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Lipid Class Composition of the Microalga *Pavlova lutheri*: Eicosapentaenoic and Docosahexaenoic Acids

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The lipid classes of *Pavlova lutheri*, cultivated in semicontinuous mode, were studied by thin-layer chromatography and gas chromatography in attempts to describe the distribution of fatty acid residues within its lipid pool, with special emphasis on eicosapentaenoic (C20:5*n*−3, EPA) and docosahexaenoic (C22:6*n*−3, DHA) acids. Neutral lipids and glycolipids were the major constituents and accounted for ∼57 and 24% of the total fatty acid residues (TFA), respectively. Phospholipids accounted for ∼10% of TFA. Two lipid classes, acylated steryl glycosides (SG) and diphosphatidylglycerols (DPG), were eventually identified in *P. lutheri* for the first time. The nonpolar fraction was mainly composed of triacylglycerol (TAG), whereas the polar fraction was mainly composed of monogalactosylacylglycerols (MGDG). The distribution of total EPA and DHA within the lipid pool was calculated in attempts to ascertain the quality of said microalgae as a feed source, as well as the possibility of enhancement of individual fatty acid production and extraction thereafter. EPA was especially concentrated in MGDG (∼45%) and TAG (∼33%); conversely, DHA was dispersed through various classes, especially within TAG (∼27%), DPG (∼22%), and betaine lipids (21%).

KEYWORDS: DHA; EPA; lipid classes; thin-layer chromatography; aquaculture

INTRODUCTION

Microalgae are one of the most important feed sources in aquaculture and are currently used in mariculture hatcheries to feed crustacean and bivalves. They are also used to feed rotifers, copepods, and brine shrimps, which will in turn be used to feed fish (*1*, *2*). The nutritional quality of the nourishment is of crucial importance in the rate of survival and final quality of the animals at harvest; it has also been claimed that the polyunsaturated fatty acid (PUFA) contents of microalgae is one of the major factors that affect their quality as feed in aquaculture (*3*). Several microalga species, including the flagellate *Pavlova lutheri*, have been claimed to possess a great nutritional value owing to their ability to synthesize and accumulate great amounts of PUFA (*3*−*5*). Furthermore, the use of *P. lutheri* as feed has been reported (*6*) to enhance the growth rate of fish larvae.

It is important to recall that PUFAs have been recognized to play nuclear roles on human health. Although their major source in the human diet is fish, it is believed (*7*) that marine fish do not synthesize C20:5(*n*−3), eicosapentaenoic acid (EPA), and C22:6(*n*−3), docosahexaenoic acid (DHA), themselves, but instead obtain those compounds as such from their feed, especially via inclusion of PUFA-rich microalgae therein. The production of fish containing substantial amounts of PUFA is therefore a step forward in increasing their nutritional quality, and hence their market value for aquaculture.

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Lipids contributed by microalgae have been widely studied (*8*), and their importance as a source of PUFA has been often claimed (*9*). There are a number of detailed studies on the various lipid classes for some species, including *P. lutheri* (*2*, *10*−*15*); however, no emphasis has to date been placed upon the total distribution of such important PUFA as EPA and DHA. It is known that PUFAs esterified in polar lipids are more effective than those esterified in nonpolar ones as growth promoters in aquaculture, so knowledge pertaining to PUFA distribution among the various lipid classes is crucial for the better use of microalgae in feed formulations for aquaculture. On the other hand, it is important to know the distribution of EPA and DHA within the lipid pool to correctly optimize culture conditions using production yield as objective function.

The main goal of this work was to describe the lipid classes of *P. lutheri* and their composition in terms of esterified fatty acids, with a special focus on total EPA and DHA contents. This work also held as a secondary goal the generation of experimental data on lipids present in high-density cultures, because these cultures are commercially the most interesting ones for aquaculture.

MATERIALS AND METHODS

Culture Conditions. The (monoalgal) strain SMBA 60 of *P. lutheri* was kindly supplied by IPIMAR [Instituto Português de Investigação Marítima (Lisboa, Portugal)]; the culture medium was artificial seawater (ASW) (*16*), without glycylglycine, buffered with 1 g L^{-1} Tris-HCl (Merck) (*17*). Cultures of 1.5 L were performed in a 2-L Biostat B (Braun) under semicontinuous mode, with a renewal rate of ∼40% every

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2 days. Temperature was maintained at 20 ± 0.5 °C via a refrigeration jacket; stirring was at 50 rpm; reconstituted air enriched with 0.3% (v/v) CO_2 was bubbled at the bottom of the reactor, at a flow rate of 0.8 L min^{-1}; pH was kept at 8.0, by automatic addition of 2 M NaOH or 2 M HCl (whenever appropriate); and light was continuously supplied at 20 W m^{-2}.

Three consecutive (replicate) batches were harvested, spray-dried, and stored under nitrogen at −30 °C, for subsequent analysis of fatty acid distribution among lipid classes. Cell number determinations were performed using a hemocytometer Neubauer Improved EM Techcolor (Hirchimann).

Chemical Analysis. Total lipid extracts were obtained from duplicate, spray-dried samples (200 mg), after five to seven sequential extractions, each with a 2 mL mixture of *n*-hexane/2-propanol (3:2 v/v), following the method described by Hara and Radin (*18*). Samples were then dried in a rotavapor, weighed, resuspended in a mixture of chloroform/methanol (2:1 v/v), and finally stored at −30 °C under nitrogen to prevent lipid oxidation.

The aforementioned lipid extracts were separated into polar and nonpolar fractions, using solid-phase extraction (*19*): 500 mg LC-Diol cartridges (Sigma) were equilibrated with 2 mL of methanol and 2 mL of chloroform; ∼50 mg of the oil sample dissolved in chloroform was placed in the cartridge using a micropipet; and the nonpolar fraction was then eluted with 2.5 mL of chloroform, whereas the polar fraction was eluted with 7 mL of a 100:0.5 (v/v) mixture of methanol/25% (v/v) aqueous ammonia.

Both polar and nonpolar fractions were subjected to one-dimensional thin-layer chromatography (TLC) for lipid class separation and identification, using TLC plates (20 × 20 cm) coated with silica gel 60 (Merck). Polar lipids were eluted with a five-component mixture of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 v/v), and nonpolar lipids were eluted with a complex mixture of petroleum ether (boiling point range = 60−80 °C)/diethyl ether/acetic acid (80:20:1 v/v). Two-dimensional TLC (*20*) was later performed to confirm the tentative identification of polar lipid classes, using chloroform/methanol/water (65:25:4 v/v) as first solvent, a mixture of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:4 v/v) as second solvent, and the same type of TLC plates. Iodine vapors were used as general staining (and immediately removed under low-pressure nitrogen). Bands were identified by cochromatography with pure standards (Sigma) and by (as specific as possible) staining when it was deemed necessary: phospholipids (PL) and glycolipids (GL) were distinctly stained with 50% H_2SO_4 (*20*), glycolipids with α-naphthol (*21*), betaine lipids (BL) with Dragendorff reagent (*14*, *22*), sulfolipids (SL) with cresyl violet (*20*), and sterols with a mixture of H_2SO_4/acetic acid (50:50 v/v) (*23*).

After visualization, bands were immediately and carefully scraped out, and fatty acids were analyzed by gas chromatography (GC) after direct transmethylation, according to the acidic method described by Lepage and Roy (*24*) after the modifications introduced by Cohen et al. (*25*), using heptadecanoic acid as internal standard and acetyl chloride as catalyst. The resulting esters were then analyzed in a gas chromatograph (Perkin-Elmer) using detection by flame ionization, resolution by a 60-m fused silica, capillary column Supelcowax-10 (Supelco), with helium as carrier gas in splitless mode, sample injection and detection at 250 and 270 °C, respectively, and an oven heating program that increased the column temperature linearly from 170 to 250 °C at a rate of 1 °C min^{-1}. Pure standards of fatty acids (Sigma) were used for tentative identification, based on comparison of retention times.

RESULTS AND DISCUSSION

Biomass Growth. The biomass growth in terms of cell number is presented in **Figure 1**; after a batch period, the culture was maintained under semicontinuous mode during several days to guarantee stabilization. The cultures did not reach a true stationary phase; the growth rate was 0.218 ± 0.026 day^{-1}. Three consecutive similar stages were used to perform the lipid analysis.

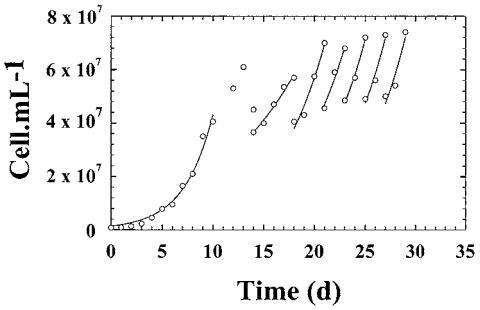

Figure 1. Biomass growth of *P. lutheri* under batch and semicontinuous mode.

Lipid Class Composition. Following the commonly accepted heuristic rule, two chromatographic systems were employed as identity was based solely on retention factor comparison. The distribution of total fatty acids present in each lipid class is depicted in **Table 1**. The triacylglycerol (TAG) and GL families accounted together for ∼76% of the total fatty acids (TFA). The nonpolar fraction, composed by TAG, diacylglycerols (DAG), monoacylglycerols (MAG), and free fatty acids (FFA), accounted for 55.7 mg g^{-1}_{AFDW}, which represents ∼57% of TFA (TAG was the major constituent). The polar fraction was mainly composed by GL (24.7 mg g^{-1}_{AFDW}), which accounted for ∼24% of TFA, with minor amounts of PL (10.0 mg g^{-1}_{AFDW}), which represented ∼10%; SL and BL were also found in the polar fraction.

TAG had already been reported (*13*) as the major lipid constituent in *P. lutheri*; it is known that this class of lipids may accumulate when the microalga is exposed to nutritional excess, or else subject to stress conditions usually associated with nutrient depletion (*10*). It has also been reported (*11*) that decreasing growth rate influences positively the amount of neutral lipids (including TAG). In our case, cultures could be maintained in a semicontinuous mode, with high densities at moderate growth rates, which in turn led to accumulation of TAG. However, higher amounts of TAG (∼70%) have been reported for continuous cultures of *Phaeodactylum tricornutum* when maintained at small growth rates (*11*). Despite the high amount of EPA found esterified on TAG, such an accumulation may be undesirable, because PUFAs esterified in polar lipids are more effective as growth promoters in aquaculture (*13*).

The identification of MAG and DAG is somewhat controversial and is often considered to be an artifact derived from hydrolysis of TAG following lipid extraction; however, there are authors (*10*) who claim their actual presence and justify their absence as a matter of low amount as compared with the analytical detection limit. Our results support the latter assumption, as those classes were detected only when high amounts of lipid extract were loaded on TLC plates.

In what concerns polar classes, the GL are mainly constituted by monogalactosylacylglycerols (MGDG) (16.5 mg g^{-1}_{AFDW}), which accounts for ∼16% of TFA; this lipid class is second only to TAG, as expected in microalgae. The total digalactosylacylglycerol (DGDG) amount was 3.02 mg g^{-1}_{AFDW} and represents ∼3% of TFA, which is below previous findings (*10*, *26*) encompassing *P. lutheri* and other microalgal species. The SG were detected at relatively high levels, 5.13 mg g^{-1}_{AFDW} (or ∼5%); although those compounds are usually found in plants, and their presence in algae has been reported, this is apparently the first time that this class was ever detected in *P. lutheri*. The presence of this lipid class was confirmed by cochromatography with a pure standard, by sterol staining, and by GL staining.

Table 1. Fatty Acid Profile and Total Fatty Acid Amounts of Individual Lipid Classes in *P. lutheri* (Average ± Standard Deviation)

fatty acid residue	lipid class[a]											
(%, w/w)	SQDG	BL	DGDG	PG[b]	UN	DPG	SG	MGDG	MAG[b]	DAG	FFA	TAG
C14:0	10.73 ± 4.57	13.65 ± 1.06	13.17 ± 0.77	6.33	7.00 ± 0.77	4.89 ± 0.30	19.34 ± 4.34	4.11 ± 0.13	3.75	15.54 ± 2.03	0.00 ± 0.00	8.46 ± 0.31
C16:0	31.66 ± 1.77	23.41 ± 2.87	20.21 ± 4.10	18.65	27.31 ± 1.72	9.05 ± 0.41	19.10 ± 4.25	8.07 ± 1.16	7.23	20.53 ± 5.46	28.22 ± 5.79	29.20 ± 0.09
C16:1(*n*–7)	5.72 ± 0.44	8.41 ± 0.17	7.79 ± 2.92	9.06	5.45 ± 0.88	3.29 ± 0.74	24.04 ± 4.37	9.48 ±0.34	4.41	12.08 ± 2.34	0.00 ± 0.00	38.02 ± 1.06
C18:0	6.45 ± 3.27	2.36 ± 1.16	4.78 ± 0.21	4.34	5.41 ± 1.28	3.78 ± 2.79	3.23 ± 1.58	1.32 ± 0.03	3.97	12.39 ± 3.06	18.63 ± 7.26	0.77 ± 0.00
C18:1(*n*–9)	8.47 ± 1.74	6.22 ± 0.28	5.77 ± 1.72	19.27	8.47 ± 4.74	4.10 ± 0.09	8.60 ± 1.96	3.16 ± 2.48	3.75	9.11 ± 0.21	0.00 ± 0.00	3.53 ± 0.02
C18:1(*n*–7)	6.92 ± 2.44	1.60 ± 0.91	0.80 ± 0.03	3.75	2.51 ± 0.00	2.57 ± 2.07	0.91 ± 0.42	0.87 ± 0.19	0.00	0.00 ± 0.00	0.00 ± 0.00	1.16 ± 0.00
C18:2(*n*–6)	8.70 ± 1.30	1.08 ± 1.50	0.80 ± 0.02	3.89	1.15 ± 0.15	0.00 ± 0.00	1.00 ± 1.42	3.39 ± 0.12	1.92	0.00 ± 0.00	0.00 ± 0.00	3.87 ± 0.11
C18:3(*n*–6)	21.35 ± 8.52	1.44 ± 0.55	3.83 ± 2.26	1.51	1.73 ± 0.75	1.88 ± 0.72	0.00 ± 0.00	1.85 ± 0.61	49.04	27.01 ± 11.68	53.15 ± 13.05	0.57 ± 0.35
C18:3(*n*–3)	0.00 ± 0.00	1.75 ± 0.54	4.66 ± 0.69	2.38	3.13 ± 2.19	3.82 ± 1.40	6.09 ± 0.91	3.34 ± 2.84	0.00	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.19
C18:4(*n*–3)	0.00 ± 0.00	2.42 ± 1.05	9.26 ± 2.77	6.94	7.30 ± 5.26	6.31 ± 1.45	7.78 ± 0.64	21.75 ± 0.51	16.04	0.00 ± 0.00	0.00 ± 0.00	1.85 ± 0.11
C20:5(*n*–3)	0.00 ± 0.00	13.09 ± 0.86	20.94 ± 2.68	9.56	7.88 ± 0.50	5.82 ± 2.00	1.71 ± 1.22	36.67 ± 1.32	9.88	3.33 ± 4.70	0.00 ± 0.00	8.42 ± 0.25
C22:6(*n*–3)	0.00 ± 0.00	24.58 ± 1.82	7.99 ± 0.23	14.32	22.65 ± 4.23	54.49 ± 8.48	8.20 ± 3.12	6.01 ± 4.10	0.00	0.00 ± 0.00	0.00 ± 0.00	3.31 ± 0.16
TFA ($mg\ g^{-1}_{AFDW}$)	2.05 ± 0.896	7.58 ± 1.44	3.02 ± 0.87	1.30 ± 0.35	5.14 ± 1.61	3.57 ± 1.36	5.13 ± 0.33	16.5 ± 1.51	1.38 ± 0.80	1.09 ± 0.60	0.60 ± 0.40	55.7 ± 3.23

[a] Based on three biological replicates, each analyzed in triplicate. BL, betain lipids; DAG, diacylglycerols; DGDG, digalactosylacylglycerols; DPG, diphosphatidylacylglycerols; FFA, free fatty acids; MAG, monoacylglycerols; MGDG, monogalactosylacylglycerols; PG, phosphatidylglycerols; SG, acylated sterol glycosides; SQDG, sulfoquinovosyldiacylglycerols; UN, unidentified phospholipid class. [b] Insufficient replicates for accurate calculation of standard deviation.

The amounts of sulfoquinovosyldiacylglycerols (SQDG) were lower than those reported previously in *P. lutheri* (*13*, *26*); this observation might be accounted for by the fact that the amount of SQDG is often inversely proportional to the amount of TAG, and this amount was considerably higher in our case (probably owing to the culture conditions utilized).

As expected, the PLs were found in minor amounts; the absence of phosphatidylethanolamine (PE) or phosphatidylcholine (PC) has been reported previously (*13*−*15*, *26*) and was corroborated by our results. However, the presence of diphosphatidylglycerols (DPG) as major constituent of PLs had not been reported previously. Furthermore, the major PL constituent is probably a still unidentified class, which, as shown below, presents an interesting fatty acid profile. The presence of DPG was once more confirmed by cochromatography with a pure standard and by specific PL staining.

The presence of BLs in *Prymnesiophytes* was previously reported (*14*, *26*); it was even suggested that these compounds might replace PC in terms of metabolic role. The detection, in our experiment, of a betaine lipid, which is consistent with previous work and which is similar to PC (*14*, *27*), supports that theory. Although we assumed that the unknown class is a phospholipid, on the basis of the staining results on TLC, it might also be another betaine lipid owing to its similar fatty acid profile; in fact, there are references (*14*, *26*, *27*) that refer to the presence of other BLs in *P. lutheri*.

It is believed that FFAs cannot be abundant in photosynthetic cells because they would lead to the formation of toxic products—detergents—that would disrupt their membranes; hence, their presence in high amounts is probably an artifact that indicates poor lipid extraction (*22*). In our case, the existence of a very small amount of FFA suggests that no extensive lipid degradation took place.

Fatty Acid Profile of Individual Classes. The fatty acid profile of each lipid class is presented in **Table 1**; the overall distribution of EPA and DHA in the lipid pool is depicted in **Table 2**. The TAGs are typically composed of medium-chain saturated and monounsaturated fatty acid residues; C16:0, C16:1(*n*−7), and C14:0 represent together >70% of TFA in this class. Although the fraction of EPA is not high (∼8%), the large amount of total TAGs makes this class account for ∼33% of the total EPA. The fact that the PUFAs in polar lipid classes are more suitable for aquaculture (*13*), combined with the high amounts of saturated fatty acids present in TAGs, suggests that the modulation of culture conditions toward optimization of the total TAG production and the percentage of EPA in this class are of the utmost importance.

Table 2. Overall Distribution of Total Eicosapentaenoic (EPA) and Docosahexaenoic (DHA) Acids among Various Lipid Classes in *P. lutheri*

	% (w/w) total fatty acid	
lipid class[a]	EPA	DHA
BL	7.79	21.3
DGDG	5.20	2.37
PG	1.34	3.35
UN	3.18	12.2
DPG	2.17	26.8
SG	1.17	3.94
MGDG	44.5	8.40
MAG	1.03	0.00
DAG	0.22	0.00
TAG	33.4	21.7

[a] BL, betaine lipids; DAG, diacylglycerols; DGDG, digalactosylacylglycerols; DPG, diphosphatidylacylglycerols; FFA, free fatty acids; MAG, monoacylglycerols; MGDG, monogalactosylacylglycerols; PG, phosphatidylglycerols; SG, acylated sterol glycosides; SQDG, sulfoquinovosyldiacylglycerols; UN, unidentified phospholipid class.

The fatty acid profile of MGDG is unique and mainly composed by PUFA, with high expression of EPA (which accounts for ∼37% of this class and ∼45% of the total EPA). The claim that C18:4(*n*−3) is present in MGDG is not unanimous; although some authors (*13*) described very small amounts, others (*26*) reported large amounts under similar conditions. In our case, C18:4(*n*−3) accounted for ∼22% of MGDG; together with EPA, they accounted for almost 60% of MGDG, which yields a rather favorable lipid profile. The DGDG class exhibits an identical profile, but is more concentrated in saturated fatty acid residues.

The largest differences within the glycolipids occurred with regard to acylated steryl glycosides (SG), with predominance of saturated fatty acid residues and with a DHA residue with much higher content than its EPA counterpart.

Although in relatively small amounts, the PLs exhibited a quite interesting fatty acid profile in terms of appearance of DHA, where they account for 44% of the total DHA. In

particular, DPG is composed by ~55% DHA and only 6% EPA. The lipid profile of PG is similar to that found in *Isochrysis galbana* (*10*). The unknown class identified as PL entertains a unique profile, with ~50% of fatty acid residues accounted for by C16:0 and DHA.

The GLs are, in particular, major constituents of chloroplast membranes, which are usually rich in EPA and C18 PUFA but do not contain DHA (*28*, *29*), whereas the PLs are associated mainly with cytoplasm membranes with high levels of DHA (*22*); our results confirm that pattern. Hence, by changing photosynthetic parameters (e.g., light intensity), it might be possible to optimize the overall production and/or the fatty acid profile of GL. Moreover, temperature affects the membrane (*13*, *22*), so the concentration of both PL and GL should be modulated by processing temperature, as has already been attempted elsewhere (*13*). Modulation of both light and temperature together might thus be crucial in attempts to optimize phospho- and glycolipid production and, concomitantly, their fatty acid profile.

The SQDG profile recorded is typical for this class; the total absence of long-chain fatty acids is noteworthy, but similar profiles have been described (*10*, *13*, *26*) for several species including *P. lutheri*.

Concerning BL, the lipid profile of DGCC observed agrees with that reported by Kato et al. (*26*, *27*); both EPA and DHA appeared among the major fatty acid residues in this class.

EPA and DHA Distribution among Classes. The distributions of EPA and DHA (**Table 2**) are very different among the various lipid classes resolved, which may be a step forward if their effective separation in large scale extraction procedures is sought. The EPA is poorly distributed within two classes (viz., TAG and MGDG), which account for almost 80% of total EPA; conversely, DHA is more widely distributed.

The most concentrated fraction of EPA occurs in MGDG, with 45% of the total EPA; moreover, the GL family contains ~51% of the total EPA, but only 15% of the total DHA. In terms of purity, EPA is the major constituent of MGDG, which means that isolation of this class would lead to recovery of ~45% of the total EPA (already bearing a higher purity than the crude algal extract). On the other hand, the PL family contains 42% of the total DHA, but only 7% of the total EPA. These results unfold tendencies of EPA and DHA to accumulate in glyco- and phospholipids, respectively.

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