# Concentration and Purification of Stearidonic, Eicosapentaenoic, and Docosahexaenoic Acids from Cod Liver Oil and the Marine Microalga *Isochrysis galbana*

**A. Robles Medina*, A. Giménez Giménez, F. García Camacho, J.A. Sánchez Pérez, E. Molina Grima and A. Contreras Gómez**

Departamento de Ingeniería Química, Facultad de Ciencias Experimentales, Universidad de Almería, E-04071 Almería, Spain

**ABSTRACT:** n-3 Polyunsaturated fatty acids (n-3 PUFA) from the marine microalga *Isochrysis galbana* were concentrated and purified by a two-step process—formation of urea inclusion compounds followed by preparative high-performance liquid chromatography. These methods had been developed previously with fatty acids from cod liver oil. By the urea inclusion compounds method, a mixture that contained 94% (w/w) stearidonic (SA), eicosapentaenoic (EPA), plus docosahexaenoic (DHA) acids (4:1 urea/fatty acid ratio and 4°C crystallization final temperature) was obtained from cod liver oil fatty acids. Further purification of SA, EPA, and DHA was achieved with reverse-phase $C_{18}$ columns. These isolations were scaled up to a semi-preparative column. A PUFA concentrate was isolated from *I. galbana* with methanol/water (80:20, w/w) or ethanol/water (70:30, w/w). With methanol/water, a 96% EPA fraction with 100% yield was obtained, as well as a 94% pure DHA fraction with a 94% yield. With ethanol/water as the mobile phase, EPA and DHA fractions obtained were 92% pure with yields of 84 and 88%, respectively.
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**KEY WORDS:** Cod liver oil, docosahexaenoic acid, eicosapentaenoic acid, HPLC purification, microalgae, stearidonic acid, urea inclusion method.

n-3 polyunsaturated fatty acids (n-3 PUFAs) have been recognized for their important role in health since 1972 (1). Eicosapentaenoic acid (EPA) can affect the circulatory system and can help prevent atherosclerosis and thrombosis (2,3). Furthermore, recent central nervous system research indicates that docosahexaenoic acid (DHA) is important in early human development. In infants, DHA is highly concentrated in brain and retinal tissues and accumulates during late fetal and early neonatal life. Diets deficient in DHA could promote abnormalities that may be irreversible (4,5).

*To whom correspondence should be addressed.

Isolation and purification methods for stearidonic (SA), EPA, and DHA acids are being developed to supply highly purified PUFAs for medical research on humans (6) and on animals (7), and for chemical research on autoxidation and stability (8,9). The lack of adequate amounts of purified materials for nutritional and clinical trials seriously retards systematic investigation of the preventative and therapeutic roles of EPA and DHA (10). These fatty acids are commonly administered as ethyl esters, free fatty acids, or as triacylglycerols. Clinical trials carried out in humans have shown that free fatty acids were absorbed fivefold more efficiently than ethyl esters. The mildly irritating effect of free fatty acids on the esophagus is easily avoided by the use of gelatin capsules, which are also necessary to prevent autoxidation of PUFA (11,12).

PUFA can be obtained from fish oil or microorganisms. The only current commercial source of n-3 PUFA is fish oil (13). Fish oil n-3 PUFA content and EPA/DHA ratios fluctuate widely. Microalga biomass is particularly suitable for extraction and purification of individual PUFA, due to its stable and reliable composition. In addition, PUFA from cultured microalgae are cholesterol-free (14), contamination-free, and taste good.

Several techniques have been developed for producing highly concentrated SA, EPA, and DHA. They all include an initial fatty acid preconcentration stage, followed by isolation of the specific PUFA of interest. Concentrates of PUFA are generally most efficiently prepared by urea adduction or low-temperature fractional crystallization techniques (15). Furthermore, fractionation of fatty acids by urea adduction has been studied on a laboratory scale (16). The urea fractionation of the fatty acids is mainly based on the degree of unsaturation. There is an inverse correlation between unsaturation and formation of urea crystals (17). Fractions rich in PUFA can also be obtained by supercritical $CO_2$ extraction of neutral lipids or the fatty acid alkyl esters (18). An alternative approach for enrichment of n-3 PUFA is based on enzymatic techniques, such as lipase-catalyzed alcoholysis (19) or inter-

esterification of triglycerides (20) and esterification of glycerol (21). To obtain even higher EPA and DHA concentrations, the preconcentration method has been combined with other fractionation procedures, such as solvent fractionation, distillation of the corresponding methyl esters, preparative gas chromatography, fractional distillation, or preparative high-performance liquid chromatography (HPLC) (6). In recent years, the latter has become a powerful tool for the isolation of compounds from complex mixtures. As fish oil has a complex matrix, preparative HPLC is the best technique for this purification (22).

This paper investigates a two-step method for isolation of SA, EPA, and DHA from cod liver oil and *Isochrysis galbana* fatty acids by the urea method and semi-preparative HPLC.

## EXPERIMENTAL PROCEDURES

*Cod liver oil saponification.* The fatty acid profile of the commercial cod liver oil (Acofarma, Barcelona, Spain) is given in Table 1. Cod liver oil (350 g) was saponified by agitation under nitrogen in 700 g of an aqueous–alcohol solution of NaOH [120 g NaOH and 1.25 g ethylenediaminetetraacetic acid (EDTA) dissolved in 400 mL water and 400 mL ethanol, 96%]. Fatty acid recovery was as described by Molina Grima *et al.* (23).

**TABLE 1**
**Influence of Urea/Fatty Acid Ratio on Fatty Acid Composition of Urea Concentrates Obtained at 4°C from Fatty Acid Extract from Cod Liver Oil (CLO)**

| | | Urea/fatty acid ratio | | | |
|---|---|---|---|---|---|
| Fatty acid[a] | CLO | 1:1 | 2:1 | 3:1 | 4:1 |
| 14:0 | 4.2 | 2.7 | 0.7 | 0.5 | 0.7 |
| 16:0 | 10.6 | 2.0 | 0.2 | 0.5 | 0.0 |
| 16:1n-7 | 7.8 | 9.6 | 6.9 | 2.5 | 3.2 |
| 18:0 | 2.6 | 0.9 | 0.1 | 0.0 | 0.0 |
| 18:1n-9 | 17.0 | 17.6 | 3.2 | 2.9 | 0.7 |
| 18:1n-7 | 4.6 | 5.9 | 1.4 | 1.0 | 0.0 |
| 18:2n-6 | 1.5 | 2.0 | 1.6 | 0.7 | 0.7 |
| 18:3n-6 | 0.2 | 0.2 | 0.4 | 0.5 | 0.5 |
| 20:0 | 0.2 | 0.2 | 0.2 | 0.0 | 0.1 |
| 18:3n-3 | 0.8 | 1.1 | 1.0 | 0.6 | 0.6 |
| 20:1n-9 | 10.8 | 9.0 | 1.3 | 0.6 | 0.8 |
| 18:4n-3 (SA) | 2.4 | 3.3 | 6.3 | 8.0 | 8.5 |
| 20:3n-6 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 |
| 22:0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:4n-6 | 0.5 | 0.8 | 1.0 | 0.9 | 1.0 |
| 22:1n-11 | 8.3 | 3.8 | 0.4 | 0.0 | 0.0 |
| 22:1n-9 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:5n-3 (EPA) | 9.4 | 13.0 | 22.6 | 24.8 | 25.6 |
| 24:0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 22:4n-6 | 0.5 | 0.7 | 1.5 | 1.7 | 1.8 |
| 22:5n-3 | 1.2 | 1.6 | 2.4 | 1.4 | 1.6 |
| 22:6n-3 (DHA) | 11.0 | 15.8 | 45.4 | 58.2 | 59.9 |
| Total saturated (sat.) | 17.6 | 5.8 | 1.2 | 0.9 | 0.8 |
| Total monounsaturated (mono.) | 48.5 | 45.9 | 13.2 | 7.0 | 4.6 |
| Total sat., mono. | 66.1 | 51.7 | 14.5 | 8.0 | 5.4 |
| Total SA, EPA, DHA | 22.7 | 32.1 | 74.3 | 91.0 | 94.0 |
| Total fatty acid yield | 100 | 33.3 | 26.4 | 20.8 | 19.8 |
| SA, EPA, and DHA Yield | 100 | 47.1 | 86.2 | 83.2 | 82.1 |

[a]SA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

*Microalgal biomass.* Lyophilized biomass of the marine microalga *I. galbana* was used as an oil-rich substrate, which contains a high proportion of PUFA (24,25). Culture conditions were as previously described (23).

*Fatty acid extraction from biomass.* Lyophilized biomass (5 g) was treated with 380 mL of freshly prepared hexane/ethanol (96%) (1/2.5, vol/vol) containing 8 g of KOH, for lipid extraction and simultaneous saponification. Extraction/saponification was carried out at room temperature for 8 h with constant agitation in a nitrogen atmosphere. Unsaponifiable separation, fatty acid obtention, and recovery were as described elsewhere (23).

*Fractionation with urea.* Fatty acids (25 g) were added while stirring constantly to a hot (65–70°C) solution of 25, 50, 75, or 100 g of urea (urea/fatty acid ratios of 1:1, 2:1, 3:1, and 4:1, respectively) and 67, 133, 200, and 267 mL methanol, respectively (urea/methanol ratio of 373 g/L). The solution was heated and stirred until clear. Urea and urea compounds were allowed to crystallize overnight at a constant temperature between –36°C and 36°C. After filtration under vacuum, the liquid phase was evaporated to a small volume in a vacuum rotary evaporator at room temperature. The concentrate was then mixed with 125 mL 0.1 N HCl and 125 mL hexane, and the hexane layer was separated. The lower layer was extracted again with 50 mL hexane, and the combined hexane phases were evaporated. Concentrates dissolved in hexane, with octyl gallate (0.01%, on a fatty acid base) as a stabilizer, were stored in nitrogen at -20°C until use.

*PUFA fractionation by HPLC.* Analytical and semi-preparative HPLC was performed with a Beckman instrument (Beckman Instruments, Inc., San Ramon, CA) with "diode array" detector and Pharmacia (Uppsala, Sweden) fraction collector. A 217-nm wavelength was selected, based on maximal absorption of spectra obtained with EPA and DHA samples from Sigma Chemical Co. (St. Louis, MO), which were also used as standards to obtain the retention time for each fatty acid.

Separation methods were developed in a Beckman (Ultrasphere) reverse-phase (RP), $C_{18}$, 5-μm particle size, 8-nm pore, 4.6 mm i.d. × 25 cm (analytical) column. The semi-preparative isolation was performed on a Beckman RP, $C_{18}$, 5-μm, 8-nm pore, 10 mm i.d. × 25 cm column. A 4.6 × 45 mm precolumn was sometimes used to protect the main columns. Isocratic elution with methanol/water (1% AcH) ratios of 90:10, 85:15, and 80:20 (w/w), and ethanol/water (1% AcH) ratios of 80:20, 75:25, and 70:30 (w/w) was used. All products were HPLC quality (Merck, Darmstadt, Germany), and water was distilled and further purified by adsorption, deionization and filtration in a Milli-Q system (Millipore Co., Bedford, MA). Aqueous and organic solvent were filtered through 0.2- and 0.5-μm Millipore filters, respectively, and degassed prior to use. The analytical-scale flow rates ranged from 0.25

to 1.25 mL/min for methanol/water and 0.15–0.4 mL/min for ethanol/water. Semi-preparative column flow rates were up to 3.5 mL/min and 2 mL/min for methanol/water and ethanol/water, respectively. Prior to fatty acid analysis, the methanol/water phase was removed in a vacuum evaporator or in nitrogen. For ethanol/water, fatty acids were extracted from the mobile phase (20 mL) four times with 5 mL of hexane.

The ultaviolet (UV) spectra of fatty acid standards and isolated fractions 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) (26).

*Analysis of the fatty acids.* Fatty acids in feedstock and fractions were analyzed by capillary gas chromatography (GC) to determine purity (% w/w of fatty acid) and yield recovered (fatty acid in fraction/fatty acid in sample). Methylation and methyl ester analysis have been described elsewhere (23).

## RESULTS AND DISCUSSION

The fatty acid profile of *I. galbana* is given in Table 2. The total fatty acid content of biomass is 9.5% of dry weight. The profile of fatty acid solution obtained by extraction–saponification from the biomass, which is similar to the biomass profile, is also given in Table 2. The fatty acid extraction yield was 75%, based on the ratio between the content of all fatty acids obtained and the total fatty acid content of the initial biomass, as described elsewhere (23).

*Enrichment in n-3 PUFA by urea fractionation.* The goal of this study has been to produce PUFA concentrates rich in SA, EPA, and DHA, and to obtain the largest overall yields possible. This was optimized with cod liver oil, and the best experimental conditions (urea/fatty acid ratio and final temperature of crystallization) were applied to fatty acids from the marine microalga *I. galbana*.

**TABLE 2**
**Fatty Acid Composition (A) and Yield (B) of *Isochrysis galbana* Biomass, Fatty Acid Extract, Urea Concentrate, and HPLC Fractions[a]**

A

| | | | | HPLC fractions | | |
|---|---|---|---|---|---|---|
| Fatty acid | Biomass | Extract | Urea concentrate | SA | EPA | DHA |
| 14:0 | 10.1 | 10.7 | 0.3 | — | — | — |
| 16:0 | 20.3 | 18.9 | 0.2 | 1.1 | — | — |
| 16:1n-7 | 21.4 | 23.3 | 4.3 | — | — | — |
| 18:0 | 0.7 | 0.5 | 1.5 | 1.1 | — | — |
| 18:1n-9 | 1.4 | 1.7 | 0.2 | — | — | — |
| 18:1n-7 | 3.6 | 3.2 | 0.8 | — | — | — |
| 18:2n-6 | 0.9 | 0.9 | 0.2 | — | — | 1.4 |
| 18:3n-6 | 0.2 | 0.2 | 0.4 | — | 2.1 | — |
| 20:0 | 0.0 | 0.1 | 0.2 | — | — | — |
| 18:3n-3 | 1.2 | 1.3 | 0.8 | — | 2.0 | — |
| 20:1n-9 | 0.2 | 0.3 | 0.3 | — | — | — |
| 18:4n-3 (SA) | 6.4 | 7.3 | 22.6 | 94.8 | — | — |
| 20:3n-6 | 0.4 | 0.2 | 0.2 | — | — | — |
| 22:0 | 0.0 | 0.1 | 0.1 | — | — | — |
| 20:4n-6 | 0.7 | 0.7 | 1.1 | — | — | 3.7 |
| 22:1n-11 | 0.1 | 0.1 | 0.1 | — | — | — |
| 22:1n-9 | 0.0 | 0.1 | 0.1 | — | — | — |
| 20:5n-3 (EPA) | 22.6 | 22.4 | 39.4 | — | 96.0 | — |
| 24:0 | 0.0 | 0.1 | 0.1 | — | — | — |
| 22:4n-6 | 1.3 | 1.3 | 3.6 | — | — | — |
| 22:5n-3 | 0.2 | 0.1 | 0.1 | — | — | — |
| 22:6n-3 (DHA) | 8.4 | 6.8 | 23.4 | — | — | 94.9 |

B

| Yield (%) | SA | EPA | DHA |
|---|---|---|---|
| Extract[b] | 92.5 | 80.1 | 65.2 |
| Urea concentrate[c] | 71.6 | 54.3 | 100 |
| SA fraction[d] | 100 | — | — |
| EPA fraction[d] | — | 99.6 | — |
| DHA fraction[d] | — | — | 94.0 |
| Overall yield (%) | 66.2 | 43.3 | 61.3 |

[a]Abbreviations as in Table 1. HPLC, high-performance liquid chromatography.
[b]Obtained by direct saponification of biomass (Ref. 25).
[c]Urea/fatty acid ratio 4:1 and crystallization temperature, 4°C.
[d]Mobile phase MeOH/$H_2O$ (80:20, w/w), mass load 9.49 mg and flow rate 3 mL/min.

Enrichment of SA, EPA, and DHA depends on the urea/fatty acid ratio (Table 1). Concentration of n-3 PUFA and overall yield vary inversely with increasing urea/fatty acid ratios. While 4:1 is the most appropriate, no significant differences are observed between 3:1 and 4:1. These results are in agreement with those of Traitler *et al.* (27), Wille *et al.* (6) and Ratnayake *et al.* (16), and are reinforced by the fact that urea binds fatty acids in a ratio of approximately 3:1 by weight. An increase in this ratio favors the formation of urea compounds all the more as their stability increases (28).

In another set of fractionations, temperature variation showed that 4°C seemed to be optimum (see Table 3). A further decrease in the crystallization temperature was accompanied by a decrease in concentration and in yield. The overall yield of fatty acids increases with temperature, which is a logical result because the tendency to form urea compounds increases when the temperature decreases (28). The low n-3 PUFA recovery at low temperature demonstrates that below −4°C, n-3 PUFAs also form urea compounds in high proportions. Among the three fatty acids of interest, it is noteworthy that the EPA concentration factor (fatty acid concentration ratio after and before urea) is lower than that of SA and DHA in all the concentrates prepared. These results are in agreement with those of Haagsma *et al.* (17) and Ratnayake *et al.* (16), who pointed out the strong tendency of EPA to form urea compounds.

The best experimental conditions (4°C and 4:1 urea/fatty acid ratio) led to a concentrate with 8.5, 25.6, and 59.9% of SA, EPA, and DHA, respectively (Tables 1 and 3). These conditions were then applied to a concentration of fatty acid from the marine microalga *I. galbana* (Table 2), yielding a crude fatty acid concentrate of 22.6, 39.4, and 23.4% of SA, EPA, and DHA, respectively. Absorption peaks were observed in the microalga concentrate, but not in the cod liver oil, at 408 nm due to carotenoids and at 697 nm due to chlorophylls, because these were not bleached during the process.

## OPTIMIZATION OF THE CHROMATOGRAPHIC SEPARATION: ISOLATION OF SA, EPA, AND DHA FROM COD LIVER OIL UREA CONCENTRATES IN AN ANALYTICAL COLUMN

The goal of the isolation is to obtain chromatographic separation, the resolution of which was around one, to scale it up, and to produce SA, EPA, and DHA fractions of purities better than 90–95%. The initial chromatographic steps in the isolation development process were performed in the analytical column with methanol/water as the mobile phase. To evaluate the degree of difficulty of the HPLC separation of two components, the resolution ($R$) has to be determined. This was chosen as the determining para-

**TABLE 3**
**Influence of Temperature on Fatty Acid Composition of Urea Concentrates Obtained with a 4:1 Urea/Fatty Acid Ratio from Fatty Acid Extract from CLO[a]**

| | | Temperature (°C) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | CLO | −36 | −28 | −20 | −12 | −4 | 4 | 12 | 20 | 28 | 36 |
| 14:0 | 4.2 | 3.0 | 3.7 | 0.7 | 1.6 | 1.5 | 0.7 | 1.0 | 0.3 | 0.9 | 0.4 |
| 16:0 | 10.6 | 1.5 | 2.7 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.3 | 0.0 |
| 16:1n-7 | 7.8 | 10.8 | 9.3 | 5.4 | 0.0 | 2.9 | 3.2 | 1.4 | 0.8 | 2.5 | 8.6 |
| 18:0 | 2.6 | 0.6 | 0.3 | 0.9 | 1.6 | 1.3 | 0.0 | 0.8 | 2.1 | 0.9 | 0.6 |
| 18:1n-9 | 17.0 | 21.2 | 17.7 | 3.9 | 0.7 | 0.6 | 0.7 | 0.7 | 0.5 | 0.7 | 3.5 |
| 18:1n-7 | 4.6 | 6.9 | 6.6 | 2.2 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 | 1.6 |
| 18:2n-6 | 1.5 | 2.1 | 1.8 | 1.6 | 0.0 | 0.0 | 0.7 | 0.7 | 0.7 | 1.7 | 2.9 |
| 18:3n-6 | 0.2 | 0.4 | 0.2 | 0.8 | 0.0 | 0.0 | 0.5 | 0.5 | 0.6 | 0.4 | 0.4 |
| 18:3n-3 | 0.8 | 0.2 | 0.9 | 1.9 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 1.3 | 1.7 |
| 20:1n-9 | 10.8 | 1.1 | 1.5 | 1.2 | 0.0 | 0.0 | 0.8 | 0.2 | 0.6 | 0.4 | 0.4 |
| 18:4n-3 (SA) | 2.4 | 8.2 | 8.4 | 9.2 | 13.1 | 11.5 | 8.5 | 8.8 | 8.5 | 8.1 | 7.2 |
| 20:4n-6 | 0.5 | 0.8 | 0.6 | 2.7 | 0.0 | 0.5 | 1.0 | 0.8 | 1.2 | 1.1 | 1.2 |
| 22:1n-11 | 8.3 | 3.3 | 4.5 | 0.3 | 0.6 | 0.6 | 0.0 | 0.0 | 0.0 | 0.3 | 0.3 |
| 22:1n-9 | 0.1 | 0.0 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:5n-3 (EPA) | 9.4 | 12.4 | 11.2 | 16.4 | 17.7 | 14.9 | 25.6 | 26.1 | 29.5 | 28.7 | 25.4 |
| 22:4n-6 | 0.5 | 0.8 | 0.7 | 1.2 | 1.7 | 1.3 | 1.8 | 2.3 | 1.8 | 1.7 | 1.4 |
| 22:5n-3 | 1.2 | 1.6 | 1.4 | 1.6 | 0.0 | 0.5 | 1.6 | 1.2 | 1.8 | 2.5 | 2.8 |
| 22:6n-3 (DHA) | 11.0 | 14.5 | 18.0 | 31.8 | 58.5 | 54.7 | 59.9 | 41.5 | 45.1 | 38.2 | 31.6 |
| Total sat. | 17.6 | 5.1 | 6.8 | 1.9 | 4.5 | 2.7 | 0.8 | 2.0 | 2.7 | 2.1 | 1.0 |
| Total mono. | 48.5 | 43.1 | 40.4 | 12.9 | 1.3 | 4.1 | 4.6 | 2.9 | 1.9 | 3.9 | 14.3 |
| Total SA, EPA, and DHA | 22.7 | 35.0 | 37.7 | 57.4 | 89.3 | 81.2 | 94.0 | 76.4 | 83.0 | 75.0 | 64.2 |
| Total fatty acid yield | 100 | 9.2 | 10.3 | 11.3 | 13.6 | 19.1 | 19.8 | 22.3 | 22.5 | 24.7 | 26.4 |
| SA, EPA, and DHA yield | 100 | 14.1 | 17.1 | 28.6 | 53.7 | 68.2 | 82.1 | 75.0 | 82.3 | 81.6 | 74.6 |

[a]Abbreviations as in Table 1.

meter in selecting the optimum experimental conditions for scale-up, as for $R = 1$, separation is around 98% and for $R = 1.5$ it is complete (29). Resolution may be calculated by:

$$R = 2(t_{R2} - t_{R1})/(\Delta t_1 + \Delta t_2) \quad [1]$$

where $t_{R1}$ and $t_{R2}$ are the retention times of two compounds and $\Delta t_1$ and $\Delta t_2$ are the corresponding peak widths, measured at the base line. Resolution thus takes into account thermodynamic and kinetic effects, which affect the separation between peaks.

*Influence of flow rate.* Flow rate had no effect on $R$ in the separations carried out with methanol/water (85:15) (Table 4). $R$ remained constant within the range of flow rates tested, so that although differences of retention time decreased with increasing flow rate, peak width also decreased in the same proportion (Eq. 1). Consequently, as long as the resolution is acceptable, a high flow rate will be useful to reduce operation time. So operation at the highest flow rate (given by maximum column pressure) is advisable.

*Mass load study.* A load study was performed to assess the maximum possible load that could be scaled up. Table 4 shows the effect of mass load on resolution. A logical decrease in resolution was observed at increasing mass loads. Resolutions lower than 1 for mass loads over 0.42, 1.11, and 1.39 mg with methanol/water at 90:10, 85:15, and 80:20, respectively, indicate overlapping, which reduces fraction purity. Because the aim of this research is to obtain maximum SA, EPA, and DHA yields and purities, the mass load scale-up was approximately that mentioned above for each mobile phase. Of course, it would be possible to scale-up to higher mass loads, but at the cost of resolution, yield, and purity.

*Mobile-phase composition.* Logically, resolution increased with decreasing methanol concentration, i.e., proportional to the eluent power of the mobile phase. At 80:20, the highest resolutions were obtained (Table 4) and the greatest loads could be separated.

*Ethanol/water mobile phase.* First of all, flow rates were lower than those used for methanol/water (Table 4), which is due to the higher viscosity of ethanol as well as to to the higher pressure required. The mass loads were much lower in this mobile phase, that is, the resolution at similar experimental conditions was lower than with methanol/water. This was due to the fatty acid partitioning equilibrium between the stationary phase and the mobile phase, which is displaced more toward the mobile phase with ethanol/water. Moreover, in this case, the SA, EPA, and DHA partitioning constants were low and, thus, retention times were also low, as were resolutions.

**TABLE 4**
**Effect of Mobile Phase Flow Rate (top), Mass Load and Mobile Phase Composition (middle), and Mobile Phase Type (bottom) on Resolution**

Mobile phase MeOH[a]/$H_2O$ (1%AcH) (85:15 w/w) load, 1.11 mg

| Flow rate (mL/min) | Resolution SA–EPA | Resolution EPA–DHA |
|---|---|---|
| 0.50 | 1.4 | 2.2 |
| 0.75 | 1.4 | 2.1 |
| 1.00 | 1.2 | 2.1 |
| 1.25 | 1.3 | 2.1 |

| Mobile phase composition | Flow rate (mL/min) | Load (mg) | Resolution SA–EPA | Resolution EPA–DHA |
|---|---|---|---|---|
| MeOH/$H_2O$ (90:10, w/w) | 0.75 | 0.42 | 1.2 | 1.4 |
| | | 0.74 | 0.8 | 0.7 |
| | | 1.11 | 0.8 | 0.6 |
| MeOH/$H_2O$ (85:15, w/w) | 0.75 | 1.11 | 1.5 | 2.1 |
| | | 1.39 | 0.8 | 1.0 |
| | | 1.86 | 1.1 | 0.9 |
| MeOH/$H_2O$ (80:20, w/w) | 0.60 | 1.11 | 2.0 | 2.0 |
| | | 1.39 | 1.4 | 1.7 |
| | | 2.22 | 0.9 | 1.3 |

| Mobile phase composition | Flow rate (mL/min) | Load (mg) | Resolution SA–EPA | Resolution EPA–DHA |
|---|---|---|---|---|
| MeOH/ $H_2O$ (80:20, w/w) | 0.6 | 1.11 | 2.0 | 2.0 |
| EtOH[b]/ $H_2O$ (80:20 w/w) | 0.3 | 0.42 | 0.9 | 0.6 |

[a]Methanol.
[b]Ethanol. All other abbreviations as in Table 1.

*Semi-preparative scale separation.* After determining the maximum analytical column loads, direct mathematical scale-up was calculated with the following formulae for the sample load ($m$) and flow rate ($f$) scale-up ratio:

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

$$f = D_2^2/D_1^2 \quad [3]$$

where $D_2$ is the diameter of the larger column and $L_2$ is its length; $D_1$ is the diameter of the analytical column and $L_1$ is its length.

Separations in which mobile-phase resolution was close to 1 and loads were greatest were scaled up. Yields and purities are shown in Table 5, including experimental conditions of the corresponding analytical and semi-preparative scales. The flow rates were the highest permissible, given the pressure drop in the column in each mobile phase. Figure 1 shows these chromatograms for the methanol/water mobile phase and the corresponding cut-off point for each fraction obtained with the criteria that yields should be close to 100%, with purity as high as possible and resolution close to 1. Yields with ethanol/water seem abnormally low, which can only be due to nonquantitative extraction of fatty acids from HPLC fractions.

The largest yields, purest fractions, and greatest loads were obtained with methanol/water 80:20. The most outstanding is one fraction which had an EPA yield of 99.3% and purity of 94.3%. The purities obtained are among the highest reported by several authors (6,14,22,27,30,31) (Table 6). However, the comparison is incomplete because only Tokiwa *et al.* (30)

**TABLE 5**
**Scale-Up of Polyunsaturated Fatty Acids Isolation by HPLC[a]**

| | Analytical scale | | | | Semi-preparative scale | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Resolution | | | | SA | | EPA | | DHA | |
| Mobile phase | Flow rate (mL/min) | Load (mg) | SA–EPA | EPA–DHA | Flow rate (mL/min) | Load (mg) | Purity | Yield | Purity | Yield | Purity | Yield |
| MeOH[b]/$H_2O$ (90:10) | 0.75 | 0.416 | 1.19 | 1.40 | 3.5 | 1.94 | 93.2 | 93.3 | 88.1 | 89.8 | 80.5 | 84.4 |
| MeOH/$H_2O$ (85:15) | 0.75 | 1.386 | 0.80 | 1.01 | 3.5 | 6.65 | 85.5 | 81.5 | 93.6 | 97.6 | 84.9 | 95.4 |
| MeOH/$H_2O$ (80:20) | 0.60 | 2.218 | 0.94 | 1.32 | 3.0 | 10.53 | 91.9 | 98.4 | 94.4 | 99.3 | 84.6 | 96.6 |
| EtOH[c]/$H_2O$ (80:20) | 0.30 | 0.138 | 1.11 | 1.06 | 1.5 | 0.693 | 87.3 | 87.7 | 87.4 | 88.6 | 72.2 | 81.7 |
| EtOH/$H_2O$ (75:25) | 0.30 | 0.416 | 1.74 | 0.96 | 1.5 | 1.386 | 84.5 | 78.2 | 90.3 | 79.7 | 79.6 | 70.5 |
| EtOH/$H_2O$ (70:30) | 0.40 | 0.564 | 2.05 | 0.71 | 2.0 | 2.771 | 69.7 | 76.4 | 91.3 | 85.1 | 79.5 | 100 |

[a]On the left, analytical-scale resolutions to be scaled-up. On the right, yields (%) and purities (%) for SA, EPA, and DHA fractions isolated at semipreparative scale from a concentrate of cod liver oil. Experimental conditions have been included. Abbreviations as in Tables 1 and 2.
[b]Methanol.
[c]Ethanol.

**TABLE 6**
**Comparison of Purities of Polyunsaturated Fatty Acids-Enriched Fractions Obtained by HPLC[a]**

| | Tokiwa *et al.* (30) | Wille *et al.* (6) | Perrut (31) | Grant (22) | Traitler *et al.* (27) | Cohen and Cohen (14) |
|---|---|---|---|---|---|---|
| Source | Fish oil | Fish oil | Fish oil | Fish oil | Seed oil | Microalgal lipids |
| Concentrate | Urea method | Urea method | Urea method | Urea method | Urea method | Urea method |
| Sample | Methyl esters | Fatty acids | Ethyl esters | Derivatized fatty acids | Fatty acids | Methyl esters |
| Load (g) | 10 | 90 | 136 | 30 | 2 | 0.001 |
| SA (%) | — | 7.2 | — | — | 16.6 | — |
| EPA (%) | 30 | 36.8 | 46.7 | — | — | 81.9 |
| DHA (%) | 30 | 40.9 | 30.5 | — | — | — |
| GLA (%)[b] | | | | | 80.2 | — |
| Column | $C_{18}$ reverse | $C_{18}$ reverse | $C_{18}$ reverse | $C_{18}$ reverse | $C_{18}$ reverse | $C_{18}$ reverse[c] |
| DixL (cm) | 5.7 × 30 | 20 × 60 | 30 × 30 | 10 × 60 | 5.7 × 30 (two columns) | |
| Particle size (μm) | 50–100 | 55–105 | 12–45 | 15–30 | 55–105 | |
| Mobile phase | THF[d]/MeOH/$H_2O$ | MeOH[e]/$H_2O$ | MeOH/$H_2O$ | EtOH[f]/$H_2O$ | MeOH/$H_2O$ | $CH_3CN/H_2O$ |
| Composition | (25:55:20, vol/vol/vol) | (90:10, w/w) | (90:10, w/w) | (80:20, w/w) | (90:10, w/w) | (gradient) |
| Flow (mL/min) | 150 | 1200 | 3333 | 225 | 150 | 5 |
| Separation (min) | 50 | 50 | 19 | 160 | 20 | |
| Detector | RI[g] | RI | RI | UV at 245 nm | RI | GC[h] |
| Fractions (%) | | | | | | |
| SA | — | 93.1 | — | — | 46.3 | — |
| EPA | 91.0 (66.7%)[i] | 85.6 | 91–96 | 97 | — | 97.3 |
| DHA | 85.5 (53.3%)[i] | 83.1 | 75–85 | 92 | — | — |
| GLA | | | | — | 95.4 | — |

[a]Abbreviations as in Tables 1 and 2. References in parentheses.
[b]γ-Linolenic acid.
[c]Elution at atmospheric pressure.
[d]Tetrahydrofuran.
[e]Methanol.
[f]Ethanol.
[g]Refractive index.
[h]Gas chromatography.
[i]Values in parentheses corresponding to fatty acids yields for HPLC separation.

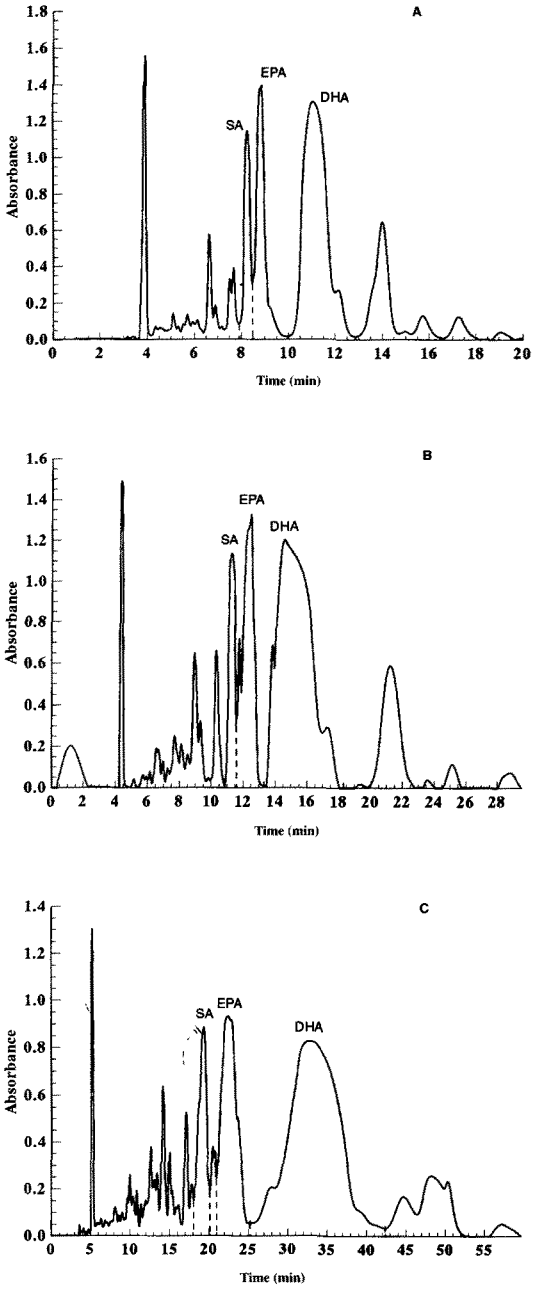


**FIG. 1.** Separation of stearidonic acid (SA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) from cod liver oil. Semi-preparative column (1 x 25 cm, 5 µm, reverse phase $C_{18}$). Mobile phase: MeOH/water (1% AcH): A, 90:10 w/w. Flow rate 3.5 mL/min. Mass load 1.94 mg of polyunsaturated fatty acids (PUFAs). B, 85:15 w/w, 3.5 mL/min, 6.65 mg. C, 80:20 w/w, 3 mL/min, 10.53 mg. Purities and yields are shown in Table 5.

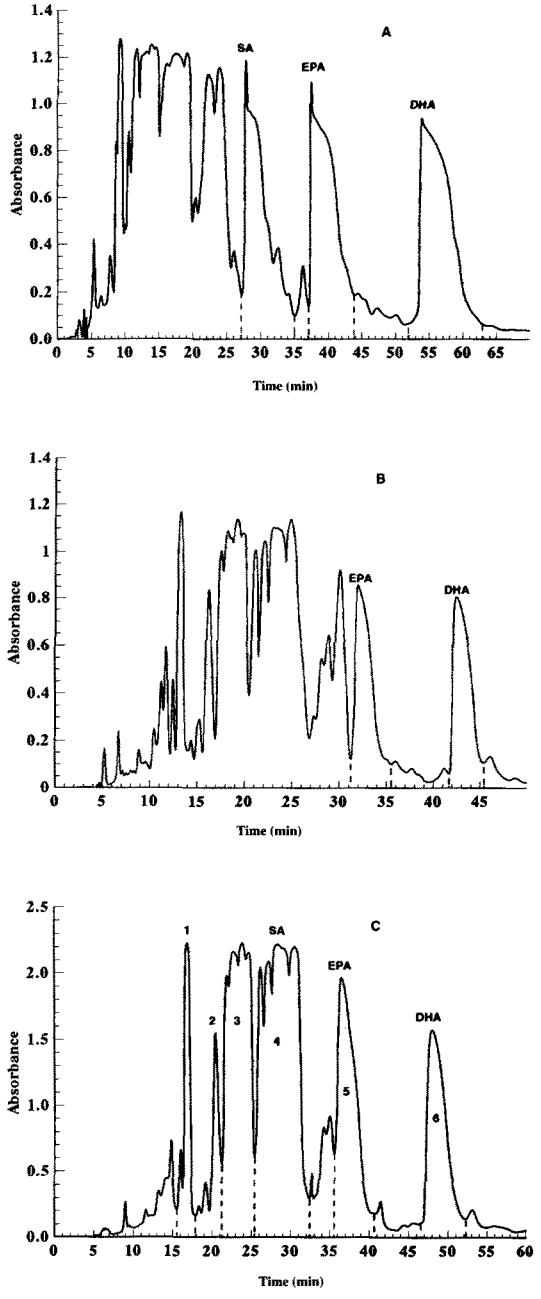


**FIG. 2.** Separation of SA, EPA, and DHA from the marine microalga *Isochrysis galbana* biomass. Semi-preparative column (1 x 25 cm, 5 µm, reverse-phase $C_{18}$). A, MeOH/water (1% AcH) 80:20 w/w, flow rate 3.0 mL/min, mass load 9.49 mg of PUFA B EtOH/water (1% AcH) 70:30 w/w, 2.0 mL/min, 2.77 mg. C, EtOH/water (1%AcH) 70:30 w/w, 1.5 mL/min, 2.77 mg. Purities and yields are shown in Table 7. Ultraviolet spectrum of the enriched fraction on C is compared with those of sigma patterns in Figure 3. The difference between SA, EPA, and DHA retention time from cod liver oil and *I. galbana* biomass under identical chromatographic conditions (Figs. 1C and 2A) is due to the use of a precolumn (4.6 x 45 mm) to protect the semi-preparative column. Abbreviations as in Figure 1.

gave fatty acid yields for HPLC separations, and these were low. From their chromatograms, yields obtained by the rest of authors cited in Table 6 might be expected to be even lower. Unfortunately, there are few publications that report the yield as well as the purity, making a comparative study difficult. A low yield is not important if the concentrate is cheap, as is the case with fish oil PUFA concentrates, but the situation is different when the source material is expensive.

**TABLE 7**
**Yields and Purities for SA, EPA, and DHA Fractions Isolated at Semi-Preparative Scale from a Concentrate of *Isochrysis galbana* Fatty Acids[a]**

| | | | SA | | EPA | | DHA | |
|---|---|---|---|---|---|---|---|---|
| Mobile phase | Flow rate (mL/min) | Load (mg) | Purity | Yield | Purity | Yield | Purity | Yield |
| MeOH/$H_2O$ (80:20) | 3.0 | 9.49 | 94.8 | 100.0 | 96.0 | 99.6 | 94.3 | 94.0 |
| EtOH/$H_2O$ (70:30) | 2.0 | 2.77 | — | — | 90.8 | 80.0 | 90.7 | 84.0 |
| EtOH/$H_2O$ (70:30) | 1.5 | 2.77 | 88.7 | 76.3 | 92.0 | 84.0 | 92.0 | 87.7 |

[a]Experimental conditions have been included. Abbreviations as in Tables 1 and 2.

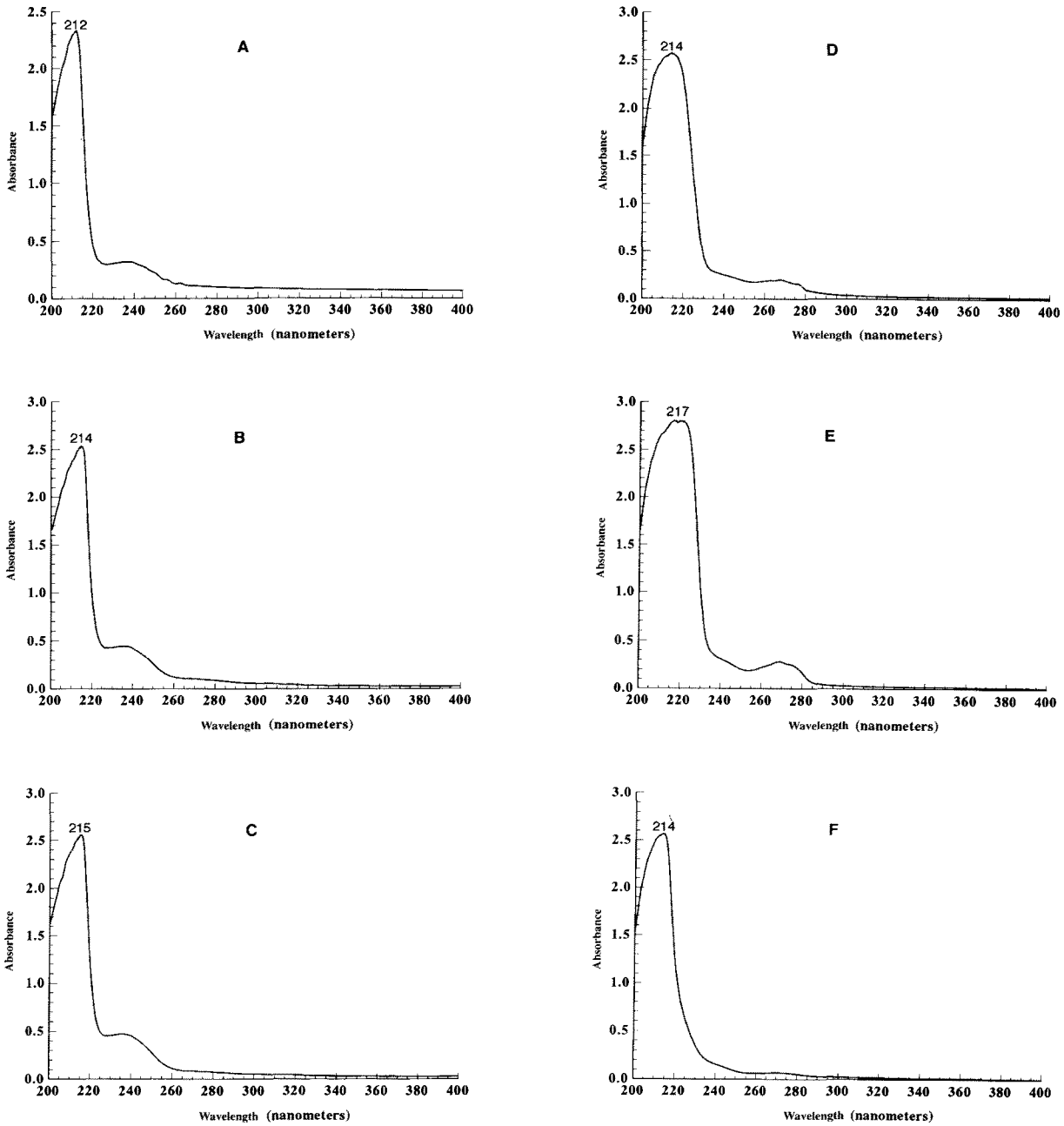


**FIG. 3.** Comparison between ultraviolet spectrum of sigma standards and the enriched fractions of stearidonic acid (SA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as shown in Figure 2C. Sigma patterns: A, SA; B, EPA; C, DHA: Fractions: D, SA; E, EPA; F, DHA.

## SA, EPA, AND DHA CHROMATOGRAPHIC ISOLATION FROM PUFA CONCENTRATE OF THE MARINE MICROALGA *I. GALBANA*

These separations were carried out with mobile phases of lower eluent capacity (methanol/water, 80:20, and ethanol/water, 70:30) to facilitate the separation of the greater amounts of PUFA in highly pure fractions with large yields. Table 7 shows the results obtained and Figure 2, the chromatograms. The chromatograms show a large number of compounds at short retention times. However, the GC analysis of these concentrates does not reveal any fatty acids other than those found in cod

liver oil concentrates. Therefore, other components, mainly pigments, are strongly absorbent at the wavelength used (32). The UV spectrum of fractions obtained were, in almost all cases, like the patterns (Fig. 3), which shows that during the purification process there was no degradation of the fatty acids.

Table 7 shows the good results obtained with methanol/water (80:20), producing an almost 100% yield of 96% pure EPA. However, purities and yields obtained with ethanol/water (70:30) were slightly lower. Nonetheless, in general, yields and purities obtained from microalga biomass were higher than those from cod liver oil (compare Table 5 and Table 7).

Table 2 summarizes fatty acid composition of the fractions obtained from the semi-preparative separations, as well as the composition of the initial material (biomass, extract and urea concentrate). It is difficult to compare these results with those found in the literature because there is little published on PUFA purification from microalga biomass. Cohen and Cohen (14) (Table 6), obtained EPA from *Porphiryridium cruentum*, although their fractionation was done on a chromatographic column at normal pressure and the mass load was only 1 mg, which is low compared to ours.

Table 2 summarizes fatty acid yields in the extraction (23), urea concentration, and HPLC fractionation steps, as well as the overall yield in n-3 PUFA. The low overall EPA yield is due to the low urea concentration EPA yield. This urea concentrate was obtained at 4°C, and the optimum crystallization temperature for EPA is 20–28°C (see Table 3).

These are the first experiments with lipids from *I. galbana* (results shown in Table 2), and major research is still necessary. By using Equations 2 and 3 for the scale-up ratio of sample mass and flow rate, a commercial preparative column with the same packing material as the semi-preparative column used in this study would render several grams of SA, EPA, and DHA daily. Such productivities are required to produce the large amounts of highly purified PUFA required for clinical and other studies.

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