiles of the various eluent fractions are noted in Fig. 7. As shown in Fig. 7, the saturated fatty esters (C14 : 0, C16 : 0, C18 : 0) elute with the first solvent fraction because they do not complex with the Ag⁺ ion. Similarly, the monounsaturated esters (C16 : 1n7, C18 : 1n9), that complex relatively less strongly with silver, are eluted mostly in the first solvent fraction; however, as expected, these esters elute after the saturated ones,

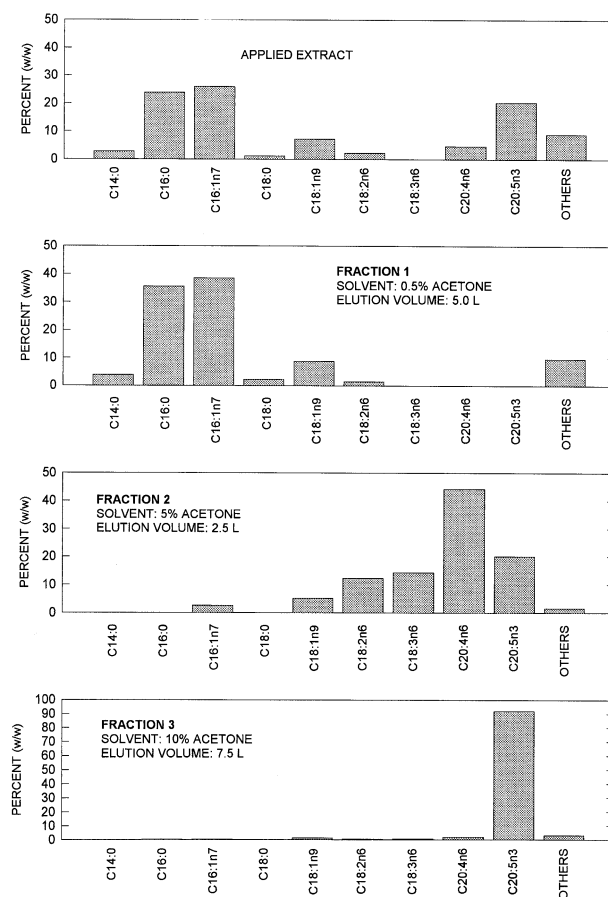


Fig. 7. The fatty ester profiles of the three solvent fractions obtained at the silver chromatography stage during EPA ester recovery from *M. subterraneus* dry biomass. The recovery scheme is given in Fig. 3. The profile of the crude extract applied to the column is also shown.

i.e. near the tail end of the 0.5% acetone fraction. This is confirmed by the slight presence of C16 : 1n7 and C18 : 1n9 also in the 5% acetone containing eluent. The polyunsaturated esters elute predominantly in the second solvent fraction. Only the EPA, the most unsaturated and, hence, a strongly held component, elutes substantially in the last solvent fraction. Complete removal of the contaminating polyunsaturates in the solvent fraction 3 is of course possible by increasing the volume of the solvent used in the second elution, or by delaying collection of the final solvent fraction. Both these approaches will enhance the purity of the final EPA, but the loss of EPA in the discarded solvent fractions will also increase.

The fatty ester profiles of the whole transesterified fish oil and the various solvent fractions obtained during chromatographic separation are shown in Fig. 8. The corresponding separation scheme is illustrated in Fig. 4. The elution pattern in Fig. 8 is consistent with expectations. Again, the unsaturated (C14 : 0, C16 : 0, C18 : 0) and the monounsaturated (C16 : 1n7, C18 : 1n9, C18 : 1n7) esters elute predominantly in the first two solvent fractions.

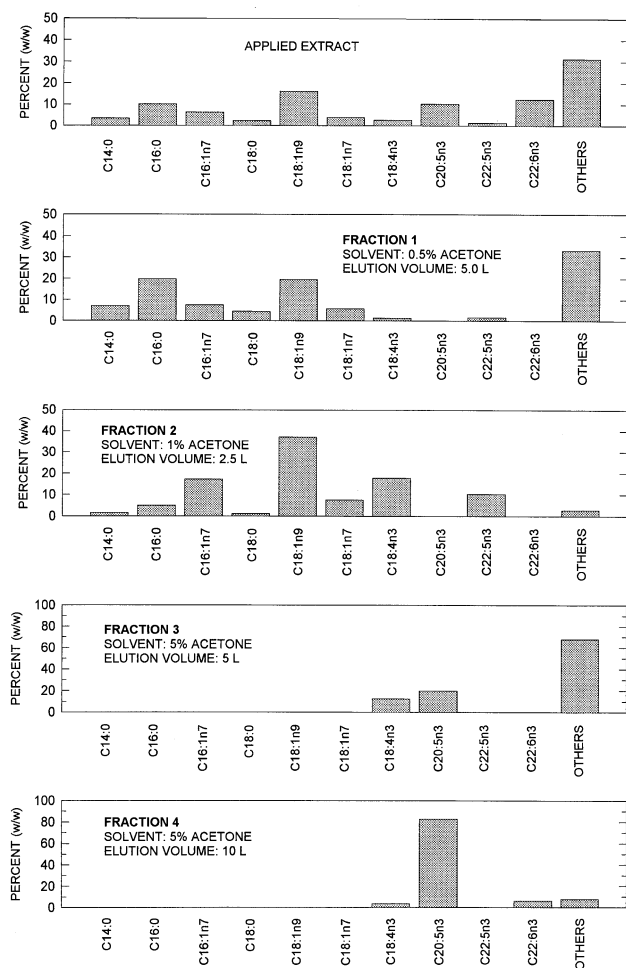


Fig. 8. The fatty ester profiles of the four solvent fractions obtained at the silver chromatography stage during EPA ester recovery from fish oil. The recovery scheme is given in Fig. 4. The profile of the transesterified fish oil applied to the column is also shown.

Clearly, most of the other unidentified esters in the solvent Fraction 4 are of the highly unsaturated type. The ester C18 : 4n3 commences eluting in the tail end of the second solvent fraction. Elution of EPA (C20 : 5n3) begins in the tail end of the first 5 liter of the 5% acetone fraction (i.e. Fraction 3 in Fig. 8). Again, the C22 : 5n3 elutes earlier than EPA even though these compounds have an identical number of carbon-carbon double bonds. These differences in the elution patterns of these two esters were observed also during purification of extracts of *P. tricoratum* (Fig. 6) and were explained earlier.

Because of contamination with the highly unsaturated fatty esters, the EPA purity in the final solvent fraction (Fig. 8) was lower than the purities achieved when EPA was isolated from extracts of the two algae. The ester C22 : 6n3, eluting in the tail end of the final solvent fraction, can be eliminated by reducing the collection volume. This will enhance EPA purity but will reduce its recovery to below 70%.

3.3. Process economics

Based on the process schemes illustrated in Figs. 2 through 4, it is easily shown that producing 1 kg of EPA ester from the three noted raw materials would require 15.2 kg of fish oil, 56.3 kg (dry basis) of *P. tricoratum*, and 70.0 kg of *M. subterraneus*. Depending on the source, the purity of the EPA will be 83%, >96%, or >91% (Figs. 2–4). If, for simplicity, it is assumed initially that the downstream product recovery costs are identical irrespective of the source used, then for a microalgae based EPA process to compete with a fish oil based one, the earlier noted quantities of algal biomass will have to be obtained at the same price as fish oil. Fish oil (pharmaceutical grade Cod liver oil) currently sells for about \$10/kg; hence, for 1 kg of EPA, \$152 worth of oil will be needed. If the same amount (\$152) is paid for 56.3 kg of *P. tricoratum* or 70 kg of *M. subterraneus*, the cost per kg of biomass will be \$2.7 (*P. tricoratum*) and \$2.2 (*M. subterraneus*).

In reality, the downstream EPA recovery from microalgae and fish oil are significantly different. Again, based on the information in Figs. 2 through 4, recovering 1 kg EPA will require different volumes of solvent: 37.5 m³ for fish oil, 9.4 m³ for *P. tricoratum*, and 15 m³ for *M. subterraneus*. Consequently, the solvent recycle costs will be different for the three processes. Assuming, reasonably, that the cost of recycling is directly proportional to the amount of solvent that needs to be recycled, the recycle costs would be in the ratio 1 : 1.6 : 4 for *P. tricoratum*, *M. subterraneus*, and fish oil processes. That ratio of costs disregards differences in the initial investment capital requirements for the solvent recycle equipment in the three processes. If the cost of the recycle equipment depends on its capacity according to the well-known 'six-tenths rule' [19], then, for an equal processing time, the corresponding capital costs would be in the ratio 1 : 1.3 : 2.3.

Because identical chromatography columns were used in the three separations shown in Figs. 2, 3, and 4, and the total solvent volumes processed were different, the processing times were in the ratio 1 : 1 : 1.5 for *P. tricoratum*, *M. subterraneus*, and fish oil. The higher processing time for fish oil in the chromatographic step will reduce annual productivity unless the chromatographic columns used for the oil process are 50% wider. Using wider columns will not affect the ratio of fatty ester loading with respect to the stationary phase, hence, only the initial capital investment would be different.

The total cost *T* of production from a given bioprocess may be approximated as

$$T = T_R + fT, \quad (6)$$

where *T_R* is the cost of the raw material (e.g. biomass or fish oil) and *fT* is the cost of downstream recovery expressed as a fraction *f* of the total processing costs *T*. For bioprocesses the factor *f* is typically 0.7 to 0.8 [3,20], i.e. downstream processing contributes between 70 and 80% to the total cost

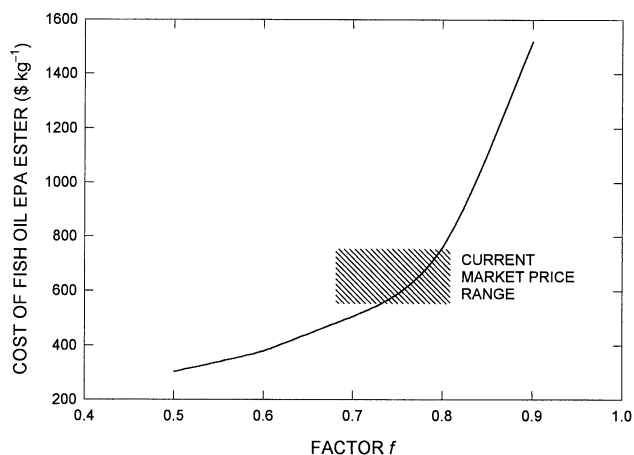


Fig. 9. Effect of the f -value on the cost of production of fish oil derived EPA ester.

of producing. Thus, for various assumed values of f , the total expense of recovering 1 kg of EPA ester from fish oil can be estimated readily using the equation

$$T = \frac{T_R}{1 - f} \quad (7)$$

where T_R , the cost of fish oil needed, was earlier estimated to be \$152. The total estimated cost of producing EPA from fish oil are shown in Fig. 9 as a function of f . The figure also shows the current market price of fish oil derived EPA ester (\$650/kg). Clearly, this analysis strongly supports a f -value of between 0.7 and 0.8 for the fish oil process.

If the microalgal biomass needed to produce a kilogram of EPA can be purchased for the same price as the fish oil required for making a kilogram of EPA, then the total cost of producing microalgal EPA will be

$$T_m = \$152 + (yfT) \quad (8)$$

where T_m is the total cost (\$/kg), T is the cost of making EPA from fish oil, y is factor correcting for the differences in the solvent recovery costs (y is 0.25 for *P. tricornutum* and 0.4 for *M. subterraneus*). Unfortunately, the necessary microalgal biomass cannot be purchased for \$152. Thus, we need to estimate the maximum acceptable price of algal biomass for the EPA-from-biomass process to still remain competitive with EPA derived from fish oil. To establish this, the cost difference between the microalgal and the fish oil processes for a kilogram of EPA has to be added to the price of fish oil and the resulting number divided by the amount of biomass needed. Thus,

$$\text{cost of biomass} = \frac{T - T_m + \$152}{\text{amount of biomass}} = \frac{T(1 - yf)}{M_A} \quad (9)$$

where M_A is 56.3 kg for *P. tricornutum* and 70 kg for *M. subterraneus*. The maximum acceptable cost of biomass (dry basis) as a function of the factor f are shown in Fig. 10 for the two algae. Realistically, the cost of producing bio-

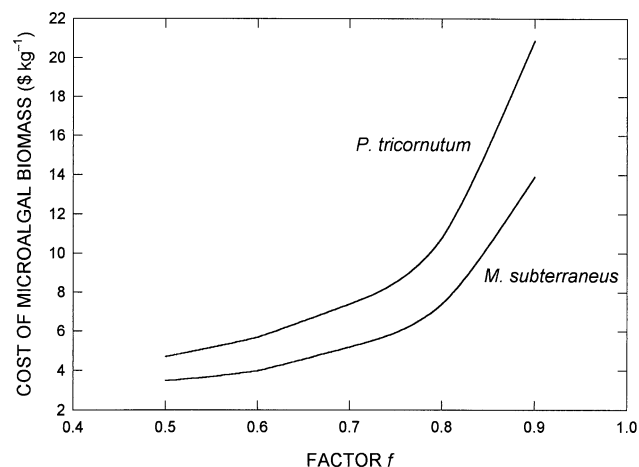


Fig. 10. Effect of the f -value on the maximum acceptable cost of microalgal biomass if alga derived EPA ester is to remain competitive with EPA from fish oil.

mass would have to be even lower than in Fig. 10. The relative positioning of the two curves in Fig. 10 may misleadingly suggest that *M. subterraneus* is preferable for use in recovering EPA. This is not so. Compared to *M. subterraneus*, a higher acceptable cost of procuring the *P. tricornutum* biomass implies that *P. tricornutum* is the preferred source material. A process utilizing *P. tricornutum* is economically more profitable or feasible relative one using *M. subterraneus*. In addition, because the scheme in Fig. 3 demands freeze-dried *M. subterraneus* biomass, this alga will need to be procured in a dry state at or below prices shown in Fig. 10. Drying is expensive; therefore, obtaining *M. subterraneus* in a suitable state at less than the price of *P. tricornutum* is impossible. Unfortunately, there has been little success in recovering most of the EPA from moist frozen biomass of *M. subterraneus*. Apparently, drying disintegrates the cell, making extraction possible. To circumvent expensive drying and improve recovery of EPA, the *M. subterraneus* cells need to be mechanically disrupted [17] before the extraction-transesterification step. This additional processing will undoubtedly add to costs. Unlike with *M. subterraneus*, extraction-transesterification of wet biomass of *P. tricornutum* is not a problem. The relative costs in Fig. 10 also do not take into account the significantly higher purity of the EPA from *P. tricornutum*. In view of all these factors, *P. tricornutum* is clearly the superior microalgal source of EPA.

The foregoing analysis is based on the observed 2.5% (w/w) EPA content (as ester) in the algal biomass. The effect of increasing the EPA content in the algal biomass on the maximum price that can be paid for such biomass is shown in Fig. 11 for the two algae. For biomass prices at or below the shown levels, an EPA-from-microalga process should remain competitive with fish oil derived EPA. Fig. 11 also shows the typical EPA content of the two algal strains used. The acceptable price depends on the factor f as shown (Fig. 11). The acceptable biomass price increases

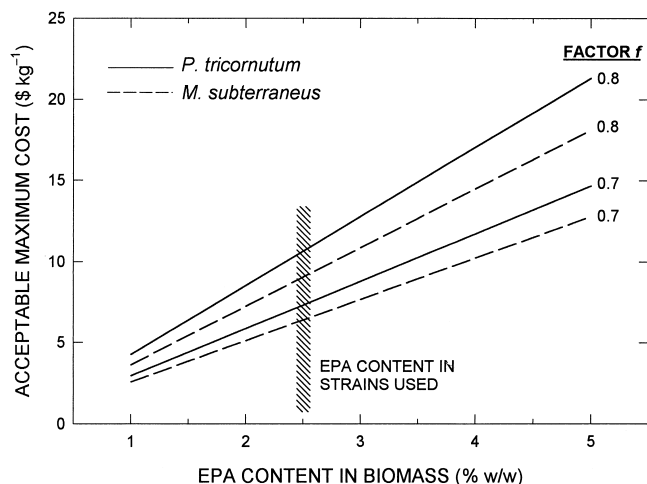


Fig. 11. Effect of the EPA content (as ester) of the algal biomass on the maximum price that can be paid for the biomass. Results are shown for two plausible f -values.

with increasing f -value because the downstream processing costs contribute more to the overall price of producing EPA and, proportionately, the contribution of the biomass purchase to the final price declines. The plots in Fig. 11 are based on the equation

$$\text{acceptable price of biomass} = 0.702zT(1 - yf), \quad (10)$$

where z is the mass fraction of EPA in the biomass. The factor 0.702 is based on the experimentally observed 70.2% recovery of the EPA ester for both algae.

Compared to the fish oil process, the microalgal processes for EPA have several additional expenses. First, the biomass needs to be preserved by freezing and held in cold storage until processed. Secondly, a solid-liquid separation step is necessary for removing the biomass before evaporative concentration of the extract. Disposal of biomass waste is an additional expense and, finally, a nonargentated silica gel chromatographic step is required to remove pigments, mainly chlorophyll, from the product. Furthermore, the extraction-transesterification regimen for the microalgal biomass is more severe than for fish oil and this affects processing costs.

Silver nitrate impregnated silica gel stationary phase and columns are easily prepared. The columns can be slurry packed and unpacked in situ following the methods developed for industrial chromatography [3]. Reusability of columns is a crucial factor in determining the economic feasibility of the proposed purification. The Ag-silica gel columns are fairly stable and can be used repeatedly [18, 21]; however, the performance declines eventually because the silver is gradually leached out. In this work, the reusability of columns was confirmed for up to three cycles after extensive cleaning between cycles. The separation performance was not affected.

Silver leaching may be eliminated completely by replacing the silver-silica gel with silver loaded aluminosilicate

[22]. Potentially, argentated polymer resin beads may also be used in fractionating polyunsaturated fatty esters [21,23, 24]. Resin columns are more expensive but more stable in long term use [21]. The resolving performance of these other matrices remains to be proved for microalgal fatty ester mixtures. If, as expected, up to 50 purification cycles are feasible before the need for regeneration of the Ag-silica gel stationary phase, then only 2.6 kg of the stationary phase will be needed for each kg of EPA ester recovered from *P. tricornutum* (Fig. 2). Thus, recovering 1 kg of EPA ester will require a mere 238 g of silver nitrate corresponding to 151 g silver. The quantity of the stationary phase needed to recover 1 kg EPA equals $131/n$ kg where n is the number of purification cycles between regeneration. Similarly, the amount of silver nitrate needed for obtaining each kg of EPA ester amounts to $11.9/n$ kg. Clearly, maximizing the number of cycles is important. Obtaining the same 1 kg of EPA ester from *M. subterraneus* and fish oil requires 311 and 689 g silver nitrate, respectively, for a 50-cycle column. In consequence, the silver consumption costs of the chromatography step for recovering a unit mass of EPA from *P. tricornutum*, *M. subterraneus*, and fish oil are in the ratio 1 : 1.3 : 2.9. In all cases, the loading of the crude fatty ester mixture was 4% (w/w) of the stationary phase.

Based on the work described, an overall schematic process flowsheet for producing microalgal EPA ester is shown in Fig. 12. The process operates in batches. Although the flowsheet shows a reactor and a second optional extractor for extended overnight extraction detailed in Section 2, a single vessel may be designed to serve both functions. In any event, the overnight cold storage step, required for quantitative extraction from a relatively static wet biomass slurry, may be eliminated all together by using a normal level of agitation in the reactor. The cooler located after the reactor (Fig. 12) would be unnecessary if the reactor is jacketed or provided with a cooling coil. Unlike in present work, the spent biomass from the reactor-extractor would be separated in a disc stack centrifuge [3]. The extract would be further polished by filtration before being concentrated in the evaporator for application to chromatography columns. The waste stream in Fig. 12 is spent biomass. The flowsheet also shows a cell disruption step that is optional for *P. tricornutum*, but necessary for processing *M. subterraneus*.

An indication of the maximum acceptable purchase price for the microalgal biomass can be obtained through another line of reasoning: If the biomass has x percent recoverable EPA, then processing of 1 kg of biomass yields $x/100$ kg EPA, assuming 100% recovery. If all this EPA was sold at the current market price of \$650/kg, the money earned would be $\$650x/100$. If all gross earnings were used to purchase the original 1 kg of biomass, then the absolute maximum biomass price would be $\$6.50x/\text{kg}$, or $\$16.25/\text{kg}$ for biomass with 2.5% (w/w) EPA. The real price that can be paid will be much lower ($<25\%$ of the calculated value) because a profit would need to be made and the downstream recovery of EPA from the biomass would need to be accounted for.

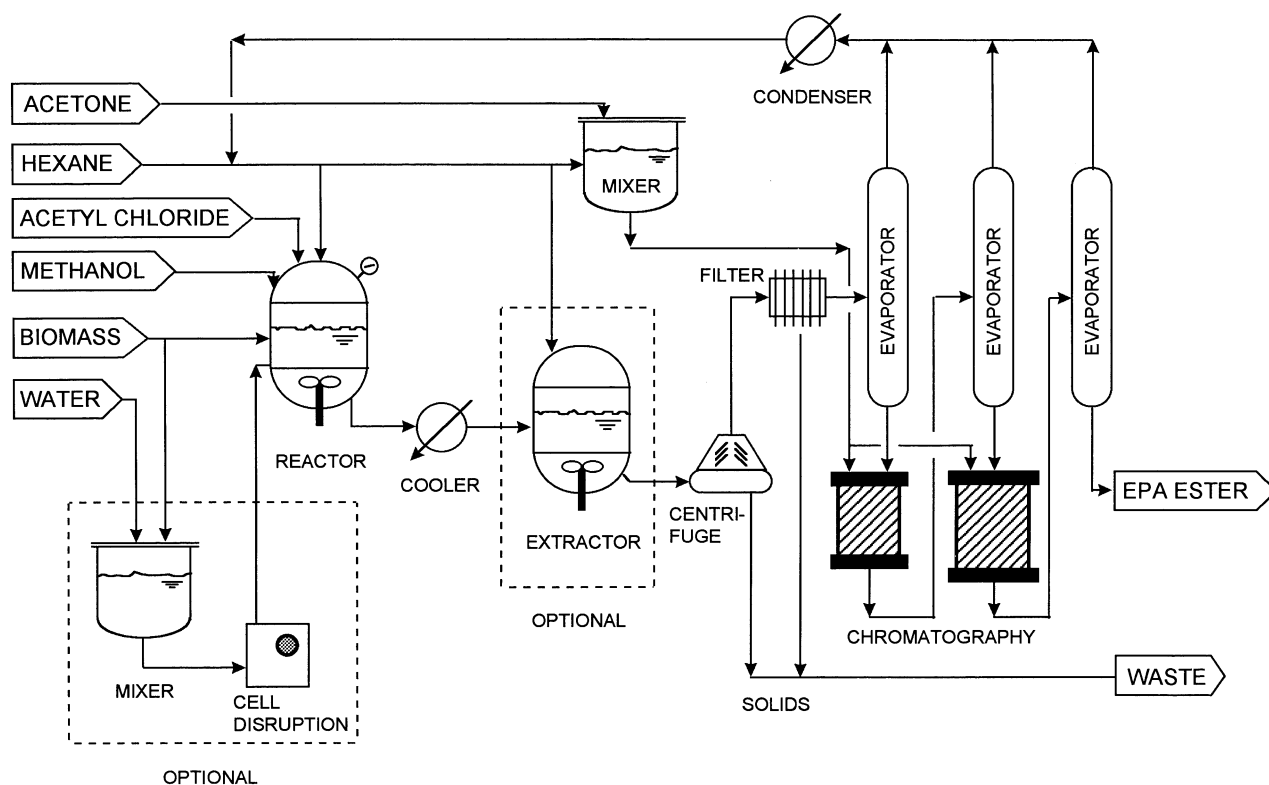


Fig. 12. Flowsheet of EPA-from-microalgae process.

3.4. Quality of purified microalgal EPA ester

Among the most important quality parameters for the microalgal EPA ester are purity and the extent of peroxidation of the product. In addition, because of the peculiarities of the processing scheme, presence of any contaminating silver in the product is also a relevant parameter. As indicated in Figs. 2 and 3, the gas chromatographic purity of *P. tricorutum* derived EPA ester exceeded 96% whereas the purity of *M. subterraneus* derived material approached 92%. The UV spectrum of the product (Fig. 13) showed no contaminating chlorophyll and carotenes. The latter compounds absorb at 630–645 nm [25] and 452 nm [26], respectively. The other quality attributes are discussed next.

3.4.1. Silver contamination

The results of silver analyses are shown in Table 1. The minimum amount of total fatty esters loaded in a small scale column (0.5 cm diameter, 8 cm height) was 7.40 mg and the fractions eluted with the same solvent sequence as used in silver analysis contained 4.65, 0.74, and 2.46 mg total esterified fatty acids, respectively. (The solvent with 10% acetone was not used in resolving fatty acids.) Using this data and that in Table 1, the maximum silver contamination of the three fatty acid ester fractions was 0, <182, and <210 ppb, respectively. The total silver leached from the column during an entire process run was negligibly small. Any leaching of silver from silica gel can apparently be

prevented by treating the silica gel with sodium aluminate before loading with silver [22].

3.4.2. Peroxide value

The amount of hydroperoxides in a lipid sample is a direct measure of the extent of peroxidation; hence, the evolution of the peroxide value with time indicates a lipid's stability against oxidative degradation. Stability characteristics of EPA are important in determining both the suitable processing conditions and the regimen for storage of the

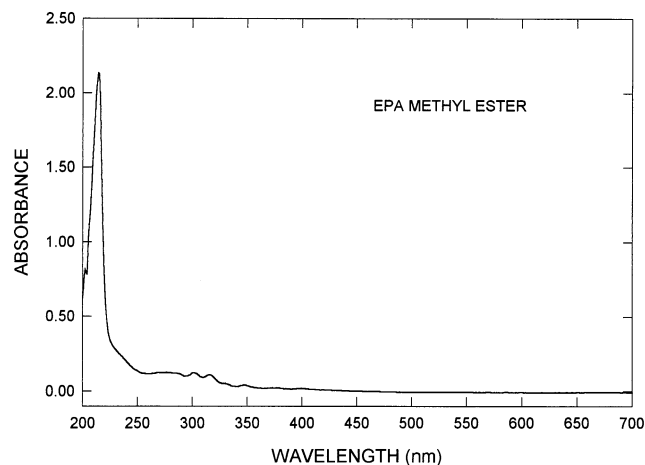


Fig. 13. The UV spectrum of EPA methyl ester of *M. subterraneus*.

Table 1
Silver concentration in solvent fractions

Solvent Fraction (% acetone)	Solvent Sample Mass (g)	Silver Content (ppb)
0.5	2.106	0.00
1	2.040	<0.01
5	2.099	<0.01
10	1.955	0.37

final product before use. As shown in Fig. 14, EPA methyl ester appears to be less susceptible to peroxidative degradation, i.e. the peroxide value does not increase significantly with storage time, when the sample is kept dissolved in hexane even at room temperature. In contrast, the peroxide value increases rapidly when the sample is stored in pure form at room temperature (Fig. 14). Storing the pure sample at 4°C does not prevent degradation, but the rate of peroxidation declines relative to when the pure sample is held at room temperature (Fig. 14). The evolution of peroxide values in Fig. 14 could be described with the cubic polynomials

$$P_{\text{ox}} = 3.260 + 19.963t - 4.721t^2 - 0.4332t^3 \quad (11)$$

and

$$P_{\text{ox}} = 8.038 + 4.723t - 0.5793t^2 - 0.0309t^3 \quad (12)$$

for samples held at room temperature and at 4°C, respectively, in the absence of hexane and under normal atmosphere. In Eq. (11) and (12), t is time in days. The regression coefficients for both equations exceeded 0.995. For Eq. (12), regression disregarded the outlier at Day 30.

As shown in Fig. 14, the minimum peroxide value even when a sample remained stable was rather high at 9.55 mEq O₂/kg EPA probably as a consequence of the processing conditions. For some context, a maximum peroxide value of

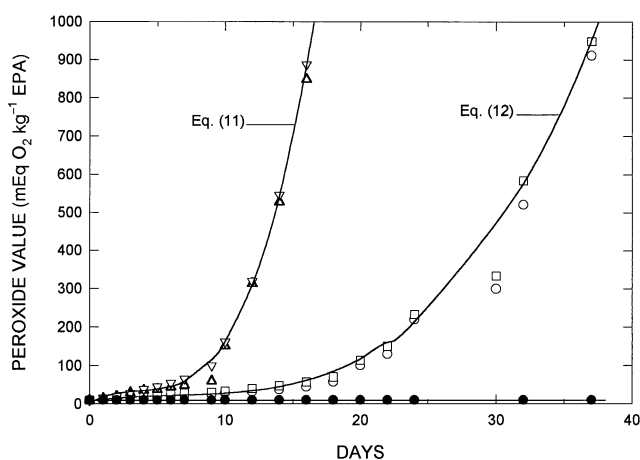


Fig. 14. Stability of *P. tricornutum* EPA methyl ester stored variously: at room temperature, without hexane, and under argon (Δ) or under normal atmosphere (∇); at 4°C, without hexane, and under argon (\circ) or under normal atmosphere (\square); and dissolved in hexane (\bullet) and stored at 4°C, or at room temperature, under normal atmosphere, or under argon.

around 10 mEq O₂/kg is considered acceptable for refined edible vegetable oils. The improved stability of hexane solvated EPA is associated with two factors. First, a mere dilution of the sample assures that the EPA molecules are so far apart that peroxide radicals formed by degradation of a molecule do not readily affect another molecule. Secondly, presence of hexane may also have a quenching effect on any peroxide radicals that are generated.

Of course, the EPA for direct consumption by patients could not be supplied dissolved in hexane. In such cases, alternative methods of preventing peroxidation would be needed. Potentially, antioxidants may be used to stabilize purified EPA. Some antioxidants have proved effective in EPA formulations. For example, in mixtures of EPA and DHA at 60°C, Ganga et al. [5] reported an induction period for oxidation of about 1 h in the absence of any added antioxidant. Antioxidants such as BHT, BHA, quercetin, and boldine added separately (0.5% w/v of antioxidant), variously increased the time to induction of oxidation. The most effective antioxidant was a 2 : 1 (w/w) mix of quercetin and boldine. This mixture increased the induction period to about 4.5 h [5]. Quercetin and boldine suppress oxidation by scavenging free radicals and excited forms of oxygen that initiate oxidation of fatty acids.

4. Concluding remarks

This work demonstrates the technical feasibility of recovering EPA ester from microalgae using a simple and scalable process. The process consists of a one-step extraction-transesterification of fatty acids in the microalgal biomass followed by fractionation on a silver-silica gel chromatography column. The one-step extraction-transesterification is practicable with moist and freeze-dried biomass of *P. tricornutum*; the procedure works also with dried *M. subterraneus* but not for its paste. EPA ester of up to 96% purity can be recovered in yields exceeding 70%. The process can be applied also to recover EPA ester from fish oil. In all cases, the maximum acceptable fatty ester loading on the silver-silica gel stationary phase is 4% (w/w). Higher loadings reduce purity of the recovered EPA. The recovery and purity are little affected even when the process is scaled up by a factor of >320. Further process improvements are expected by replacing the silver-silica gel stationary phase with longer-life silver loaded aluminosilicate columns. Based on available data, recovering EPA ester from *P. tricornutum* is substantially less expensive relative to EPA recovery from *M. subterraneus*. Enhancing the EPA content of algal strains by selection, genetic manipulation, or improved cultivation regimens can further improve the EPA-from-microalgae process economics. For microalgal EPA to be competitive with the fish oil derived material, the price of microalgal biomass (dry basis) must not exceed about \$5/kg.

Acknowledgments

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