Comparison Between Extraction of Lipids and Fatty Acids from Microalgal Biomass

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Seven solvent mixtures have been used to extract the lipid fraction of lyophilized biomass of *Isochrysis galbana*. Six of them were composed of biocompatible solvents. Each method was carried out under relaxed operating conditions (i.e., one hour at room temperature) with extraction in a nitrogen atmosphere to prevent autooxidation and degradation of polyunsaturated fatty acids (PUFAs). Apart from the well-established Bligh and Dyer method [*Can. J. Biochem. Physiol. 37*:911 (1959)] ($Cl_3CH/MeOH/H_2O$, 1:2:0.8, vol/vol/vol), which rendered the highest yield of lipids (93.8%), ethanol (96%) and hexane/ethanol (96%), 1:2.5 vol/vol produced the best results (84.4 and 79.6%, respectively). To obtain free fatty acids, KOH was added to the solvent mixtures used to extract the total lipids, except for $Cl_3CH/MeOH/H_2O$, and direct saponification was carried out at 60°C for 1 h or at room temperature for 8 h. The highest yields obtained by direct saponification were 81% with hexane/ethanol (96%), 1:2.5, vol/vol and 79.8% with ethanol (96%). Partial yields of the main *n*-3 PUFAs found in *I. galbana*, stearidonic acid (SA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were calculated for both extraction methods. For lipid extraction with ethanol (96%), yields of 91, 82 and 83% were obtained for SA, EPA and DHA, respectively. When direct saponification was used, hexane/ethanol (96%; 1:2.5, vol/vol) produced the best yields of (91, 79 and 69% for SA, EPA and DHA, respectively).

KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, extraction, lipids, microalgae, polyunsaturated fatty acids.

Polyunsaturated fatty acids (PUFAs) have attracted increased attention due to their pharmaceutical properties. *Isochrysis galbana* is a marine microalga from which an oil rich in PUFAs can be obtained (1). However, pharmaceutical and clinical applications require higher concentrations than those normally produced by microalgae lipids.

Rapid and reliable methods of extraction and purification of PUFAs from microalgal biomass are required for the further development in this area of microalgal biotechnology. At the same time, relaxed treatment must be used to minimize autoxidative degradation and the presence of artifacts. On the other hand, if PUFAs are to be used in baby food or in pharmacological applications, solvents should be selected that are acceptable in terms of toxicity, handling, safety and cost.

Several already commonly used methods have been considered for the extraction of lipids from microalgae. The best was the recovery from algae in nitrogen and continuous agitation in the monophasic Bligh and Dyer solvent system (2) ($Cl_3CH/MeOH/H_2O$, 1:2:0:8, vol/vol/vol). The resulting homogenate could then be diluted with water and/or chloroform to produce a biphasic system, where the chloroform layer would contain the lipids and the methanol/water layer the nonlipids. In this way, a purified lipid extract would be obtained when the chloroform layer is isolated. However, chloroform is a toxic solvent prohibited in the food industry, and biocompatible solvents must be used. Other promising solvents for lipid extraction from microalgal biomass, such as *n*-butanol, ethanol and hexane/isopropanol, were used by Nagel and Lemke (3) due to their low carcinogenicity, although the aim of these works was the production of methyl ester fuel.

Although this lipid extract is valuable in itself, there is a demand for free fatty acids or fatty acids bound as triglycerides for clinical analysis, making saponification of lipid extracts necessary. Direct saponification during extraction would be a faster method. The resulting homogenate could then be diluted with one of the solvents to produce two phases, separating fatty acids from the polar layer. Literature regarding extraction of lipids from microalgae is rather limited and, as far as we known, no yields have been reported, nor have comparisons among different systems been done. This paper presents the results of a comparative study of lipid and fatty acid extraction from the lyophilized biomass of *I. galbana* with seven solvent mixtures.

EXPERIMENTAL PROCEDURES

Microalgal biomass. Lyophilized biomass of the marine microalga *I. galbana* was used as an oil-rich substrate that contained a high proportion of PUFAs (1,4). Its fatty acid profile is given in Table 1, and Table 2 shows the composition of neutral and polar lipids. Cells were grown in laboratory cultures, harvested by centrifugation and then lyophilized and stored at −18°C until used.

Lipid extraction. Seven solvent mixtures have been used to extract the lipid fraction (Table 1). With systems A, B and C, extraction was carried out by following the method of Kates (5), based on the Bligh and Dyer (2) procedure with microorganism biomass. In this way, crude lipids are initially extracted in a monophasic system from which a two-phase system (one phase, the extract, with a high lipid content and the other, the raffinate, with a low lipid content) later separates and purifies the lipids. The procedure for systems D, E, F and G does not include the final purification step.

In each experiment, 5 g of lyophilized biomass were treated with 380 mL of the corresponding extraction system in a 2.5-L reactor with an outer sleeve that permits the circulation of water for temperature control. Extraction at room temperature was carried out for 1 h in nitrogen atmosphere with constant agitation. The mixture obtained was then filtered through glass filters (100–160 μm bore). The residue was washed with 190 mL of the extraction system, and this filtrate was added to the first one.

System A ($Cl_3CH/MeOH/H_2O$, 1:2:0.8, vol/vol/vol, monophasic) was made biphasic by the addition of 150 mL Cl_3CH and 150 mL water ($Cl_3CH/MeOH/H_2O$, 1:1:0.9, vol/vol/vol, biphasic), giving rise to an upper hydromethanolic layer (raffinate) and a lower layer of chloroform (extract) in which the lipids were dissolved.

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TABLE 1

Fatty Acid Profiles of Extracts and Raffinates (in parentheses) Obtained by Lipid Extraction from *Isochrysis galbana* (% of total fatty acids)

Fatty acid	Biomass	Solvent mixtures[a]						
		A	B	C	D	E	F	G
14:0	10.1	10.1(18.1)	6.9(16.5)	6.4(6.5)	9.8	9.9	10.8	8.5
16:0	20.3	18.3(25.1)	20.2(23.2)	19.2(13.1)	21.4	20.3	18.8	19.7
16:1n7	21.4	19.7(19.5)	25.7(16.3)	27.0(20.7)	22.3	23.2	26.2	25.0
18:1n-7,9	5.0	5.4(4.5)	5.3(4.7)	4.4(4.1)	4.6	4.0	3.6	4.0
18:4n-3	6.4	7.5(8.3)	5.7(7.2)	6.5(8.9)	6.5	7.0	8.3	7.2
20:5n-3	22.6	24.8(16.7)	21.4(20.5)	22.3(29.9)	22.1	22.0	23.4	22.9
22:6n-3	8.4	8.4(4.8)	8.2(5.2)	8.5(10.0)	7.5	8.3	4.3	7.0
Total others	7.2	7.4(4.5)	8.0(6.9)	7.1(7.8)	6.0	6.3	5.7	5.7
Total saturated	31.2	29.2(44.5)	28.7(41.4)	26.6(21.2)	32.0	31.1	30.6	29.4
Total monounsaturated	26.6	25.4(24.2)	31.1(21.0)	31.6(25.4)	28.6	27.5	30.5	29.0
Total n-3 PUFAs[b]	38.8	42.1(31.0)	36.8(34.2)	38.6(50.3)	36.5	38.6	37.1	38.8

[a]A, Cl_3CH/MeOH/H_2O (1:2:0.8, vol/vol/vol); B, hexane/EtOH (96%) (1:2.5, vol/vol); C, hexane/EtOH (96%) (1:0.9, vol/vol); D, butanol; E, EtOH (96%); F, EtOH (96%)/H_2O (1:1, vol/vol); G, hexane/isopropanol (1:1.5, vol/vol).
[b]PUFAs, polyunsaturated fatty acids.

TABLE 2

Fatty-Acid Composition of Neutral and Polar Lipids of *Isochrysis galbana* Biomass (%)

Fatty acid	Neutral lipids	Polar lipids
14:0	1.5	5.2
16:0	5.7	15.0
16:1n-7	5.5	14.8
18:1n-7,9	1.5	5.4
18:4n-3	1.4	4.6
20:5n-3	5.7	14.2
22:6n-3	1.8	4.7
Total others	2.9	8.3
Total fatty acids	26.0	72.2
Total saturated	8.1	22.7
Total monounsaturated	6.9	21.0
Total n-3 PUFAs[a]	9.4	25.0

[a]See Table 1 for abbreviation.

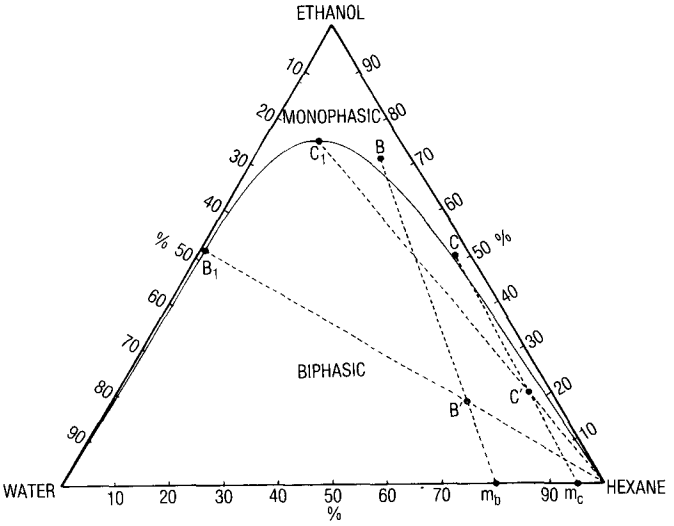

FIG. 1. Hexane/ethanol/water phase diagram (% w/w).

Systems B and C are represented in the hexane/ethanol/water phase diagram (Fig. 1), based on equilibrium data for this system from Bonner (6) (expressed as percentages of dry weight). The composition of system B was modified to obtain two phases by addition of 1480 mL hexane and 250 mL water (point M_B), the new proportion being located at point B′ (Fig. 1). Thus, the lower layer, hydroethanolic (approximately 575 mL, point B_1), and the upper layer, hexane (approximately 1650 mL), could be separated. For system C, 885 mL hexane and 30 mL water were added (point M_C, Fig. 1), giving rise to a heavier phase (ethanol/water, point C_1) of 276 mL and a lighter phase of 1180 mL hexane.

The remaining lipids in the hydroalcoholic phases, as well as lipids extracted with systems D, E, F and G, were determinated by extracting five times with 100 mL chloroform. The total volume was finally reduced to 100 mL in a vacuum evaporator at 30°C. Fatty acid content was always quantified by gas chromatography.

Calculation of lipid extraction yield. Yield is commonly determined by the ratio between extracted lipids with a given method and those extracted with a control method (3). In this study, interest is mainly focused on saponifiable lipids instead of total lipids, and the former are expected to be in a direct relationship with fatty acid content. Consequently, yield of saponificable lipid extraction might be obtained by:

$$R = (A_L/A_B)\ 100 \quad [1]$$

where A_L is the total fatty acid content of the extracted lipids and A_B is the total fatty acid content of the initial biomass, which was 9.5% of dry weight. The yield of each fatty acid (R_F) may be calculated by considering its proportion in the initial biomass (X_{FB}) and its corresponding proportion in the lipid extract (X_{FL}):

$$R_F = (A_{FL}/A_{FB})\ 100 = X_{FL}\ A_L/X_{FB}\ A_B = (X_{FL}/X_{FB})\ R \quad [2]$$

where A_{FL} and A_{FB} are the content of the fatty acid in the extracted lipids and in the biomass, respectively. X_{FL} and X_{FB} values are shown as percentages in Table 1. Finally, R is the abovementioned yield of total lipids.

Fatty acid extraction. KOH (8 g) was added to 380 mL of each extraction mixture for the lipid extraction and

simultaneously for the fatty acid saponification. The different systems used are shown in Table 3. In each experiment, 5 g of lyophilized biomass was treated with 380 mL of the corresponding extraction/saponification system, freshly prepared in the abovementioned reactor. Extraction/saponification at room temperature was carried out for 8 h or at 60°C for 1 h, with constant agitation in a nitrogen atmosphere. After saponification, 100 mL water was added (except for *n*-butanol; because of its low solubility in water, 9 g/100 g, 3400 mL water had to be added). Unsaponifiables were separated by five extractions with 200 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 200 mL hexane, which ensured high depletion of fatty acids.

Yield calculation of fatty acid extraction. Yield was calculated by an expression analogous to Equation 1, in which A_L now represents the content of all fatty acid obtained from the biomass as free fatty acids, and A_B has the same meaning as in Equation 1. In the same way, yield of each fatty acid may be calculated by Equation 2, where A_{FL} is now the content of the fatty acid in the total free fatty acid solution, and X_{FL} is the proportion of this fatty acid in the free fatty acid solution (values of which are shown in Table 4).

Fatty acid determination. Fatty acids were determined by gas chromatography. Methylation was done by direct transesterification with acetyl chloride/methanol (1:20) by following the method of Lepage and Roy (7), which is an acid hydrolysis (8). The analysis of methyl esters was carried out by gas chromatography in a 30-m capillary column of fused silica (SP2330; Supelco, Bellefonte, PA), 0.25 mm i.d., 0.20 μm standard film, split ratio 100:1, and a flame-ionization detector. Sigma Lipid Standard 189-15 (Sigma Chemical Co., St. Louis, MO), Supelco rapeseed oil mixture and Supelco PUFA-1 and PUFA-2 patterns were used for the determination. Nonadecanoic acid was used as an internal standard to quantitate fatty acid content in biomass dry weight.

The following fatty acids were recognized and taken into account for yield calculations although only the main fatty acids will be considered in the discussion that follows: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, 18:3n-6, 18:3n-3, 18:4n-3 (stearidonic acid, SA), 20:0, 20:1n-9, 20:3n-6, 20:4n-6, 20:5n-3 (eicosapentaenoic acid, EPA), 22:0, 22:1n-1, 22:1n-9, 22:4n-6, 22:5n-3, 22:5n-3, 22:6n-3 (docosahexaenoic acid, DHA) and 24:0.

Lipid fractionation. Lipids were fractionated in a chromatography column with 250–400-mesh silica gel and eluted with chloroform, acetone and methanol at 10, 40 and 10 times the bed volume of each solvent, respectively (5). The volume of each solvent was then collected and analyzed by gas chromatography, as indicated previously. The described experiments were at least carried out twice, the yields being mean values with deviations below 3%.

RESULTS AND DISCUSSION

Lipid extraction. Fatty acid profiles of *I. galbana* obtained by direct transesterification of the biomass and those obtained with the seven extracting solvent mixtures are

TABLE 3

Yields (%) of Extracts and Raffinates (in parentheses) Obtained by Lipid Extraction from *Isochrysis galbana*

Fatty acid	Solvent mixtures[a]						
	A	B	C	D	E	F	G
18:4n-3	108.9(1.2)	46.5(30.8)	49.0(11.6)	70.7	91.3	82.0	74.1
20:5n-3	101.4(0.7)	49.4(24.8)	53.2(11.0)	67.3	82.2	65.7	67.0
22:6n-3	92.9(0.5)	51.0(17.4)	59.6(9.9)	60.8	83.0	32.3	55.1
Total n-3 PUFAs[b]	100.0(0.7)	49.5(24.2)	49.2(10.8)	66.2	84.0	60.5	66.0
Total fatty acids	92.9(0.9)	52.2(27.4)	49.5(8.3)	70.4	84.4	63.3	66.0

[a]As in Table 1.
[b]See Table 1 for abbreviation.

TABLE 4

Fatty Acid Profiles of Extracts Obtained by Direct Saponification from *Isochrysis galbana* (% of total fatty acids)

Fatty acid	Biomass	Saponification systems[a]										
		B^1	B^2	C^1	C^2	D^1	E^1	E^2	F^1	F^2	G^1	G^2
14:0	10.1	10.7	10.4	9.2	13.1	11.9	8.5	11.0	12.1	12.3	12.1	12.1
16:0	20.3	21.0	16.7	17.2	16.5	15.1	20.3	21.4	16.9	15.6	17.7	13.7
16:1n-7	21.4	21.9	23.9	26.3	20.4	18.0	21.4	22.2	22.9	22.3	21.7	24.7
18:1n-7,9	5.0	4.8	3.1	3.1	5.0	7.8	5.7	5.4	3.9	4.0	4.3	2.5
18:4n-3	6.4	6.5	7.6	8.0	9.1	12.1	6.5	6.2	7.9	8.2	7.8	9.1
20:5n-3	22.6	22.3	23.5	22.1	22.0	18.8	22.7	20.8	22.5	22.2	20.9	24.6
22:6n-3	8.4	7.5	8.0	6.9	5.9	4.9	7.8	7.3	7.0	6.9	7.0	6.8
Total others	5.8	5.3	6.8	4.7	8.0	11.4	7.1	5.7	6.8	8.5	8.5	6.5
Total saturated	31.2	32.7	27.9	27.6	32.6	29.5	30.9	33.3	30.2	29.4	32.2	26.7
Total monounsaturated	26.6	26.7	27.0	29.6	25.9	29.3	27.8	28.1	27.0	26.6	26.4	26.6
Total n-3 PUFAs	38.8	38.0	40.7	38.3	38.8	36.7	38.4	35.6	38.7	40.6	38.1	42.0

[a]Superscript 1 denotes 1 h at 60°C. Superscript 2 denotes 8 h at room temperature. Systems (B–G) and abbreviation as in Table 1.

shown in Table 1. Although, in general, all fatty acid profiles of the extracts are analogous to that of the initial biomass, it is interesting to note the slight variations found among them. This must be borne in mind when comparing microalgal fatty acid profiles from different laboratories, as the extraction method affects the results.

Table 1 shows that for systems A, B and C, fatty acid profiles of the raffinates are different from that of the biomass, although a general trend is not observed. Overall lipid extraction yields with the seven solvent mixtures tested are shown in Table 2. The highest yield (93.8%, extract plus raffinate) was obtained with Cl_3CH/MeOH/H_2O (1:2:0.8, vol/vol/vol), followed by 84.4% with ethanol (96%) and 79.6% (extract plus raffinate) with hexane/ethanol (96%; 1:2.5, vol/vol).

Ethanol and hexane, systems B and C, are biocompatible solvents commonly used in the food industry, thereby avoiding the problem of toxicity due to the chloroform and methanol of system A (9,10). In spite of the high yield attainable, the solvent mixtures used by Folch (11), Bligh and Dyer (2) and Kochert (12) are unadvisable when the final product is to be used in the food or pharmaceutical industries. System B is less advantageous than system A because of the high proportion of lipids remaining in its raffinate (27.4%; Table 3). In system C, this proportion is lower (8.3%; Table 3), although the overall yield of lipids extracted is also lower (57.8%).

The steps undergone in systems B and C are schematized in Figure 1. Partitioning (point B′) of lipids between hexane and ethanol/water phases (point B_1) was unfavorable (52.2:27.4; Table 3) in system B, which initially produced a high yield of lipid extraction (79.6%; Table 3). On the other hand, yield of lipid extracted with system C is low (57.8%), although partitioning (point C′) is more favorable (49.5:8.3; Table 3). This would seem to point out that lipid extraction may be carried out with a system such as B, or with even greater ethanol content [bearing in mind the yield of 84.4% reached with the ethanol (96%) extracting system] and followed by formation of a biphasic system similar to C′, that is, with a high proportion of hexane to enhance lipid partitioning in the extract.

More research should be aimed at lowering solvent volumes, although in the present study volumes equivalent to those used by Kates (5) with system A have been used. Furthermore, improvement in the coefficient of partition, by adding electrolytes or varying the temperature and period of extraction, could all contribute to higher yields.

The decreasing order of yields, i.e., systems E, B, D, G, F and C, shows how the alcohol content of the solvent mixture also decreases, with the exception of *n*-butanol. It thus seems that the main factor in lipid extraction is the alcohol content. Furthermore, the higher the polarity, the higher the yield (*n*-butanol has low polarity). This may account for the high yield of system A, as methanol has a slightly higher polarity than ethanol, as does chloroform over hexane. These results are in agreement with the fractionation of the lipid extract from *I. galbana* into neutral lipids (26.0%) and polar lipids (72.2%) (Table 2), as polar solvents better extract the polar lipids that form the higher proportion of total lipids. This argument is in accordance with the low yield reported by Ahlgren and Merino (9) in the extraction of lipids from *Spirulina* with hexane/isopropanol (1:0.7), due to the low polarity of the solvent mixture that was therefore not recommended for lipid extraction.

Yields of SA, EPA, DHA and n-3 PUFAs (also given in Table 3) show that the hypothetical maximum yield would be obtained in two steps: first the extraction of lipids and then free fatty acid recovery by acid hydrolysis of the lipid extract [Lepage and Roy method (7)]. Yields of SA are higher than those of total lipids; for EPA, yields are similar and for DHA somewhat lower, demonstrating that those lipids allocated in membrane structures (polar lipids) are more difficult to extract than those found in storage lipid droplets (neutral lipids): DHA is mainly found in the polar fraction of the total lipids (Table 2).

Fatty acid extraction by direct saponification. Direct saponification of the microalgal biomass enables fatty acids to be obtained as potassium salts instead of as crude lipids in a first step. This could reduce cost and operating time as compared to lipid extraction and further saponification.

Fatty acid profiles and overall yields, obtained with each solvent mixture and operating condition tested, are shown in Tables 4 and 5, respectively. All profiles were similar to those presented in Table 1, even under the different saponification conditions. Operating conditions did not affect yields; however, those of fatty acid extraction were somewhat lower than for lipid extraction. Hexane/ethanol (1:2.5, vol/vol) at 60°C for 1 h produced the highest yield (81%), close to that obtained with ethanol (96%) regardless of temperature or duration of extraction (≈80%). Decreasing yields were obtained with hexane/isopropanol (1:1.5, vol/vol) (≈60%), followed by hexane/ethanol (96%); 1:0.9, vol/vol) and ethanol (96%)/water (1:1, vol/vol), yields of which were below 50%. This is the same order as that discussed for lipid extraction, with the exception of *n*-butanol, which will be considered later.

TABLE 5

Yields (%) of Extracts Obtained by Direct Saponification from *Isochrysis galbana*

Fatty acid	Saponification systems[a]										
	B^1	B^2	C^1	C^2	D^1	E^1	E^2	F^1	F^2	G^1	G^2
18:4n-3	82.3	91.4	60.0	65.5	17.4	81.0	77.3	55.9	57.6	94.9	90.7
20:5n-3	79.9	79.0	48.2	46.1	7.5	80.2	72.9	45.1	44.5	63.9	66.5
22:6n-3	72.3	68.8	40.0	33.1	6.1	74.1	68.8	37.6	37.4	46.5	48.0
Total n-3 PUFAs[b]	79.3	78.7	47.4	47.1	8.5	79.0	72.7	46.4	47.6	60.9	66.1
Total fatty acids	81.0	75.0	48.0	47.1	9.0	79.8	79.2	46.5	45.5	62.0	61.1

[a]As in Table 4.
[b]See Table 1 for abbreviation.

From an overall view of these results, three facts arise: (i) fatty acid profiles are similar in both methods (lipid or fatty acid extraction); (ii) an analogous order of efficiency is found when comparing yields of the different solvent mixtures; and (iii) for each solvent system, the yield of lipid extraction is higher than that of fatty acid extraction. It may thus be assumed that direct saponification of biomass could ideally be induced in two stages, the first being a lipid extraction by solvent, and the second being alkaline hydrolysis of the extracted lipids rendering fatty acid salts. With this scheme, the limiting step would be the lipid extraction, which is supported by the fact that 1 h of lipid extraction at room temperature is enough to reach somewhat higher yields than at 60°C for 1 h or 8 h at room temperature when saponification takes place. Thus, the yields obtained from fatty acid extraction will depend on the suitability of the extraction solvent. Therefore, although all those considerations for lipid extraction are appropriate in this case, it must be borne in mind that the last step of the total procedure is the recovery of free fatty acids, which may lower the overall yield (see systems C and F). Similar comments were reported by Gellerman and Schlenk (13). The extremely low yield obtained with *n*-butanol/KOH (9%) is due to following the same protocol for each solvent mixture tested. In this case, a large amount of water was necessary to obtain the hydrobutanolic solution (see Experimental Procedures section) and later extraction of free fatty acids with the same volume of hexane as in the other solvent systems.

With regard to fatty acid obtention, when direct saponification is carried out, system B is adventageous in comparison with lipid extraction and later saponification, as it avoids the formation of a hydroethanolic phase with a high proportion of unpurified lipids (27.4%; see Table 3). Furthermore, by direct saponification, purified fatty acids are initially found in a hydroalcoholic phase from which extraction with hexane is enhanced.

Partial yields of the main n-3 PUFAs found in *I. galbana* SA, EPA and DHA are given in Table 5. Hexane/ethanol (96%, 1:2.5, vol/vol) and ethanol (96%) produced the best yields for SA, EPA and DHA, regardless of the operating conditions.

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