Eicosapentaenoic Acid (20:5n-3) from the Marine Microalga *Phaeodactylum tricornutum*

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ABSTRACT: Eicosapentaenoic acid (EPA, 20:5n-3) was obtained from the marine microalgae *Phaeodactylum tricornutum* by a three-step process: fatty acid extraction by direct saponification of biomass, polyunsaturated fatty acid (PUFA) concentration by formation of urea inclusion compounds, and EPA isolation by semipreparative high-performance liquid chromatography (HPLC). Alternatively, EPA was obtained by a similar two-step process without the PUFA concentration step by the urea method. Direct saponification of biomass was carried out with two solvents that contained KOH for lipid saponification. An increase in yield was obtained because the problems associated with emulsion formation were avoided by separating the biomass from the soap solution before adding hexane for extraction of insaponifiables. The most efficient solvent, ethanol (96%) at 60°C for 1 h, extracted 98.3% of EPA. PUFA were concentrated by the urea method with a urea/fatty acid ratio of 4:1 at a crystallization temperature of 28°C and by using methanol and ethanol as urea solvents. An EPA concentration ratio of 1.73 (55.2:31.9) and a recovery yield of 78.6% were obtained with methanol as the urea solvent. This PUFA concentrate was used to obtain 93.4% pure EPA by semipreparative HPLC with a reverse-phase, C_{18}, 10 mm i.d. × 25-cm column and methanol/water (1% acetic acid), 80:20 w/w, as the mobile phase. Eighty-five percent of EPA loaded was recovered, and 65.7% of EPA present in *P. tricornutum* biomass was recovered in highly pure form by this three-step downstream process. Alternatively, 93.6% pure EPA was isolated from the fatty acid extract (without the PUFA concentration step) with 100% EPA recovery yield. This two-step process increases the overall EPA yield to 98.3%, but it is only possible to obtain 20% as much EPA as that obtained by three-step downstream processing.
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KEY WORDS: Eicosapentaenoic acid (EPA), *Phaeodactylum tricornutum*, downstream processing of microalgae, urea inclusion method, HPLC purification.

The n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) has attracted increased attention, due to its important role in human health. EPA has potential uses for prevention or treatment of medical disorders in heart and circulatory diseases (1,2), inflammation (3), and cancer (4).

Pharmaceutical and clinical applications require high PUFA concentrations. The lack of adequate amounts of purified materials for nutritional and clinical trials seriously retards systematic investigation on the preventive and therapeutic roles of n-3 PUFA (5). Therefore, rapid and reliable methods of extraction and purification of PUFA are required.

Many microalgae species are rich in oils that contain various amounts of PUFA. Their commercialization depends on their competitiveness with fish oil as a source of these fatty acids and, therefore, require improving the fat and PUFA content (by selecting overproducing clones), reducing cultivation costs, exploiting the added value of a controlled production process, and developing a higher-quality oil with large amounts of a single bioactive fatty acid (fish oils contain a mixture of many metabolically active fatty acids) (6). *Phaeodactylum tricornutum* is a potential source of EPA because it is a fast-growing alga; Molina Grima *et al.* (7) have attained an outdoor EPA production of 47.8 mg. day^{-1}. $liter^{-1}$. An additional advantage of this species is that the content of docosahexaenoic acid (DHA) and arachidonic acid (AA) is low or negligible, which has important advantages in simplifying EPA recovery (8).

There is little published on PUFA purification from microalga biomass, and it is difficult to compare extraction conditions and results due to different microalgae cell structures. Cohen and Cohen (9) obtained EPA from *Porphyridium cruentum* through a five-step procedure: lipid extraction with $Cl_3CH/CH_3OH/H_2O$ (2:1:0.8, vol/vol/vol), separation into neutral lipid, glycolipid and phospholipid classes, transmethylation of the glycolipid fraction, fractionation with urea of fatty acid methyl ester, and fractionation by reverse-phase chromatography at atmospheric pressure. In this way, Cohen and Cohen (9) obtained 97 and 80% pure EPA and AA fractions, respectively. Through a similar process, Cohen *et al.* (10) obtained 90% pure γ-linolenic acid from the cyanobacterium *Spirulina platensis*. EPA has also been obtained from the fungus *Pythium irregulare* (11) and a microorganism of the genus *Echinosporangium* through a process that included lipid extraction, methylation, column chromatography, low-temperature crystallization, urea adduction, centrifugal partition chromatography, and a final hydrolysis step to obtain the free fatty acid from the isolated methyl esters (12).

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The extraction solvent system most used is the Bligh and Dyer (13) mixture, $Cl_3CH/CH_3OH/H_2O$ (1:2:0.8, vol/vol/vol) (9,10,12). However, chloroform can produce tumors in animals, and methanol damages vision (14). Therefore, lipids must be extracted with nontoxic solvents, such as ethanol, *n*-butanol, isopropanol, or hexane (14,11) to facilitate their use in nutritional and clinical studies.

In previous works (15–17), we have developed a three-step method to obtain highly pure PUFA from the marine microalgae *Isochrysis galbana* (22.6% of EPA of total fatty acid and 2.7% of biomass dry weight). These steps are: (i) fatty acid extraction by direct saponification of biomass (15); (ii) enrichment of PUFA by urea fractionation (16,17); and (iii) isolation of PUFA through high-performance liquid chromatography (HPLC) on a semipreparative scale (16). In the latter study, EPA was isolated with 96% purity, but this only represented a 43.3% recovery of EPA contained in the initial *I. galbana* biomass. This low overall EPA yield was due to the low-yield urea-concentration step. This paper reports investigation of this three-step EPA isolation method in a comparative study of *P. tricornutum* and *I. galbana*, and describes attempts to increase EPA recovery by modifying the experimental conditions of extraction and urea steps, or by avoiding urea concentration.

EXPERIMENTAL PROCEDURES

Microalgal biomass. Lyophilized biomass of the marine microalga *P. tricornutum* was used as an oil-rich substrate that contains a high proportion of EPA (7). The total fatty acid content of biomass is 10% of dry weight. Cells were grown in laboratory cultures, harvested by centrifugation, and then lyophilized and stored at −18°C until used.

Fatty acid extraction from biomass. Two solvents were used to extract the fatty acids: hexane/ethanol (96%) (1:2.5, vol/vol) and ethanol (96%). Lyophilized biomass (15 g) was treated with 1140 mL of freshly prepared solvent containing 24 g of KOH for lipid extraction and simultaneous saponification, in a 2.5-L reactor with an outer sleeve that permits the circulation of water for temperature control. Extraction/saponification was carried out at room temperature during 8 h or at 60°C for 1 h, with constant agitation in a nitrogen atmosphere. The mixture obtained was then filtered through glass filters (100–160 μm bore) to remove the biomass residue. This residue was washed with 300 mL of ethanol (96%), and the filtrate was added to the first. Then 300 mL water was added to the combined filtrate, and unsaponifiables were separated by five extractions with 600 mL hexane. The hydroalcoholic phase, containing the soaps, was made acid by HCl 1:1 addition to pH 1, and the fatty acids obtained were recovered with eight extractions of 300 mL hexane (which ensured high depletion of fatty acids). Yield calculation of fatty acid extraction was as in Reference 15.

Fractionation with urea. Fatty acids (25 g) were added to a hot (65–70°C) solution of 100 g urea and 267 mL methanol or 532 mL ethanol (urea/fatty acid ratio of 4:1), while stirring constantly. The solution was heated and stirred until clear. Urea and urea compounds were allowed to crystallize overnight at 28°C. Extraction and preservation of PUFA were as in Reference 16.

PUFA fractionation by HPLC. Analytical and semipreparative HPLC was performed with a Beckman HPLC model "System Gold" (Beckman Instruments, Inc., San Ramon, CA) with a "diode array" detector. A wavelength of 217 nm was used for EPA detection (16). Separation was carried out in two Beckman (Ultrasphere) reverse-phase, C_{18}, 5-μm particle size, 8-nm pore columns: one 4.6 mm i.d. × 25 cm (analytical) and one 10 mm i.d. × 25 cm (semipreparative). Isocratic elution with methanol/water (1% acetic acid, AcH) (80:20 w/w) was used. The analytical and semipreparative-scale flow rates used were 0.6 and 3.0 mL/min, respectively. All products were of HPLC quality (Merck, Darmstadt, Germany), and water was purified in a Milli-Q system (Millipore Co., Bedford, MA). Solvents were filtered through 0.2-μm Millipore filters and degassed prior to use. The methanol/water of the fractions was removed in a vacuum evaporator prior to fatty acid analysis. Ultraviolet spectra of fatty acid standards (Sigma Chemical Co., St. Louis, MO) and isolated fractions were obtained with a Spectronic 3000 Array (Milton Roy Co., Milpitan, CA) spectrophotometer and compared to detect possible conjugation due to degradation (16,18).

Fatty acids analysis. Fatty acids in feedstock and fractions were analyzed by capillary gas chromatography to determine both purity (% w/w of fatty acid) and yield recovery (fatty acid in extract, urea concentrate, or HPLC fraction/fatty acid in biomass, extract, or urea concentrate, respectively). Methylation and methyl ester analysis have been described elsewhere (15). The following fatty acids were recognized and taken into account for yield calculations: 14:0, 16:0, 16:1n-7, 16:2n-4, 16:3n-4, 16:4n-1, 18:0, 18:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3 (stearidonic acid, SA), 22:0, 20:4n-6 (AA), 20:4n-3, 20:5n-3 (EPA), 24:0, and 22:6n-3 (DHA), but 18:0, 18:1n-7, 18:1n-9, 18:4n-3, and 22:0 are included in the tables as "Others."

Lipid fractionation. Lipids were fractionated in a chromatography column with 250–400 mesh silica gel and eluted with chloroform, acetone, and methanol by the method described previously (15).

RESULTS AND DISCUSSION

Fatty acid extraction from biomass. The solvents used here, ethanol (96%) and hexane–ethanol (96%) (1:2.5, vol/vol), were the most efficient solvents in fatty acid extraction by direct saponification from *I. galbana* (15).

The fatty acid profile of *P. tricornutum* biomass and those obtained with the two solvent mixtures at the two experimental conditions tested are shown in Table 1. This biomass is an excellent EPA source (30.5% of the total fatty acid and 3.1% of biomass dry weight) and contains low amounts of other PUFA, such as SA, AA, and DHA, which simplifies EPA isolation. Fatty acid profiles of all four extracts are analogous to

TABLE 1
Fatty Acid Composition (% of total fatty acids) of Extracts Obtained by Direct Saponification from *Phaeodactylum tricornutum*

		Saponification systems			
Fatty acid	Biomass	Hexane–EtOH (96%) (1:2.5, vol/vol) 8 h, room temperature	Hexane–EtOH (96%) (1:2.5, vol/vol) 1 h, 60°C	EtOH (96%) 8 h, room temperature	EtOH (96%) 1 h, 60°C
14:0	6.7	5.7	5.8	5.6	5.9
16:0	14.5	14.7	15.1	14.5	16.7
16:1n-7	20.7	20.9	21.2	20.7	19.0
16:2n-4	7.8	7.9	8.0	7.8	7.4
16:3n-4	6.8	6.8	7.0	6.8	6.4
16:4n-1	1.9	2.0	2.0	1.8	1.9
18:2n-6	2.2	2.8	2.4	3.6	2.4
18:3n-3	0.4	0.4	0.3	0.4	0.2
20:4n-6 (AA)[a]	2.8	2.8	2.8	2.7	3.6
20:4n-3	0.5	0.4	0.3	0.4	0.3
20:5n-3 (EPA)[b]	30.5	30.5	30.2	30.5	31.9
24:0	1.9	1.5	1.5	1.3	1.5
22:6n-3 (DHA)[c]	1.2	1.0	0.9	0.9	0.8
Others	2.1	2.6	2.3	3.0	2.0

[a]AA, arachidonic acid.
[b]EPA, eicosapentaenoic acid.
[c]DHA, docosahexaenoic acid.

that of the initial biomass. *Phaeodactylum tricornutum* presents a fatty acid profile less complex than *I. galbana* (15,16), which is more adequate if EPA is the only fatty acid of interest.

Overall fatty acid extraction and EPA yields are given in Table 2. These yields are higher than those obtained with *I. galbana* biomass [79.5 and 78.0% of average yields with ethanol (96%) and hexane–ethanol (96%) (1:2.5, vol/vol), respectively] (15), although *P. tricornutum* has a silica valve (the oval form) or an organic valve (the fusiform and triradiate forms) (19), whereas *I. galbana* has only an organic body-scale (20). This increase in yield was obtained by avoiding the problems associated with emulsion formation by separating the biomass from the soap solution before adding hexane for extraction of insaponifiables (obviously, biomass residue contains superficial agents that stabilize the hydroalcoholic–hexane emulsion). This fast step is easy, which was not borne in mind during fatty acid extraction from *I. galbana* (15), increases the yield and shortens operating time.

Ethanol (96%) is more efficient than hexane–ethanol (96%) (1:2.5, vol/vol) (Table 2) due to the higher polarity of the former, as shown by the fractionation of the lipid extract from *P. tricornutum* into neutral lipids (23.2%) and polar lipids (76.8%) (Table 3). The same result was obtained in fatty acid extraction from *I. galbana* (15). Partial yields of EPA are given in Table 2 and are in agreement with overall fatty acid yields. Ethanol (96%) at 60°C for 1 h produced the highest EPA extraction yield (98%), and therefore this is the extract selected to obtain EPA through the following process steps.

On the other hand, Table 3 shows that 84.9% of the EPA is isolated with the polar-lipid fraction. This was probably the reason for which Cohen and Cohen (9), on a small scale, made an intermediate step of fractionation of polar lipids from total lipids and extracted EPA only from the glycolipid fraction. We were able to obtain EPA from polar lipids; however, in this case such a procedure gave rise to a 15% loss in EPA. For industrial extraction of EPA from this microalga, EPA from neutral lipids also must be considered to maximize yield.

PUFA enrichment by urea fractionation. This step is to produce PUFA concentrates rich in EPA and to obtain the

TABLE 2
Overall Fatty Acid Extraction and EPA Yields[a]

Saponification system	20:5n-3 (EPA)	Total
Hexane–EtOH (96%) (1:2.5, vol/vol) 8 h, room temperature	86.6	86.6
Hexane–EtOH (96%) (1:2.5, vol/vol) 1 h, 60°C	85.6	86.4
EtOH (96%), 8 h, room temperature	88.6	88.6
EtOH (96%), 1 h, 60°C	98.3	96.2

[a]See Table 1 for abbreviation.

TABLE 3
Neutral and Polar Lipid Composition of Total Lipid and Distribution of EPA in *Phaeodactylum tricornutum* Biomass[a]

Lipids	Total lipids (%)	Total EPA (%)
Neutral	23.2	15.1
Polar	76.8	84.9
Glycolipid	49.1	56.7
Phospholipid	27.7	28.2

[a]See Table 1 for abbreviation.

largest possible yield. The best experimental conditions for PUFA (SA, EPA, and DHA) concentration, obtained previously (16,17), were a urea/fatty acid ratio of 4:1 and a crystallization temperature of 4°C. But, at this temperature, a low EPA recovery yield of 54% was obtained due to the strong tendency of EPA to form urea compounds, which increases at low temperatures (16,17,21). This is the major drawback of the urea method. However, since *P. tricornutum* is fundamentally of interest to obtain EPA, the best temperature (28°C) for EPA crystallization was used.

The fatty acid profile of urea concentrates are shown in Table 4. Almost all saturated and monounsaturated fatty acids were effectively removed. Methanol was more effective than ethanol, increasing the EPA content to 55.2%, while with ethanol it was 50.3%. Thus, concentration ratios (EPA in urea concentrate to EPA in fatty acid extract) of 1.73 and 1.58, respectively, were obtained. These concentration ratios are lower than those obtained with fatty acid from cod liver oil (3.1 and 2.6 with methanol and ethanol, respectively, under the same experimental conditions) (16,17) and similar to that obtained with *I. galbana* extract (1.75 at 4°C) (16). This is logical because a lower numerical concentration ratio will be obtained when the initial fatty acid mixture has a higher PUFA concentration (the urea method concentrates all PUFA).The *P. tricornutum* extract had a PUFA concentration of 54.9% (Table 4), whereas the concentration in cod liver oil and *I. galbana* extracts were 27.6 and 41.2%, respectively (16).

The EPA recovery obtained with ethanol was also greater than with methanol (83.8 and 78.6%, respectively; total was 53.0% and 45.3%, respectively). These EPA yields are higher than those obtained from the *I. galbana* extract (54.3%) (16) due to the optimum EPA crystallization temperature used (28°C). Therefore, although these yields are acceptable, they lower the overall EPA recovery of the three-step process (regardless of the yield of the HPLC step). To increase this overall EPA yield, an attempt might be made to omit the urea concentration step because the EPA extract concentration seems high enough to isolate EPA by HPLC.

Chromatographic fractionation. Therefore, two fatty acid mixtures were used to obtain highly pure EPA by chromatographic fractionation: the PUFA concentrate obtained by the urea method with methanol, with 55.2% EPA (Table 5) and the fatty acid extract before PUFA concentration by the urea method, which had 31.9% EPA (Table 6).

Because this step is to produce a fraction highly pure in EPA, by loading the maximum possible amount of PUFA concentrate or fatty acid extract that will permit a large EPA recovery yield, a mobile phase with a low eluent capacity (methanol–water, 1% AcH, 80:20, w/w) (16) was chosen.

The maximum possible load for which purity and yield are acceptable depends on the fatty acid profile of the sample. For that, a previous analytical column study was made to obtain the maximum possible load that could be scaled up. This study was made by taking into account the direct relationship between chromatographic resolution (R) and separation: for $R = 1$, separation is around 98% (16). Figure 1A shows the analytical chromatograms for PUFA concentrate separation for which EPA-peak 1 and EPA-peak 2 resolutions are 1.1 and 1.4, respectively.

This chromatographic fractionation was scaled-up by using the same surface velocity and column length but increasing the capacity by using a greater cross-sectional area. This will preserve the characteristics of the flow in the packed bed, and thereby, the concentration profiles of each solute cannot be greatly altered (22). Moreover, it does not increase

TABLE 4
Fatty Acid Composition of Urea Concentrates Obtained from *Phaeodactylum tricornutum* Fatty Acid Extract at a Crystallization Temperature of 28°C and with a 4:1 Urea/Fatty Acid Ratio[a]

Fatty acid	Extract	Urea solvent	
		Methanol	Ethanol
14:0	5.9	0.3	0.3
16:0	16.7	0.0	0.0
16:1n-7	19.0	6.9	13.4
16:2n-4	7.4	10.5	10.7
16:3n-4	6.4	12.3	10.6
16:4n-1	1.9	2.1	1.9
18:2n-6	2.4	2.1	2.9
18:3n-3	0.2	1.0	0.9
20:4n-6 (AA)	3.6	5.9	5.6
20:4n-3	0.3	1.4	1.4
20:5n-3 (EPA)	31.9	55.2	50.3
24:0	1.5	0.0	0.0
22:6n-3 (DHA)	0.8	1.5	1.3
Others	2.0	0.8	0.7
Total saturated	24.1	0.3	0.3
Total monounsaturated	19.0	6.9	13.4
Total saturated, monounsaturated	43.1	7.2	13.7
Total PUFA	54.9	92.0	85.6

[a]PUFA, polyunsaturated fatty acid. See Table 1 for other abbreviations.

TABLE 5
Fatty Acid Composition of *Phaeodactylum tricornutum* Extract [EtOH (96%), 1 h, 60°C], Urea Concentrate (MeOH), and HPLC Fraction (Fig. 1B)[a]

Fatty acid	Extract	Urea concentrate	HPLC fraction
14:0	5.9	0.3	—
16:0	16.7	0.0	—
16:1n-7	19.0	6.9	2.9
16:2n-4	7.4	10.5	—
16:3n-4	6.4	12.3	2.1
16:4n-1	1.9	2.1	—
18:2n-6	2.4	2.1	1.6
18:3n-3	0.2	1.0	—
20:4n-6 (AA)	3.6	5.9	—
20:4n-3	0.3	1.4	—
20:5n-3 (EPA)	31.9	55.2	93.4
24:0	1.5	0.0	—
22:6n-3 (DHA)	0.8	1.5	—
Others	2.0	0.8	0.0

[a]HPLC, high-performance liquid chromatography. See Table 1 for other abbreviations.

TABLE 6
Fatty Acid Composition of *Phaeodactylum tricornutum* Extract [EtOH (96%), 1 h, 60°C] and HPLC Fraction (Fig. 2B)[a]

Fatty acid	Extract	HPLC fraction
14:0	5.9	3.9
16:0	16.7	0.4
16:1n-7	19.0	0.4
16:2n-4	7.4	0.5
16:3n-4	6.4	0.1
16:4n-1	1.9	—
18:2n-6	2.4	0.2
18:3n-3	0.2	0.2
20:4n-6 (AA)	3.6	—
20:4n-3	0.3	0.1
20:5n-3 (EPA)	31.9	93.6
24:0	1.5	—
22:6n-3 (DHA)	0.8	—
Others	2.0	0.6

[a]See Tables 1 and 5 for abbreviations.

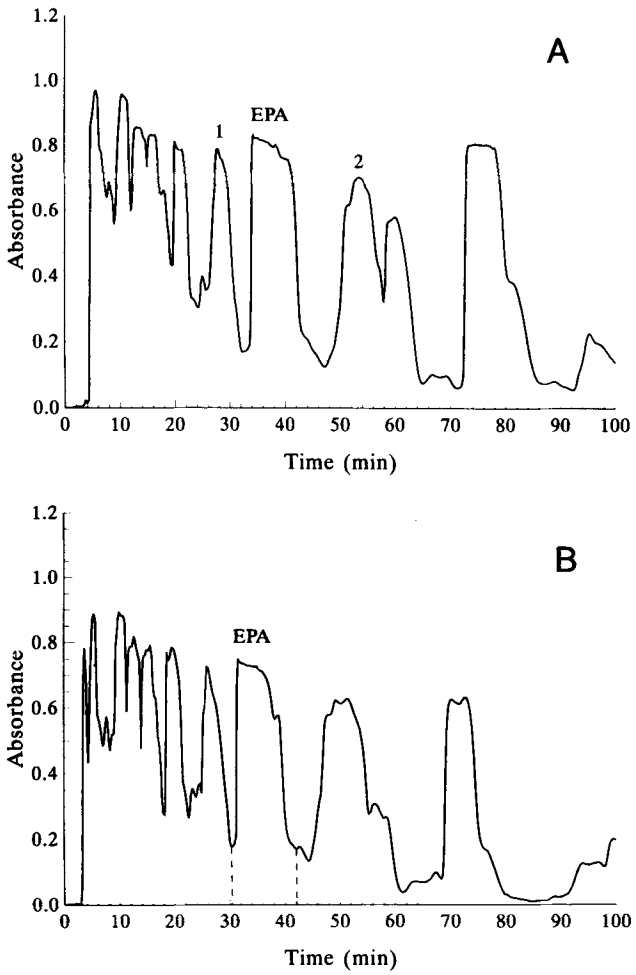

FIG. 1. Chromatographic fractionation of *Phaeodactylum tricornutum* polyunsaturated fatty acid concentrate to obtain eicosapentaenoic acid (EPA). Mobile phase: Methanol–water (1% acetic acid) 80:20 w/w. A: Analytical column (4.6 mm i.d. × 25 cm), reverse-phase C_{18}; flow rate 0.6 mL/min; mass load 7.2 mg. EPA-peak 1, resolution 1.1; EPA-peak 2, resolution 1.4. B: Semipreparative column (10 mm i.d. × 25 cm), reverse- phase C_{18}; flow rate 3.0 mL/min; mass load 37.4 mg. Purity of EPA fraction is shown in Table 5.

the pressure drop. The flow rate (f) and sample load (m) scale-up ratio is (16):

$$f = D_2^2/D_1^2 \quad [1]$$

$$m = D_2^2 \cdot L_2/(D_1^2 \cdot L_1) \quad [2]$$

where D_1 and D_2 are the diameters of the larger and analytical column, respectively, and L_1 and L_2 their lengths. Therefore, 37.4 mg of urea concentrate (Table 5) was loaded, obtaining the chromatogram shown in Figure 1B. The flow rate used (3 mL/min) was the highest possible, which is limited by the maximum allowable column pressure (3500 psig) (16). This chromatogram shows the cut-off point for the EPA fraction (purity and yield are 93.4 and 85.0%, respectively) (Table 5). It is possible to increase this purity by decreasing the mass load or the EPA recovery and to increase the yield by decreasing mass load or purity. The EPA yield in the extraction, urea concentration, and HPLC fractionation steps was 65.7%. This relatively low overall EPA yield is fundamentally due to the low urea EPA yield in the urea step (78.6%). EPA recovery from the HPLC fractionation step is relatively low also (85%), but yields over 90% have been obtained by decreasing the mass load (extract, 98.3%; Table 2).

Similarly, Figure 2 shows the analytical and semipreparative chromatograms for fatty acid extract fractionation. Now, a more complex fatty acid mixture must be fractionated. The analytical separation (Fig. 2A) gave EPA-peak 1 and EPA-peak 2 resolutions of 1.6 and 0.9, respectively, but now only 2.6 mg of fatty acid extract could be loaded and, therefore, 12.3 mg in the semipreparative column (Fig. 2B and Table 6). However, 98.3% of the EPA contained in the initial *P. tricornutum* biomass was obtained with a 93.6% purity (EPA yields were 98.3% in extraction, 100% in HPLC fractionation and, therefore, 98.3% overall EPA yield).

The ultraviolet (UV) spectra of the EPA standard and isolated fraction in Figure 1B were compared to determine possible conjugation due to degradation, which would have given an absorption displacement peak of around 234–237 nm (dienes) or 268 nm (trienes) (18). Figure 3 shows that the UV spectrum of the EPA obtained is similar to that of the standard, which shows that, during downstream processing, there was no degradation of EPA.

In conclusion, it can be noted that the fatty acid extraction yield from *P. tricornutum* biomass by direct saponification has been increased by separating the biomass residue from the soap solution by filtration. Moreover, highly pure EPA (93.6%) has been obtained in a downstream process with only two steps, suppressing the urea step. This new process reduces operating time, increases the overall yield of EPA and diminishes the possibilities of PUFA degradation. This two-step process is possible due to the high EPA concentration of *P. tricornutum* biomass and the low concentration of other PUFA (Table 1). Keeping in mind that these are only preliminary results, there was no economical advantage because lower amounts of EPA were obtained from the fatty acid extract than from the urea concentrates with the same mobile

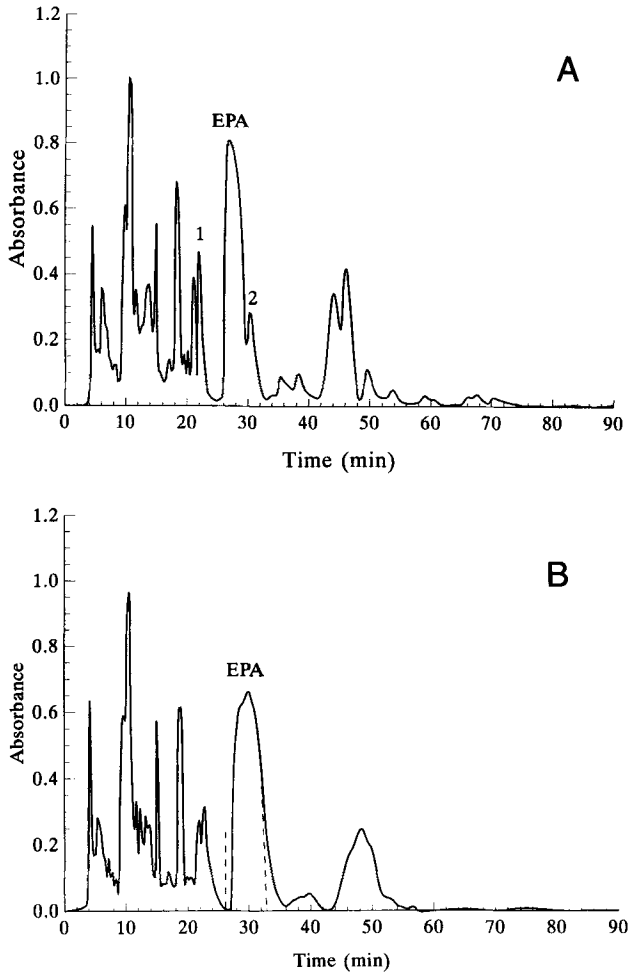

FIG. 2. Chromatographic fractionation of *Phaeodactylum tricornutum* fatty acid extract to obtain EPA. Mobile phase: Methanol–water (1% acetic acid) 80:20 w/w. A: Analytical column (4.6 mm i.d. × 25 cm), reverse-phase C_{18}; flow rate 0.6 mL/min; mass load 2.6 mg. EPA-peak 1, resolution 1.6; EPA-peak 2, resolution 0.9. B: Semipreparative column (10 mm i.d. × 25 cm), reverse-phase C_{18}; flow rate 3.0 mL/min; mass load 12.3 mg. Purity of EPA fraction is shown in Table 6. See Figure 1 for abbreviation.

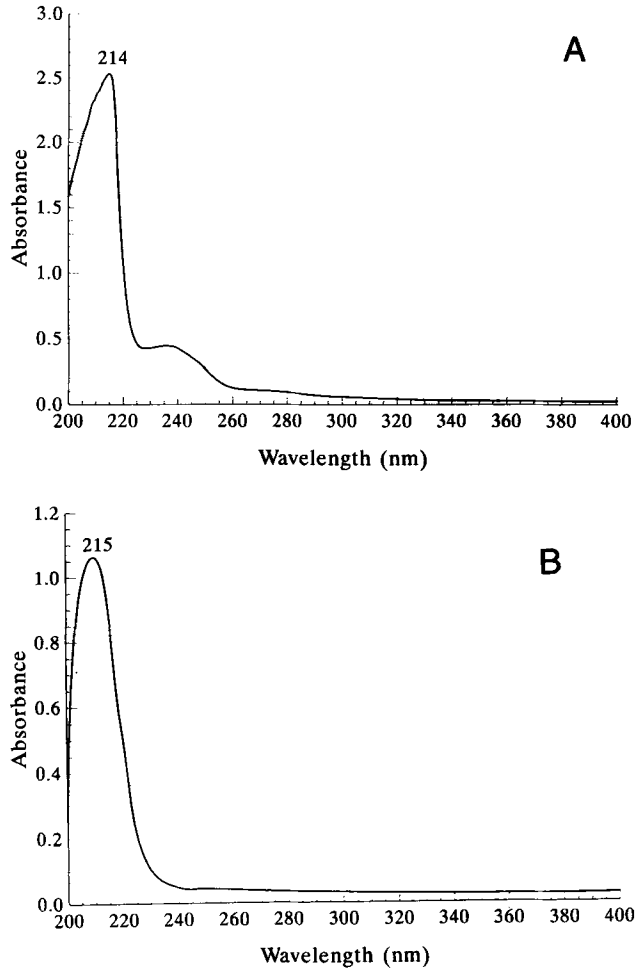

FIG. 3. Comparison between ultraviolet spectra of EPA Sigma Standard (Sigma Chemical Co., St. Louis, MO) and the fraction shown in Figure 1B. A: EPA Sigma pattern. B: EPA fraction. See Figure 1 for abbreviation.

phase consumption. More research is necessary to increase the load or to reduce the separation time, by gradient elution, for example. Other authors (9,10) have obtained high purity but did not report PUFA recovery, which was probably low. Future experiments in our laboratory must lead to scale-up these chromatographic fractionations and a relatively economical process to obtain highly pure EPA from microalgae, keeping in mind the high market price of highly pure EPA.

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