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ScienceDirect
Food Chemistry 104 (2007) 1580–1585
Food Chemistry
www.elsevier.com/locate/foodchem

Fatty acid and lipid class composition of the eicosapentaenoic acid-producing microalga, *Nitzschia laevis*

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Received 8 August 2006; received in revised form 2 January 2007; accepted 1 March 2007

Abstract

The diatom *Nitzschia laevis* is a potential producer of eicosapentaenoic acid (EPA, C20:5*n* − 3). In order to further adopt this alga in the functional food and aquaculture industries, the lipid class composition and fatty acid distribution in the lipid pool of *N. laevis* were studied using thin-layer chromatography and gas chromatography. The total lipids of *N. laevis* were fractionated into neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLs). NLs were the major constituents and accounted for 78.6% of the total lipids. Triacylglycerol (TAG) was the predominant component of NLs (87.9%). GLs and PLs accounted for 8.1% and 11.6% of the total lipids, respectively. Phosphatidylcholine (PC) was the major component of PLs (69.7%). The principal fatty acids identified in most lipid classes were tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), palmitoleic acid (C16:1) and EPA. EPA was distributed widely among the various lipid classes with the major proportion (75.9% of the total EPA) existing in TAG, monoacylglycerol and PC.

Keywords: Lipids; Fatty acids; Eicosapentaenoic acid; *Nitzschia laevis*

1. Introduction

Eicosapentaenoic acid (EPA, 20:5*n* − 3), a long chain *n* − 3 polyunsaturated fatty acid (PUFA), is essential for the regulation of some biological functions and prevention of arrhythmia, atherosclerosis, cardiovascular disease and cancer (Pulz & Gross, 2004). The intake of a sufficient quantity of EPA is therefore necessary for human health and nutrition (Ruxton, Reed, Simpson, & Millington, 2004). In aquaculture industries, EPA is also important for the growth and development of fish, crustaceans and bivalves. There is a great demand for EPA-enriching feed in aquaculture (Borowitzka, 1997).

Fish oil is currently the major source of EPA, but it is believed that fish get EPA from their feed (i.e., microalgae) instead of synthesis by themselves (Wen & Chen, 2003). Using microalgae for the production EPA has recently attracted much attention in the research field of algal biotechnology. As most of the algae studied for EPA production are strict photoautotrophs, the EPA productivity is greatly limited by insufficient supply of light due to mutual shading of cells (Chen, 1996).

Diatoms are abundant and diverse in terms of their number and distribution in nature and some species are reported to contain considerable amounts of EPA (Lebeau & Robert, 2003). The genus *Nitzschia*, namely, *N. alba* and *N. laevis*, has been found to have a great EPA production potential (Barclay, Meager, & Abril, 1994). *N. laevis* could grow well heterotrophically with glucose as the sole carbon source and its cellular EPA content was higher than that obtained under photoautotrophic conditions (Tan & Johns, 1996). Therefore, this alga has gained increasing interest in recent years. The influences of environmental conditions on its heterotrophic growth and EPA yield have been thoroughly studied (Chen & Chen, 2006; Wen & Chen, 2001a; Wen & Chen, 2001b; Wen & Chen, 2002).

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doi:10.1016/j.foodchem.2007.03.008

Lipids are the sole sources of PUFAs in microalgae. Their contents significantly are influenced by environmental conditions and can be physiologically manipulated, which results in variation of fatty acid content in the lipid pool. Furthermore, information on EPA localization in the lipid classes and the distribution of the companion fatty acids is important for successful EPA purification on an industrial scale (Alonso, Belarbi, Rodriguez-Ruiz, Segura, & Gimenez, 1998). On the other hand, it is reported that a high lipid and PUFA diet, with undefined precise constituents, in aquaculture, often creates problems in the control of nutritional quality of marine animals (Halver & Hardy, 2002). A full understanding of the lipid class composition and fatty acid distribution of the target algal species, therefore, is necessary for the application of EPA-producing microalgae in functional foods and aquaculture feeds. The aim of the present study was to investigate the lipid class composition and fatty acid distribution in the lipid pool of *N. laevis* grown under heterotrophic conditions, with a special emphasis on its EPA distribution. The results will be helpful for regulating EPA synthesis and optimizing EPA purification in *N. laevis* and will facilitate the formulation of EPA-enriching functional foods and animal feeds.

2. Materials and methods

2.1. Heterotrophic cultivation

The diatom *Nitzschia laevis* (UTEX 2047) was used in this study. The cells were cultured in 250 ml Erlenmeyer flasks, each containing 100 ml of modified Lewin's marine diatom medium (LDM) which was optimized for *N. laevis* UTEX 2047 by Wen and Chen (2001b). The flasks were incubated in an orbital shaker (at 160 rpm) at 23 °C in darkness. The initial medium pH was adjusted to 8.5 prior to autoclave.

2.2. Determination of cell dry weight

A 3 ml aliquot of the fermentation broth was sampled aseptically to determine the cell dry weight. The sample was centrifuged at 2040*g* for 5 min, and the cell pellet was washed with distilled water, twice. Cell dry weight was determined by filtering the fluid through a preweighed filter paper (Whatman GF/C) and dried at 80 °C in a vacuum oven to constant weight.

2.3. Determination of glucose concentration

Residual glucose concentration of the medium was determined by the 3,5-dinitrosalicylic acid method (Miller, 1959).

2.4. Lipid extraction and analysis

Cells were harvested in the early stationary phase for lipid and fatty acid analysis. Total lipids were extracted from 200 mg of lyophilized biomass according to the modified Folch procedure (Christie, 2003). The extract was dried in a rotary evaporator, and then weighed, resuspended in chloroform, and finally stored at −20 °C under nitrogen gas to prevent lipid oxidation.

Total lipids extracted were separated into neutral lipids (NLs), glycolipids (GLs) and phospholipids (PLs) using solid-phase extraction (Christie, 2003). A 500 mg Sep-Pak™ cartridge of silica gel (Waters) was first conditioned by elution with 5 ml of chloroform, and about 50 mg of lipid were then applied to it. Elution with 10 ml of chloroform yielded the NLs, 10 ml of acetone gave the GLs, and 10 ml of methanol yielded the PLs. Each fraction was concentrated under a stream of nitrogen gas, resuspended in 0.1 ml of chloroform.

The aforementioned lipid 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). Plates were activated in an oven at 100 °C for 2 h before use. Solvents used were hexane/diethyl ether/acetic acid (70:30:1, v/v) for neutral lipids and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 v/v) for both GLs and PLs (Touchstone, 1995). Two-dimensional TLC was performed to confirm the identification of both polar lipid classes, using chloroform/methanol/28% aqueous ammonia (65:35:5 v/v) as the first solvent, a mixture of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 v/v) as the second solvent, and the same type of TLC plates (Christie, 2003).

A 0.1% (w/v) solution of 2,7-dichlorofluorescein in 95% methanol was used as general stain, which caused lipids to show up as yellow spots under UV light. Bands were identified by co-chromatography with pure standards (Sigma) and by (as specific as possible) staining when necessary: α-naphthol for GLs, molybdenum blue spray reagent for PLs, Dragendorff's reagent for phosphatidylcholine (PC), ninhydrin for phosphatidylethanolamine (PE) and phosphatidylserine (PS), cresyl violet for sulphoquinovosyldiacylglycerol (SQDG) (Christie, 2003; Williams, 1978).

2.5. Fatty acid analysis

After visualization and identification, lipid bands were immediately and carefully scraped out, and fatty acids were analyzed by gas chromatography (GC) after direct transmethylation with sulphuric acid in methanol (Christie, 2003). The fatty acid methanol esters (FAMEs) were extracted with hexane and analyzed by HP-6890 gas chromatography (Hewlett-Packard) equipped with HP7673 injector, a flame-ionization detector and a HP-INNOWAX™ capillary column (HP 19091N-133, 30 m × 0.25 mm × 0.25 μm). Two microliters of the sample were injected in the splitless injection mode. The inlet and detector temperatures were kept at 250 °C and 270 °C, respectively, and the oven temperature was programmed from 170 °C to 230 °C increasing at 1 °C/min. High purity

nitrogen gas was used as the carrier gas. FAMEs were identified by comparison of their retention times with those of the authentic standards (Sigma), and were quantified by comparing their peak areas with that of the internal standard (C17:0).

3. Results and discussion

3.1. Heterotrophic growth characteristics

The kinetics of the cell growth and glucose consumption of *N. laevis* in heterotrophic batch culture are shown in Table 1. The alga grew well with a specific growth rate of 0.580 d^{-1}. The growth yield coefficient, based on glucose, $Y_{x/glu}$, was 0.479 g/g. The highest biomass concentration, X_{max} of 10.10 g/l was obtained in the early stationary phase (day 5). The kinetic growth parameters obtained in this study were comparable to those reported by Wen and Chen (2003).

3.2. Lipid composition

Cells of the early stationary phase were used for lipid analysis. The fractions of lipids are shown in Fig. 1. The NLs, composed of triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG), accounted for 78.6% of the total lipids. The polar lipids represented 19.5% of the total lipids, while GLs and PLs accounted for 8.1% and 11.6%, respectively. The unidentified lipid fractions (UN) were detected to be of 1.9%.

Table 1
Kinetic parameters of growth and glucose consumption of *N. laevis* in heterotrophic batch culture[a]

Parameters[b]	μ (d^{-1})	X_{max} (g/l)	$Y_{x/glu}$ (g/g)
N. laevis	0.58 ± 0.02	10.10 ± 0.02	0.479 ± 0.002

[a] All values were means of three measurements and expressed as means ± standard deviation.
[b] μ, specific growth rate (d^{-1}); X_{max}, maximum biomass concentration (g/l); $Y_{x/glu}$, growth yield coefficient based on glucose (g/g).

TAG was the most abundant lipid fraction of *N. laevis*, which represented 69.1% of the total lipids. TAG was reported to be the predominant lipid class in heterotrophic microalgae, such as *Nitzschia alba* (about 87.0%) and *Crypthecodinium cohnii* (about 55.0%) (Anderson, Livermore, Kates, & Volcani, 1978; Henderson, Leftley, & Sargent, 1988). It was known that an increase in TAG was usually associated with nutrient depletion (Alonso et al., 1998). In our studies, cells harvested at the early stationary phase had higher amounts of accumulated storage lipids (mainly in the form of TAG), mainly due to the depletion of glucose, the sole carbon source (data not shown). MAG and DAG were found at 9.7 mg/g and 2.4 mg/g of cell dry weight, respectively, and accounted for 7.6% and 1.9% of the total lipids, respectively. MAG and DAG are the key metabolites in TAG biosynthesis in living organisms. The presence of low amounts of MAG and DAG was also reported in other microalgae (Alonso et al., 1998).

The GL fraction of *N. laevis* was composed of monogalactosylacylglycerol (MGDG), digalactosylacylglycerol (DGDG) and SQDG, which together accounted for 8.1% of the total lipids. DGDG constituted the largest fraction of GLs (3.5% of the total lipids). The content of GL of *N. laevis* in this study might be influenced by the heterotrophic culture conditions, as the GL content in other heterotrophic microalgae, i.e. *N. alba*, was also low, and even no GLs were reported in *C. cohnii* (Anderson et al., 1978; Henderson et al., 1988) whereas the contents of GLs were normally much higher in phototrophic microalgae (Alonso

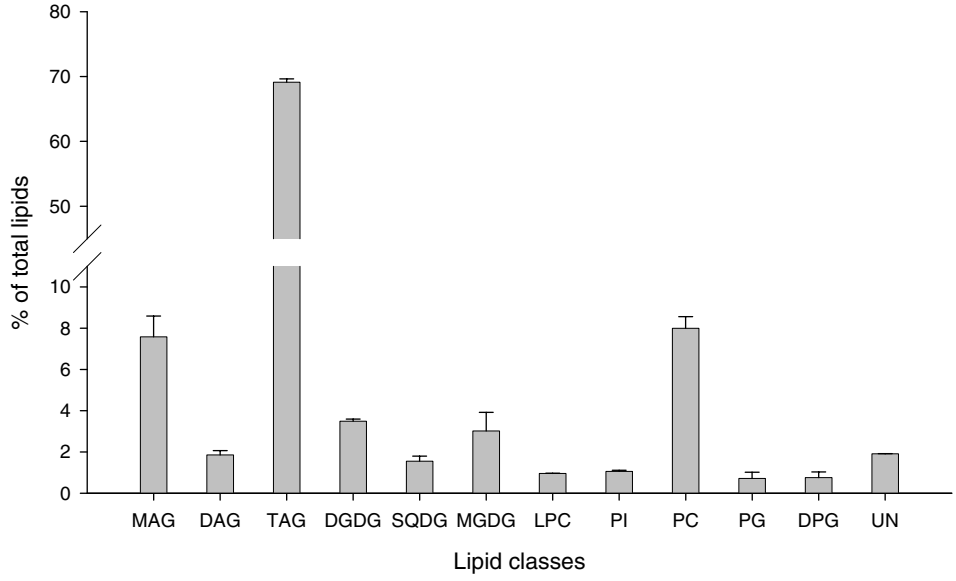

Fig. 1. Individual lipid class content (% of total lipids) of *Nitzschia laevis*. Values are represented as means ± standard deviation of triplicates.

et al., 1998). This might be because MGDG, DGDG and SQDG were highly associated with photosynthetic membranes of algae (Gurr, Harwood, & Frayn, 2002).

PL, an essential component of the cell membranes, was found at 14.7 mg/g of cell dry weight and accounted for 58.8% of polar lipids and 11.6% of the total lipids, respectively. PC was the major component of PLs and accounted for 69.7%. Lysophosphatidylcholine (LPC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) were minor components, which accounted for 8.3%, 9.2%, 6.3% and 6.6% of PLs, respectively. The results on PLs fractionation were in agreement with another report on *C. cohnii*, another heterotrophic microalga for docosahexaenoic acid production (Henderson et al., 1988). In fact, PC was reported to be the major component of PLs in many microalgae, though its content might vary due to species-specificity or culture conditions (Alonso et al., 1998; Bigogno, Khozin-Goldberg, Boussiba, Vonshak, & Cohen, 2002).

3.3. Fatty acid distribution in individual lipid classes

Fatty acid profiles of different lipid fractions of *N. laevis* are depicted in Table 2. TAG was mainly composed of mid-chain fatty acids, C14:0, C16:0 and C16:1, which altogether represented 79.5% of the total fatty acids (TFA) in this lipid fraction. Although the proportion of EPA in TAG was only 6.3%, due to highest percentage of TAG in cell lipid, the amount of EPA presented in TAG was still higher (37.4% of total EPA) than that in other lipid classes. The high content of medium-chain fatty acids and low content of EPA in TAG were also reported in *N. alba* and *N. palea* (Anderson et al., 1978; Opute, 1974). The percentage of unsaturated fatty acids of TFA (Unsatd) of TAG was 45.9%, and the degree of fatty acid unsaturation (∇/mol) was 0.9, as shown in Table 2. Both of them were the lowest values in the whole lipid class. As it has been reported that polar lipids are better than neutral lipids, such as TAG, for aquaculture feeding, combined with the high amounts of saturated fatty acids and low amounts of EPA present in TAG found in this study, the culture medium and conditions for *N. laevis* should be further optimized to reduce its content of TAG.

Fatty acid composition of DAG was similar to that of TAG, in which C14:0, C16:0 and C16:1 altogether represented 65.1% of TFA and EPA percentage was 6.2%. The fatty acid profile of MAG was quite different from those of TAG and DAG. The content of EPA in MAG was 34.8% of TFA and accounted for about 22.6% of the total EPA. The EPA in MAG, therefore represented the second major fraction of the total EPA, though MAG only represented about 7.4% of the total lipids. Similar to EPA, the contents of other PUFAs, such as C18:3$n-3$ and arachidonic acid (AA, C20:4$n-6$), were also high in this lipid fraction. As shown in Table 2, MAG had higher Unsatd (83.8%) and ∇/mol (2.7), than had the other lipid fractions.

Table 2
Fatty acid composition of individual lipid classes of *N. laevis*[a] (% of TFA)

Fatty acids	Lipid class[b]										
	MAG	DAG	TAG	DGDG	SQDG	MGDG	LPC	PI	PC	PG	DPG	UN
C14:0	7.2 ± 2.5	14.5 ± 0.3	20.0 ± 0.0	11.1 ± 0.6	6.9 ± 0.4	5.7 ± 0.1	11.2 ± 1.3	4.8 ± 0.2	14.1 ± 0.2	5.5 ± 0.4	14.0 ± 1.9	7.0 ± 1.2
C14:1	0.5 ± 0.0	1.1 ± 0.2	0.9 ± 0.0	0.7 ± 0.0	2.8 ± 2.8	0.5 ± 0.0	1.2 ± 0.4	1.6 ± 0.5	0.7 ± 0.0	2.5 ± 0.4	1.2 ± 0.1	0.5 ± 0.2
C16:0	8.8 ± 0.8	21.1 ± 1.2	34.1 ± 0.1	20.5 ± 2.8	30.5 ± 3.6	11.9 ± 1.7	29.0 ± 3.6	31.5 ± 3.4	13.5 ± 0.5	34.6 ± 2.9	25.8 ± 2.4	9.5 ± 0.2
C16:1	27.2 ± 3.1	29.4 ± 2.0	25.4 ± 0.1	30.9 ± 3.7	7.4 ± 2.5	23.6 ± 0.3	15.7 ± 1.4	16.1 ± 4.6	15.7 ± 0.3	12.8 ± 4.0	5.8 ± 0.4	7.1 ± 1.4
C18:0	0.2 ± 0.6	1.8 ± 0.4	0.1 ± 0.0	1.9 ± 1.3	9.1 ± 1.7	4.5 ± 0.9	3.6 ± 1.8	3.4 ± 2.0	0.3 ± 0.0	3.7 ± 0.6	4.1 ± 0.9	2.0 ± 0.8
C18:1	1.8 ± 0.8	7.8 ± 1.0	5.0 ± 0.0	1.3 ± 2.3	19.7 ± 2.4	3.4 ± 0.3	13.3 ± 1.0	16.8 ± 0.1	4.2 ± 0.0	20.2 ± 4.3	21.5 ± 2.7	3.4 ± 0.8
C18:2	1.7 ± 0.2	12.9 ± 0.2	3.1 ± 0.0	2.4 ± 0.0	3.6 ± 1.4	1.9 ± 0.0	3.6 ± 2.7	5.6 ± 0.7	5.6 ± 0.1	2.4 ± 0.0	4.1 ± 0.7	5.5 ± 0.4
C18:3$n-6$	3.4 ± 2.3	1.7 ± 0.5	1.7 ± 0.0	0.6 ± 3.3	4.5 ± 1.9	3.8 ± 0.7	2.0 ± 1.5	1.8 ± 2.0	3.1 ± 0.1	2.2 ± 0.6	3.1 ± 0.6	4.3 ± 0.4
C18:3$n-3$	6.9 ± 0.8	1.3 ± 0.0	1.2 ± 0.0	0.6 ± 0.0	1.8 ± 2.3	9.1 ± 0.9	1.0 ± 0.5	5.6 ± 0.2	1.5 ± 0.1	1.4 ± 1.8	2.9 ± 0.5	2.8 ± 0.1
C20:4$n-6$	7.5 ± 0.1	2.1 ± 0.2	2.4 ± 0.0	10.2 ± 3.7	5.8 ± 0.4	4.5 ± 1.8	8.7 ± 3.6	4.2 ± 2.5	18.1 ± 0.3	3.2 ± 1.2	6.0 ± 1.5	27.5 ± 0.4
C20:5$n-3$	34.8 ± 1.3	6.2 ± 0.4	6.3 ± 0.2	19.7 ± 2.2	7.9 ± 2.5	31.2 ± 2.0	10.9 ± 2.3	8.7 ± 1.2	23.3 ± 0.5	11.4 ± 0.8	11.5 ± 2.1	30.3 ± 1.1
TFA[c]	9.7 ± 1.9	2.4 ± 0.4	88.6 ± 1.0	4.5 ± 0.2	2.0 ± 0.5	3.9 ± 1.7	1.2 ± 0.0	1.4 ± 0.1	10.2 ± 1.1	0.9 ± 0.6	1.0 ± 0.5	2.5 ± 0.0
Unsatd[d]	83.8 ± 2.0	62.6 ± 0.9	45.9 ± 0.4	66.5 ± 2.6	53.5 ± 3.8	78.0 ± 1.9	56.3 ± 11.8	60.3 ± 10.4	72.1 ± 0.6	56.1 ± 3.7	56.1 ± 3.0	81.4 ± 0.4
∇/mol[e]	2.7 ± 0.1	1.1 ± 0.0	0.9 ± 0.0	1.8 ± 0.1	1.2 ± 0.2	2.4 ± 0.1	1.4 ± 0.5	1.3 ± 0.3	2.3 ± 0.0	1.2 ± 0.2	1.4 ± 0.2	3.0 ± 0.1

[a] All values were means of three measurements and expressed as means ± SD.

[b] MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; UN, unidentified lipid fractions.

[c] TFA, total fatty acids (mg)/cell dry weight (g).

[d] Unsatd, percentage of unsaturated fatty acids of the total fatty acids.

[e] ∇/mol: the degree of fatty acid unsaturation (Chen & Johns, 1991), ∇/mol = (1.0 (% monoene) + 2.0 (% diene) + 3.0 (% triene) + 4.0 (% tetraene) + 5.0 (% pentaene))/100.

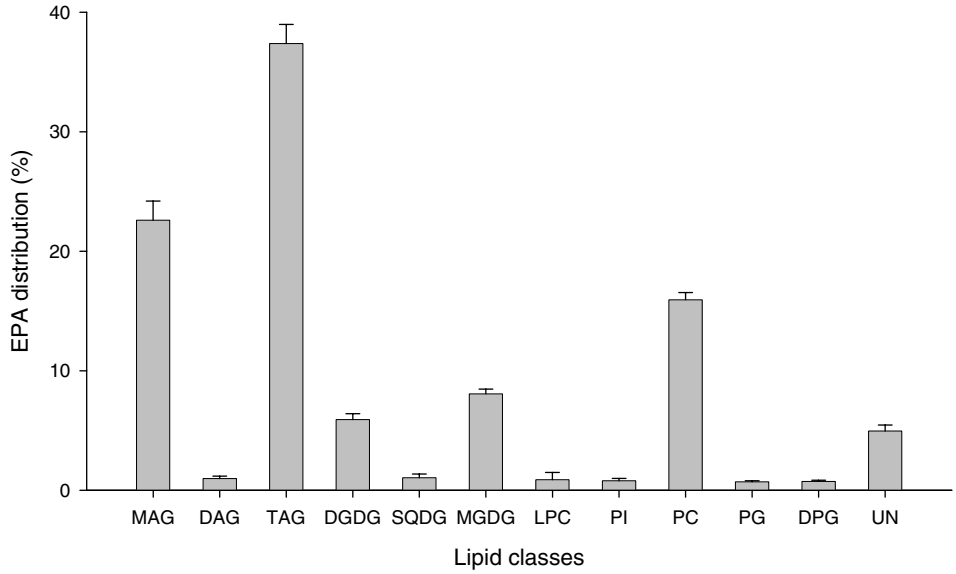

Fig. 2. Overall distribution of eicosapentaenoic acid (EPA) among various lipid classes of *Nitzschia laevis*. Values are represented as means ± standard deviation of triplicates.

Among GLs, the fatty acid profiles of MGDG and DGDG were characterized by high contents of C16 fatty acids and EPA. Similar results were also reported in *N. palea*, *I. galbana*, *P. lutheri* and *P. tricornutum*, with the exception of *N. alba*, which had a high content of C14:0, but low contents of C16 fatty acids and EPA (Alonso et al., 1998; Anderson et al., 1978; Arao, Kawaguchi, & Yamada, 1987; Opute, 1974; Tatsuzawa & Takizawa, 1995). The Unsatd and ∇/mol of MGDG and DGDG of *N. laevis* were high, due to their high contents of PUFAs (Table 2). The fatty acid profile of SQDG was unique within the GLs. The major fatty acids were C16:0 (30.5% of TFA) and C18:1 (19.7% of TFA). EPA residue was only 7.9%. A high content of C16:0 in SQDG was reported in other microalgae (Alonso et al., 1998).

As for PLs, fatty acid composition of PC was characterized by high contents of C14:0, C16:0, C16:1, AA and EPA (Table 2). EPA accounted for about 23.3% of TFA in this lipid class, which is the largest fatty acid fraction. Unsaturated and ∇/mol of PC in *N. laevis* were 72.1% and 2.3, respectively. The percentage of EPA in PC accounted for about 15.9% of the total EPA, which represented the third largest fraction of total EPA. The function and application of PC were reported elsewhere in functional food and animal feed productions (Halver & Hardy, 2002; Schneider, 2001). Therefore, a high percentage of PC in the total lipids of *N. laevis* (8%), combined with its high percentage of PUFA, was quite interesting.

PI was essentially composed of C16:0, C16:1 and EPA, which accounted for 56.3% of TFA. High contents of C16:0 and EPA/docosahexaenoic acid (DHA, C22:6$n-3$) in PI were also reported in *P. tricornutum* (Alonso et al., 1998). The fatty acid composition of PG was somewhat similar to that of PC, but had more C16:0 and less C14:0. Low content of C14:0 and high contents of C16:0, C16:1 and EPA/DHA in PG have been consistently reported in some microalgae (Alonso et al., 1998; Bigogno et al., 2002). As for other PLs, LPC and DPG also had favourable content of EPA, as shown in Table 2, but their fatty acid composition could not be compared with other microalgae since few results have been reported in microalgae.

The overall distribution of EPA in the lipid pool is depicted in Fig. 2. EPA was widely distributed in the lipid classes but varied in content, ranging from 0.7% in PG and DPG to 37.4% in TAG. Most was distributed within three classes, TAG, MAG and PC, which totally accounted for 75.9% of EPA of the cell. This result was useful for EPA purification, as isolation of these three lipid fractions would lead to the recovery of about 70% of the total EPA, with high purity.

4. Conclusion

The lipid composition and fatty acid distribution in the lipid pool of *N. laevis* under heterotrophic batch cultivation was investigated in detail in this study. NLs were the major constituents (78.6%) of lipid in *N. laevis*, in which TAG was the predominant component. PC was the major component of PLs, which contained a high percentage of PUFA. EPA, as well as tetradecanoic acid (C14:0), hexadecanoic acid (C16:0) and palmitoleic acid (C16:1), were the principal fatty acids in most lipid classes. EPA was found to be distributed in all lipid fractions with 75.9% of total EPA accumulating in TAG (37.4%), MAG (22.6%) and PC (15.9%). The lipid class and fatty acids varied remarkably in microalgae under different culture processes and conditions. In order to adequately formulate EPA-enriched functional food and animal feed, the results from this study may help to find a focus for future studies, i.e., to elucidate conditions which may enhance further

accumulation of EPA in PC as PC, instead of the TAG widely used in food and feed industries.

Acknowledgements

This research was supported by a grant from the Research Grants Council of Hong Kong and the Outstanding Young Research Award of the University of Hong Kong.

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