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The Production of Polyunsaturated Fatty Acids by Microalgae: from Strain Selection to Product Purification

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A selection programme to increase the cellular eicosapentaenoic acid (EPA) content has been carried out with the microalga Isochrysis galbana. *The selection process involved two stages of single selection. EPA content continuously increased from 2·4% dry weight (d.w.) of the 'parent' culture to an average value of 5·3% d.w. in the final stage. The proportion of total EPA variation attributable to the genetic variation (heritability in a broad sense) was 0·99 showing the importance of the genome in the determination of this fatty acid. The growth and fatty acid profile of an EPA-rich isolate grown as a chemostat in a cylindrical photobioreactor have been studied. A decrease in EPA content was observed (5·21% w/w to 2·8% w/w) at the lowest dilution rate $D = 0{\cdot}024\ h^{-1}$, up close to the maximum growth rate, $D = 0{\cdot}038\ h^{-1}$. At the same time, the biomass concentration also decreased from 1015 mg/litre to 202 mg/litre over the above-mentioned range of dilution rate (D). Nonetheless, the EPA productivity increases with D, with a maximum of 15·26 mg/litre/day at $D = 0{\cdot}0208\ h^{-1}$. Furthermore, steady-state dilution rates may be related to average internal light intensity. Reverse-phase, high-pressure liquid chromatography (HPLC) on octadecylsilyl semi-preparative columns was used to separate stearidonic acid (SA), EPA and docosohexaenoic acid (DHA) in polyunsaturated fatty acid concentrate obtained by the urea complexation method from a fatty acid solution previously obtained by direct saponification of biomass. Isolate SA, EPA and DHA fraction purity was 94·8, 96·0 and 94·9%, respectively, with yields of 100·0, 99·6 and 94·0%.*

INTRODUCTION

Microalgae are considered a potential source of a wide spectrum of chemicals, including such highly valuable products as phycobiliproteins, carotenoids, antioxidants and long-chain polyunsaturated fatty acids (PUFAs). In recent years, several companies have been developing commercial applications of these products.[1-4]

The production of lipids from microalgae produced in photobioreactors, fermenters or by pond-farming, depending on the value of the

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product, is expected to develop rapidly.[5] Among these lipids, n-3 PUFAs in particular are being studied because of their role in human health, eicosapentaenoic acid being considered of immense therapeutic value.[6] Yet, to achieve economic viability of EPA production with microalgae, an increase in EPA content is necessary and for this, genetic methods must ultimately be employed.[7,8] Beyond species/strain choice, factors affecting the growth rate and biochemical composition of the cell must be borne in mind, especially environmental and operating conditions.[9]

Another area of interest to microalgal culture is growth modelling, which is useful in the prediction of system behaviour under different conditions.[10,11] Algal biomass production is generally limited by light and therefore, for the study of its kinetics, it has been found necessary to model light distribution inside the culture.[12] One parameter exerting a notable influence on the accurate calculation of average light intensity is the biomass absorption coefficient, which originates in Lambert–Beer's law. Its estimation depends not only on chlorophyll *a* content,[13,14] but also on other pigments and light-absorbing molecules[15] and must therefore be precisely determined.

In this context, the marine microalga *Isochrysis galbana* has been the subject of a phenotypic selection programme for an EPA-rich strain, and growth conditions enhancing EPA content were studied. Furthermore, the urea complexation method was used to concentrate PUFAs in biomass fatty acids followed by SA, EPA and DHA purification by HPLC. An overview of these different areas is presented here.

MATERIALS AND METHODS

Selection process

Phenotypic selection to improve the EPA content in *I. galbana* was carried out using a 'parent' culture supplied by Dr J. Fábregas of the University of Santiago de Compostela, Spain. Fifty-nine isolates, called generation I, were successfully produced from single cells isolated with a micropipette under the microscope. The isolate richest in EPA was selected to produce a new set of 42 isolates called generation II. All isolates were cultured under identical conditions in spherical 1000-ml flasks. Cultures were harvested during the stationary phase of growth for subsequent analysis.[16,17] Quantitative genetic analysis of fatty acid variation was carried out on collection strains of *I. galbana* and *Phaeodactylum tricornutum* following the methodology of Caten & Jinks[18] and Wood *et al.*[19]

Growth conditions

An isolate (labelled II-4) selected from the second set of isolates was grown in a chemostat culture in a 5-litre computer-controlled fermenter (New Brunswick scientific Bioflo III).

The culture medium and sterilization processes have been described previously.[20] The cultures were constantly illuminated with four Osram Dulux EL (20 W) fluorescent lamps arranged around the culture vessel. Incident light intensity was measured by a laboratory quantum scalar irradiance meter QSL-100 (Biospherical Instrument Inc., San Diego, CA). All growth experiments were carried out at a temperature of 20°C, CO_2 was injection-controlled at pH = 8·00 and the air supply was sterilized by filtration through 0·22 μm millipore filters at a rate of 1·5 litre min^{-1}. Agitation was set at 150 rpm, equivalent to an agitator Reynolds number of 9750.

Biomass absorption coefficient (K_a)

K_a was determined by measuring the absorbance of six *I. galbana* cultures with different pigment content and 12 biomass concentrations between 50 and 1100 mg $litre^{-1}$. The intensity of light coming from all directions in the centre of a (4·9-cm diameter) cylindrical vessel was measured with QSL-100 for each biomass concentration assayed. Absorbance was calculated by Lambert–Beer's law. The absorption coefficients were calculated by dividing the slopes of the straight lines of absorbance vs biomass concentration by the vessel radius.

Analytical methods

Lipids were extracted by the Kochert method.[21] Fatty acid methylation was done by direct transesterification following the method of Lepage & Roy.[22] The analysis of methyl esters was carried out by gas chromatography using a 30-m capillary column of fused silica (SP2330, Supelco, Bellefonte, PA, USA), with an internal diameter of 0·25 mm, 0·20 μm standard film, split ratio of 100:1, and a flame ionization detector. SIGMA Lipid Standard 189-15, Supelco Rapeseed oil mixture and Supelco PUFA-1 patterns were used

for the determination. Nonadecanoic acid was used as an internal standard to quantify fatty acid content in biomass. Proteins were assayed as described by Lowry *et al.*[23]

SA, EPA and DHA were isolated by the following method: total fatty acid was obtained by direct saponification of 5-g samples of lyophilized biomass at 60°C for 1 h, in a hexane–ethanol (96%) 2:5 v/v system with 2·1% w/v KOH. The fatty solution thus obtained was concentrated by the urea fractionation method[24] with 375 g litre^{-1} of methanol at a urea–fatty acid ratio of 4:1. The fractionation of the PUFA solution concentrate was carried out by HPLC using a reverse phase, C18, 10 mm × 250 mm, 5 μm, 80 Å column, methanol–water (acetic acid) 80:20 (1%) w/w as eluent, at a 3 ml min^{-1} flow in the mobile phase and loading 9·49 mg of PUFA concentrate in the column.

The fatty acid composition was determined by GLC at each step of the purification process.

Table 1. Average fatty acid content in two generations of isolates of *Isochrysis galbana* and heritability, h^2, for the main fatty acids in several collection strains[a]

Fatty acid	*I*	*II*	*K–W*	h^2
14:0	2·1 ± 0·08	2·1 ± 0·08	0·01 n.s.	0·91
16:0	2·7 ± 0·10	3·0 ± 0·14	1·70 n.s.	0·97
16:1*n*7	3·0 ± 0·10	3·0 ± 0·11	0·08 n.s.	0·98
18:1*n*7	0·6 ± 0·03	0·6 ± 0·04	0·71 n.s.	0·21
18:4*n*3	1·4 ± 0·06	1·4 ± 0·05	0·31 n.s.	0·92
20:5*n*3	4·2 ± 0·15	5·3 ± 0·14	17·61[b]	0·92
22:6*n*3	1·5 ± 0·05	2·0 ± 0·08	30·46[b]	0·92
Saturated	5·1 ± 0·15	5·9 ± 0·21	8·65[c]	n.c.
Unsaturated	11·2 ± 0·34	13·1 ± 0·35	10·22[c]	n.c.
PUFAs	6·2 ± 0·21	8·8 ± 0·25	41·68[b]	n.c.
EFAs	4·7 ± 0·17	5·7 ± 0·16	14·41[b]	n.c.
Lipids	18·3 ± 3·84	21·4 ± 3·73	12·28[b]	n.c.

[a]Content is expressed as percent of dry weight; I, generation I; II, generation II; K–W, Kruskal–Wallis non-parametric test for average comparisons; n.s., not significant; n.c., not computed.
[b]$P < 0·001$.
[c]$0·001 < P < 0·01$.

RESULTS AND DISCUSSION

The selection process

The selection process involved two stages of single selection (without artificial mutagenesis) of *I. galbana*, as described above. The resulting isolates appear to follow a consistent patter, in which the fatty acids found at each stage of the selection process were 14:0, 16:0, 16:1*n*7, 18:1*n*7, 18:4*n*3, 20:5*n*3 and 22:6*n*3. Nevertheless, at the same time, the isolates differed widely in their content of specific fatty acids, within each generation as well as between generations (Table 1). Thus, for example, the EPA content ranged between 1·8–6·6% d.w. among the generation I isolates and analogous variation was observed for the other fatty acids (Table 1). Similarly, the range of variation for EPA content in generation II was 3·6–7·8% d.w.

The EPA content increased continuously from the 'parent' culture to generation II, as may be observed. EPA content improvement was evident in the increase of average amounts from the 'parent' culture to the second generation of isolates, rising from 2·4% to 5·3% d.w. This means that with only two rounds of phenotypic selection it was possible to double the original EPA content. Other lipid characters such as total PUFAs, essential fatty acids and unsaturated fatty acids, also showed a parallel, statistically significant increase (Table 1).

These results provide support for the hypothesized genetic origin of fatty acid variation.[16,17] Recently, quantitative genetic analysis attributed fatty acid variation in *I. galbana* and *Phaeodactylum tricornutum* to the genetic component (heritability) of the variation. Heritability in the broad sense was over 0·63 (0·99 for EPA) among collection strains of *I. galbana* and ranged between 0·00 and 0·96 (0·41 for EPA) among collection strains of *P. tricornutum*.[25] This means that genetic variation could explain more than 50% of fatty acid variation among microalgae strains. This significantly high variation might be susceptible to being fixed by selection which improves specific fatty acid content.

These experiments show the reliability of standard microbiological improvement methods in increasing the content of valuable products in microalgae.[26,27] Mutation/selection programmes could thus play an important role in the microalgal improvement as suggested by De la Noue & De Pauw,[26] Craig *et al.*[28] and López Alonso *et al.*[16] and as recently demonstrated in *Dunaliella*.[29]

Growth and mutual shading

In the microalgal production of fine chemicals, such as EPA in this case, a high product content obtained under stressful conditions does not necessarily mean high productivity. Therefore,

one of the isolates containing large amounts of EPA was grown in a chemostat in order to study the relationship between growth rate and biochemical composition, with special emphasis on EPA productivity.

In a first stage, growth-limiting factors were established and modelled in order to understand the kinetic behaviour of the culture. The variation of steady-state biomass concentration and productivity with dilution rate is shown in Fig. 1. These curves are characteristic of continuous light-limited cultures,[30] where steady-state growth depends on the availability of light. Light intensity, I, inside the culture is dependent on cell concentration, C. In dense cell cultures, there is a light gradient along the radius of the culture vesel due to light attenuation. Thus, as biomass concentration increases, then average internal light decreases. Furthermore, as physiological adaptation of the cells seems to respond to an average light intensity,[31,32] it is necessary to calculate the average light intensity to which cells are exposed.

Based on Lambert–Beer's law, Evers[12] described the distribution of light in a cylindrical vessel illuminated evenly from all sides obtaining the following expression for light intensity at a given point within the culture:

$$I(S,C)=\frac{I_0}{\pi}\int_0^{\pi}\exp\{-K_aC[(R-S)\cos\phi+[R^2-(R-S)^2\sin^2\phi]^{0.5}]\}\mathrm{d}\phi \qquad (1)$$

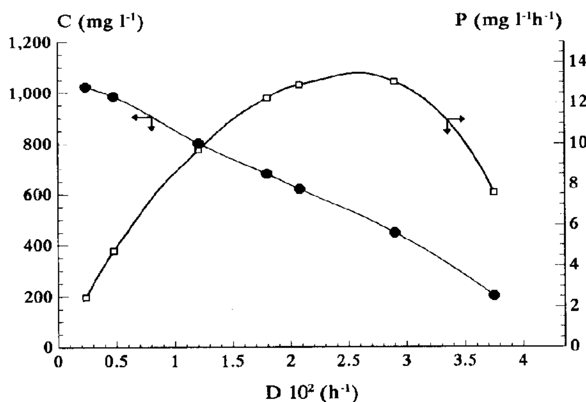

Fig. 1. Influence of dilution rate, D, on steady-state biomass concentration, C, and biomass productivity, P. The solid lines represent theoretical values obtained with the model for both variables.

where C is the biomass concentration, K_a is the biomass absorption coefficient, S is the distance from the vessel surface to an internal point, ϕ is the angle of incidence of the light path, R is the vessel radius and I_0 is the incident light intensity on the culture surface.

Up to now, K_a has been considered constant within each species, although several authors have reported that K_a strongly depends on the pigment content of the biomass.[13,33] In, a new approach, we have measured light attenuation in six cultures of the same strain of *Isochrysis galbana* grown under different lighting conditions, and therefore with different pigment contents, in order to calculate the variation in the absorption coefficient. These results are shown in Fig. 2 and details are given in Table 2. It may be observed that as pigment content is lowered, K_a decreases as much as 100%. When K_a is plotted against pigment content, X_p, a linear relationship results, making it possible to apply linear regression and rendering the equation:

$$K_a = Y'_pX_p + Y_b \qquad (2)$$

where $Y_b = 0{\cdot}0199$ m^2 g^{-1} (biomass) and $Y'_p = 1{\cdot}7357$ m^2 g^{-1} (pigments); $r^2 = 0{\cdot}967$.

Therefore, not only is the global absorption coefficient not constant, but it may also be considered the result of two contributing factors, the pigment-free biomass, Y_b, and the other pigment absorption, Y'_p (absorption coefficient normalized to total pigment content). The contribution of Y_b to biomass absorption coefficient, K_a, varies from 47 to 24% for X_p values of $1{\cdot}29\times10^{-2}$ and $3{\cdot}65\times10^{-2}$, respectively.

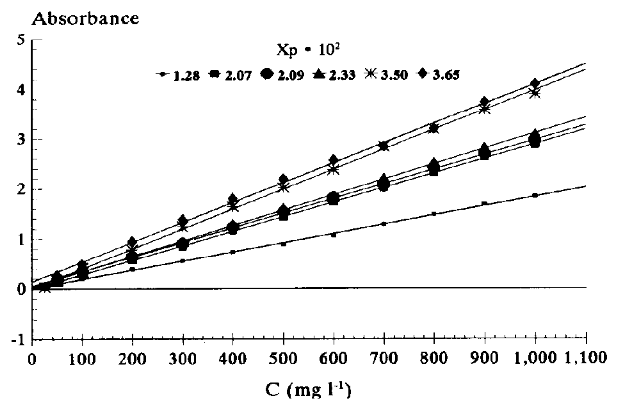

Fig. 2. Influence of biomass concentration with different pigment composition on culture absorbance.

Table 2. Effect of pigment content on absorption coefficient, K_a

	Mass fraction of total pigments, $X_p \times 10^2$					
	1·29	*2·07*	*2·09*	*2·33*	*3·51*	*3·65*
Chlorophyll *a* (mass fraction 10^2)	0·79	1·22	1·31	1·52	2·09	2·12
Chlorophyll *c* (mass fraction 10^2)	0·16	0·34	0·35	0·39	0·58	0·56
Carotenoids (mass fraction 10^2)	0·34	0·53	0·44	0·42	0·85	0·87
$K_a \times 10^2$ ($m^2\,g^{-1}$ biomass)	3·755	5·898	5·898	6·743	7·959	8·163
r^2	0·9996	0·9999	0·9999	0·9995	0·9992	0·9986

To obtain the average light intensity for the whole of the photobioreactor, it is sufficient to integrate eqn (1) between $0 < S \leq R$ to obtain the following expression:

$$I(C,X_p) = \frac{I_o}{\pi R}\int_0^R \int_0^\pi \exp\{-K_a C[(R-S)\cos\phi + [R^2-(R-S)^2\sin^2\phi]^{0\cdot 5}]\}\,d\phi\,dS \quad (3)$$

where the average light intensity is now a function of biomass concentration and its pigment content, bearing in mind the relationship between K_a and X_p (eqn (2)).

A hyperbolic model modified by an exponent, n, similar to that of Moser[34] for nutrient-limiting cultures, and analogous to Bannister's[35] 'shape parameter' describing the abruptness of the transition from low to high light region, has been used to determine the relationship between specific growth rate and light intensity:

$$\mu = \frac{\mu_{max} I^n}{I^n + I_k^n} \quad (4)$$

where μ_{max} is the maximal specific growth rate and I_k is a constant representing the affinity of cells to light.

Assuming perfect mixing and, further, that part of the absorbed energy can be utilized for cell maintenance, by steady-state material balance into a chemostat in which μ is expressed by eqn (4), the following expression is obtained:

$$D = \frac{\mu_{max} I^n}{I^n + I_k^n} - m \quad (5)$$

In the above $m = 0{\cdot}00385\ h^{-1}$ is the experimentally determined specific maintenance rate[31] when the light intensity was set at zero (darkness) in a batch culture. The slope of $\ln(C/C_o)$ over time, where C_o represents the initial biomass concentration, gives the value of m.

As mean light intensity and, consequently, the specific growth rate depend on C and X_p, one might suppose that the same steady state (D, C) could be reached with different (C, X_p) pairs. Nonetheless, in our experimental device a univocal relationship between D and C has been found as the same steady-state biomass concentration is reached when the same dilution rate is analysed at different times and different states of growth. Thus, an incident light intensity and vessel geometry remain constant, there must be a relationship between steady-state biomass concentration and pigment content which subtracts the degree of freedom in eqn (3) and makes the pairs (D, C) unique. For the system and environmental conditions assayed here, this relationship can be expressed by the following polynomial:

$$X_p = 1{\cdot}12 \times 10^{-2} - 8{\cdot}6 \times 10^{-6}C + 2{\cdot}6 \times 10^{-8}C^2$$
$$r^2 = 0{\cdot}992 \quad (6)$$

Pairs (D, I) were obtained by applying eqns (4) and (6) with experimental values of C, X_p and D. After adjustment by non-linear regression to eqn (5), the values of the parameters and regression coefficient are: $\mu_{max} = 0{\cdot}046\ h^{-1}$, $I_k = 9{\cdot}67 \times 10^{15}$ quanta $cm^{-2}\,s^{-1}$, $n = 1{\cdot}7$, $r^2 = 0{\cdot}9975$.

Figure 3 represents the real and simulated data (solid lines). The saturation intensity was 40 quanta $cm^{-2}\,s^{-1}$ as can be seen in Fig. 3. The minimum light intensity necessary to maintain the basal metabolism of the biomass is obtained at the limit $D = 0$, $1{\cdot}12 \times 10^{15}$ quanta $cm^{-2}\,s^{-1}$. This is called the compensation intensity.

Biochemical composition

It has been well known since the first publications on applied algology[36] that growth conditions determine the biochemical composition of the

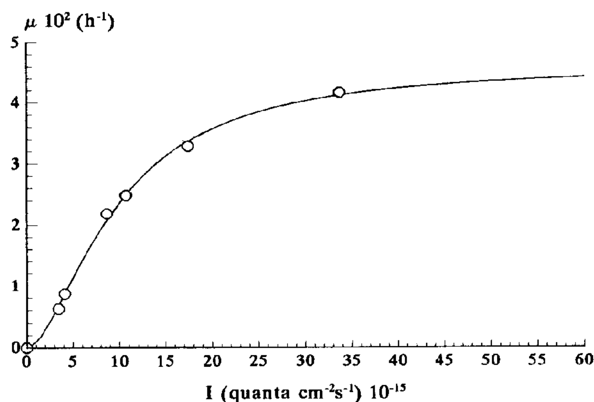

Fig. 3. Variation of specific growth rate, μ, with average light intensity, I.

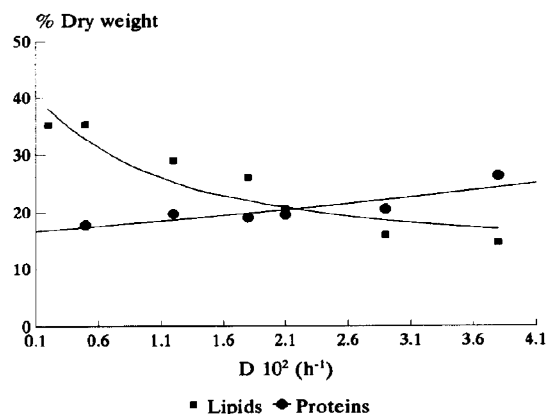

Fig. 4. Influence of dilution rate, D, on lipids and protein content.

cells. In a general sense, fast-growing cells synthesize a greater amount of proteins than lipids to maintain the cellular division rate, as the duplication period is short. At low dilution rates, that is to say, slow-growing cells, a high proportion of lipids and low protein content is obtained, making the lipid to protein ratio around 2. This variation is related to the different functions of biomolecules in cell metabolism, during which storage products, mainly neutral lipids, are synthesized when the need for the structural compound (i.e. proteins) is reduced (see Fig. 4).

Furthermore, this change in the biochemical composition has an effect on the fatty acid profile of the lipid fraction. Palmitic (16:0) and palmitoleic (16:1) acids constitute over 7% d.w., with less EPA, which is around 5% d.w., in cells grown at low dilution rates (see Fig. 5). At an intermediate D, EPA remains, proportionally, approximately the same, although 16:0 and 16:1 content fall to 3·5% and 4%, respectively. Finally, at high dilution rates, all fatty acid content decreases, with EPA the major fatty acid (see Fig. 5).

Thus, a high proportion of storage lipids, consisting mainly of 16:0 and 16:1, are found in slow-growing cells. When light limits growth, cells have to synthesize chloroplast membranes in order to collect scarce light efficiently. These membranes are composed of polar lipids which are highly unsaturated.[37,38] In *I. galbana*, around 30% of total fatty acids found in glycolipids is EPA.[39] This may explain why the EPA content remains around 5% d.w. in the range of $D = 0{\cdot}0024\ h^{-1}$ to $0{\cdot}021\ h^{-1}$, as in this interval

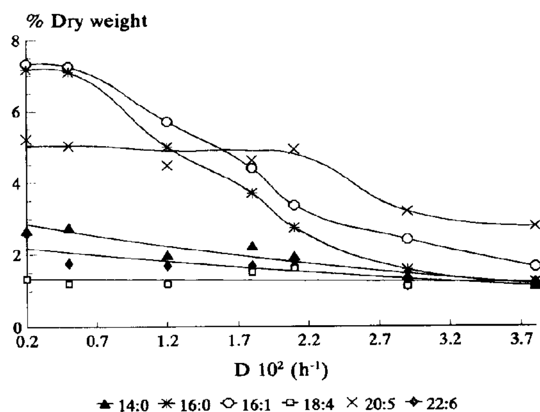

Fig. 5. Fatty acid profiles at different dilution rates, D.

growth is strongly limited by light and the proportion of glycolipids is high.

EPA and DHA separation

The focus of microalgal biotechnology on highly valuable fine chemicals requires not only mass cultivation techniques and processes, but also development of later processing methods. At this point, the commercialization of purified biomolecules (SA, EPA and DHA) or the enriched lipid fractions (glycolipids) improves the added value of the productivity system. Table 3 illustrates this, showing the different stages of fatty acid purification. The fatty acid content of *I. galbana* biomass and composition of an extract obtained by extraction and direct saponification are given in columns 1 and 2. By urea complexation, a PUFA

Table 3. Fatty acid profiles (%) of initial biomass, lipid extract, urea concentrate (methanol, 4°C, urea–fatty acid 4:1) and HPLC fractions (methanol–water 1% acetic acid 80:20; 3 ml min^{-1}; 9·49 mg)

Fatty acid	*Biomass*[a]	*Extract*[b]	*Urea*[c]	*SA*[d]	*EPA*[d]	*DHA*[d]
14:0	10·1	10·7	0·3	—	—	—
16:0	20·3	18·9	0·2	1·1	—	—
16:7*n*7	21·4	23·2	4·3	—	—	—
18:0	0·7	0·5	1·5	1·1	—	—
18:1*n*9	1·4	1·7	0·2	—	—	—
18:1*n*7	3·6	3·2	0·8	—	—	—
18:2*n*6	0·9	0·9	0·2	—	—	1·4
18:3*n*6	0·2	0·2	0·4	—	2·1	—
18:3*n*3	1·2	1·3	0·8	—	2·0	—
20:0	—	0·1	0·2	—	—	—
20:1*n*9	0·2	0·3	0·3	—	—	—
18:4*n*3	6·4	7·3	22·6	94·8	—	—
20:3*n*6	0·4	0·2	0·2	—	—	—
22:0	—	0·1	0·1	—	—	—
20:4*n*6	0·7	0·7	1·1	—	—	3·7
22:1*n*11	0·1	0·1	0·1	—	—	—
22:1*n*9	—	0·1	0·1	—	—	—
20:5*n*3	22·6	22·4	39·4	—	96·0	—
24:0	—	0·1	0·1	—	—	—
22:4*n*6	1·3	1·3	3·6	—	—	—
22:5*n*3	0·2	0·1	0·1	—	—	—
22:6*n*3	8·4	6·8	23·4	—	—	94·9
Total	100·0	99·9	100·0	97·0	100·0	100·0

[a]Lyophilized *I. galbana* biomass.
[b]Extract obtained by direct saponification at 60°C during 1 h with hexane–ethanol 96% 2:5 v/v, 8 g KOH/380 ml.
[c]Urea concentrate obtained at a MeOH–fatty acid ratio of 4:1 and successive crystallization of inclusion complexes at 4°C.
[d]SA, EPA and DHA fractions obtained by semi-preparative HPLC.

concentrate is obtained from this extract (column 3). Finally, three fractions in which the major fatty acids are SA, EPA and DHA, respectively, are obtained by semi-preparative HPLC.

As might be expected, the same fatty acid profile is observed in biomass and in the lipid extract. After that, saturated and monosaturated fatty acids were well eliminated by the urea method. So, as an example, while the palmitic acid percentage diminishes from 10·7 to 0·3%, EPA and DHA percentages increase from 22·4 to 39·4% and from 7·0 to 23·4%, respectively. Table 4 shows a comparison of data on fish oils from the literature and those reported here for *I. galbana* biomass. The inclusion complex methodology was similar in the above-mentioned studies, except for Ackman *et al.*,[40] who used ethanol as the urea solvent instead of methanol. The values of the concentration factor, *R*, reached for polyunsaturated fatty acids by Ackman *et al.*[40] are the highest, although the differences may be due to the initially high proportion of monounsaturated fatty acids, which gives rise to greater richness in PUFAs.

In Table 3, the purities in SA, EPA and DHA of the three fractions obtained by HPLC are also shown. Major fatty acid yields in each of these fractions of SA, EPA and DHA were 100, 99·6 and 94·0%, respectively. Table 5 shows that purities reached in this study are slightly higher than those reported in the literature.

It must be kept in mind that purity is susceptible to improvement only at the expense of a

Table 4. Comparison of the fatty acid composition of several fish oils and lipids in biomass in the present study, before and after urea fractionation

Fatty acid	*Lipid*	*Urea*[a]	*R*[b]	*Reference*
Saturated	9·0	1·1	0·12	40
Monounsat.	67·8	6·9	0·10	
SA	0·8	5·1	6·38	
EPA	5·4	32·5	6·02	
DHA	3·9	29·2	7·49	
Saturated	30·8	—	—	41
Monounsat.	27·7	0·3	0·01	
SA	1·7	7·2	4·20	
EPA	13·9	36·8	2·60	
DHA	13·6	40·9	3·01	
Saturated	18·3	1·0	0·05	24
Monounsat.	50·8	3·3	0·06	
SA	2·4	10·0	4·20	
EPA	12·1	27·6	2·30	
DHA	11·7	44·6	3·80	
Saturated	31·8	2·3	0·07	Present study
Monounsat.	27·4	5·8	0·21	
SA	7·0	22·6	3·22	
EPA	22·4	39·4	1·76	
DHA	7·0	23·4	3·34	

[a]Urea concentrate.
[b]Urea concentrate–lipid ratio.

Table 5. Comparison of the purity of SA, EPA and DHA obtained by HPLC separation from *I. galbana* biomass (present study) and fish oils

References	*Purity (%)*		
	18:4n3	*20:5n3*	*22:6n3*
Present study	94·8	96·0	94·9
41	93·1	85·6	83·1
42	—	91·0	85·5
43	—	91–96	75–85
44	—	>90	>90
45	—	97·3	—

decrease in yield, which depends on the collection cut-off point of fractions. However, only Tokiwa *et al.*[42] and Cohen & Cohen[45] report values for the yields obtained. Tokiwa *et al.*[42] reported 66·7% for EPA and 53·3% for DHA and Cohen & Cohen[45] reported 85% for EPA, much lower than the 99·6% and 94·0% for EPA and DHA respectively, found in this study.

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