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DHA Concentration and Purification from the Marine Heterotrophic Microalga *Crypthecodinium cohnii* CCMP 316 by Winterization and Urea Complexation

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Summary

A simple and inexpensive procedure involving saponification and methylation in wet biomass, winterization and urea complexation in a sequential way has been developed in order to concentrate docosahexaenoic acid (DHA) from *Crypthecodinium cohnii* CCMP 316 biomass. Different urea/fatty acid ratios and crystallization temperatures were tested in the urea complexation method. ANOVA test revealed that, in the studied range, the temperature had the most significant effect on the DHA concentration. The highest DHA fraction (99.2 % of total fatty acids) was found at the urea/fatty acid ratio of 3.5 at the crystallization temperatures of 4 and 8 °C. The highest DHA recovery (49.9 %) was observed at 24 °C at the urea/fatty acid ratio of 4.0, corresponding to 89.4 % DHA of total fatty acids. Considering the high proportions of DHA obtained in the non-urea complexing fractions, the current procedure was an appropriate way to concentrate and purify DHA from *C. cohnii*.

Key words: DHA, PUFAs, *Crypthecodinium cohnii,* winterization, urea complexation

Introduction

Docosahexaenoic acid (DHA, 22:6ω3) is regarded to be essential for proper visual and neurological development of infants (*1–3*). Despite being an important polyunsaturated fatty acid (PUFA) in human breast milk, in the past DHA was generally absent from infant formulae (*4*). However, the World Health Organization (WHO), the British Nutritional Foundation (BNF), the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) and the International Society for the Study of Fatty Acids and Lipids (ISSFAL) have recognized the importance of DHA and arachidonic acid (AA) and recommended that long chain PUFA should be included in preterm infant formulae (*5*). Presently, over 50 % of all infant formulae in the U.S. contain a blend of DHA and AA (*6*).

Traditional source of omega-3 fatty acids is fish oil. However, the use of fish oil as a food additive is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability. Furthermore, the presence of eicosapentaenoic acid (EPA, 20:5ω3) in fish oil is undesirable for application in infant food as this fatty acid is associated with neonate growth retardation (*7,8*). Moreover, fish oil ω3-PUFA content fluctuates widely as the fish stocks are declining. Therefore, alternative sources are of interest. Microalgae biomass is particularly suitable for extraction and purification of individual PUFA due to its stable and reliable composition. In addition, PUFA from cultured microalgae are cholesterol-free, contaminant-free (*e.g.* heavy metals, polychlorobiphenyls (PCBs)) and taste good. Attempts have been made to produce DHA phototrophically by growing microalgae in photobioreactors, but it is difficult to achieve high biomass concentration and high DHA productivity (*9*). This is due to unsolved problems, namely limited light access and oxygen accumulation, in the photo-

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autotrophic cultures (*10*). Screening of microalgae for heterotrophic production potential of DHA could therefore be of significance. The heterotrophic microalga *Crypthecodinium cohnii* is an interesting source for DHA production (*11,12*) and for research on DHA biosynthesis (*6,13–16*) due to its unique fatty acid composition. *C. cohnii* can accumulate relatively high amounts of lipids with 30–50 % DHA of the fatty acids and no other polyunsaturated fatty acid is present above 1 % (*17*). This characteristic makes the DHA purification process from this microorganism very attractive, particularly for pharmaceutical applications, since the inclusion of a PUFA as a drug component requires its purification to over 95 % (*18*).

There are several methods for concentration of ω3-PUFA, but only few are suitable for large-scale production. Winterization has been used to fraction triglycerides with different melting points that are present in edible oils and involves the chilling of the oil to allow the solid portion to crystallize and the subsequent filtration of the two phases (*19*). The melting point of fatty acids changes considerably with the type and degree of unsaturation and thus separation of mixtures of saturated and unsaturated fatty acids may become possible. At low temperatures, long chain saturated fatty acids, which have higher melting points, crystallize out and PUFA remain in the liquid form (*20*).

Urea molecules readily form solid-phase complexes with saturated free fatty acids (FFA). In this way, PUFA and branched FFA may be separated from saturated FFA. Urea complexation seems to be one of the most appropriate methods for ω3-PUFA enrichment: it allows the handling of large quantities of material in simple equipment, requires inexpensive solvents such as methanol or hexane, as well as milder conditions (*e.g.* room temperature), the separation is more efficient than with other methods such as fractional crystallization or selective solvent extraction, and the cost is lower (*21,22*). Moreover, urea complexation protects the ω3-PUFA from autoxidation (*20*).

The present work aims at the concentration and purification of DHA from the heterotrophic microalga *Crypthecodinium cohnii* using the rapid, inexpensive and simple winterization and urea complexation methods in a sequential way. Freeze-drying is a very high energy and time consuming operation (*23*), difficult to be implemented at a large-scale. In the present work the fatty acid extraction was carried out from wet biomass rather than from lyophilized cells or the extracted oil, usually reported in the literature (*9,22,24–27*), which may represent a significant economical benefit compared with the traditional procedures. It is known that urea/fatty acid ratio and crystallization temperature strongly influence the efficiency of DHA purification process (*28*). In this way, different urea/fatty acid ratios and crystallization temperatures were used in order to achieve the best concentration and purification process efficiency. As far as we are aware, this is the first work wherein winterization and urea complexation have been used in a sequential way to concentrate DHA from *C. cohnii.*

Materials and Methods

Growth conditions

Crypthecodinium cohnii CCMP 316 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) culture collection (Maine, USA) and was maintained in axenic conditions by subculturing every two weeks in the f/2+NPM medium (*29–31*) supplemented with glucose (6 g/L). Cultures were grown on 500 mL of the f/2+NPM medium suplemented with glucose (15 g/L) in 2-litre shake flasks at 120 rpm and 27 °C in the dark. Biomass was collected at the stationary phase (5 days) by centrifugation at 6000 rpm (8900 *g*) using an Avanti J-25I (Beckman Coulter, Fullerton, USA) for 15 min and then frozen for further experiments.

Saponification and transmetylation

A volume of 1116.5 mL of ethanol 96 % (by volume) and 23.5 g of KOH were added to 93.8 g of wet biomass (corresponding to 23.3 g of dry cell mass (DCM) and 20.6 g of ash free dry mass (AFDM)), according to Medina *et al*. (*22*). The mixture was incubated in an orbital shaker at 100 rpm and 20 °C overnight. Afterwards, 100 mL of distilled water were added, followed by several hexane extractions (5×200 mL) in order to separate the unsaponifiables. The hexane used in all these steps (saponification, transmethylation, winterization and urea complexation) contained 0.01 % (by mass per volume) of butylated hydroxytoluene (BHT) in order to prevent lipid degradation.

The hydroalcoholic phase, containing the soaps, was acidified to pH=1 by the addition of hydrochloride solution (1:1 by volume, HCl 37 % Merck, Darmstadt, Germany). Then the FFA were recovered by several extractions (8×200 mL) with hexane. The organic phase, containing the FFA, was dried with anhydrous sodium sulphate, and the solvent was evaporated in a vacuum rotary evaporator at 35 °C. The FFAs were then methylated according to Khozin-Goldberg *et al.* (*32*), with modifications, by adding 465.6 mL of the methylation mixture of methanol (Merck, Darmstadt, Germany) and H_2SO_4 (Merck, Darmstadt, Germany) (49:1, by volume) and heating at 80 °C for 1 hour. After cooling to room temperature, 232.8 mL of water and the same volume of hexane were added and the upper hexane layer was removed and dried with anhydrous sodium sulphate. The solvent was evaporated in a vacuum rotary evaporator at 35 °C and *n*-hexane was added (V=2.46 mL).

Fatty acid analysis

Methyl esters were analyzed by gas-liquid chromatography on a Varian 3800 gas-liquid chromatograph (Palo Alto, USA), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 μm) Supelcowax 10 (Supelco, Bellafonte PA, USA) with helium as carrier gas at a flow rate of 3.5 mL/min. The column temperature was programmed at an initial temperature of 200 °C for 8 min, then increased at 4 °C/min to 240 °C and held there for 8 min. Injector and detector temperatures were 250 and 280 °C, respectively, and split ratio was 1:50. Peak

identification was carried out using known standards (Nu-Chek-Prep, Elysian, USA). Methylheptadecanoate ester (Merck, Darmstadt, Germany) was added as internal standard.

Winterization

The mixture containing the methyl esters was stored at –18 °C overnight. Crystals were formed and afterwards the liquid fraction was separated from the crystals. The methyl esters of this fraction were quantified by GC as previously described, after the addition of 0.2 mL of methylheptadecanoate ester (5.3 mg/mL).

Urea complexation

Based on previous GC analysis of the winterized fraction containing the methyl esters, 41.0 and 35.9 µL of the liquid fraction and 0.45 mL of methanol were added to 0.105 g of urea in order to achieve an urea/fatty acid ratio of 3.5 and 4 (by mass), respectively, according to Medina *et al.* (*22*), and a methanol/urea ratio of 3.4 (by mass) (this methanol/urea ratio allowed a homogeneous and clear mixing of methyl esters with urea). The mixture was heated at 60–65 °C and stirred until the solution became clear. The urea complexes were obtained by cooling the solution at different temperatures (4, 8, 12, 20 and 24 °C) overnight, followed by an ultracentrifugation (centrifuge Sigma 2–26 K, Manchester, UK) at 14 000 rpm (20 800 *g*), at the respective complexation temperature, in order to separate the urea complexing from the non-urea complexing fractions. A volume of 0.5 mL of distilled water at 60 °C was added to both fractions and vortexed, followed by the addition of 1 mL of hexane (with 0.01 % BHT by mass per volume) to extract the methyl esters. The hexane phases from both fractions (urea complexing and non-complexing) were collected and evaporated under nitrogen atmosphere for further GC analysis. Methylheptadecanoate ester was added as internal standard, as previously described.

DHA recovery

The DHA recovery of the winterization and urea complexation steps (expressed in percentage) was calculated from the following equation:

$$\mathrm{DHA}_{\mathrm{recovery}} = \left(\frac{(m_{\mathrm{DHA}})_{\mathrm{a}}}{(m_{\mathrm{DHA}})_{\mathrm{b}}} \right) \cdot 100 \qquad /1/$$

where $(m_{\mathrm{DHA}})_{\mathrm{a}}$ is the DHA mass after the concentration/purification step and $(m_{\mathrm{DHA}})_{\mathrm{b}}$ is the DHA mass before the concentration/purification step.

Results and Discussion

In the present work, only one derivatization reaction (transmethylation rate of 98 %) was performed (Fig. 1). In this way, the methyl ester compositions of the extracts were compared under the same conditions (Tables 1 and 2).

A previous DHA concentration winterization step was carried out before the urea complexation step. According to Ganga *et al.* (*33*), this procedure is essential for subsequential urea precipitation, increasing the DHA purity. Table 1 shows *C. cohnii* cellular fatty acid composition before and after the winterization procedure in the liquid phase. The total fatty acid content of *C. cohnii* biomass was 9.9 % of DCM, which is similar to the one reported for *Isochrysis galbana* (9.5 %) (*22*). DHA was the major fatty acid (32.6 % of total fatty acids). The other major fatty acids were 12:0, 14:0, 16:0 and 18:1ω9. After the winterization procedure, the saturated fatty acid fraction dropped in the liquid phase, and 12:0 and 14:0 were the fatty acids which showed the strongest decrease (31 and 21 % of the initial values, respectively), whilst the unsaturated fatty acid fraction increased. DHA was the polyunsaturated fatty acid which showed the highest increase (26 % of the initial value) in the liquid phase and the recovery was 70.1 %.

Different urea/fatty acid ratios were used in the urea complexation, based on Medina *et al.* (*22*). These authors stated that the most efficient urea/fatty acid ratios for ω3-PUFA concentration from cod liver oil were 3 and 4. Table 2 shows the fatty acid composition of urea complexing and non-urea complexing fractions at different urea/fatty acid ratios and crystallization temperatures. Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å diameter. However, in the presence of long straight-chain molecules it crystallizes in a hexagonal structure with channels of 8–12 Å diameter within the hexagonal crystals. In the presence of long-chain unbranched molecules, the formed channels are sufficiently large to accommodate aliphatic chains. The presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea, thus monoenes are more readily complexed as compared to dienes, which, in turn, are more readily complexed than trienes (*20*,*34*). This explains why a large fraction of saturated and monounsaturated fatty acids was incorporated in the urea complexes (63.7–81.7 % of total fatty acids).

The saturated fatty acid which was present in the highest proportion in the non-urea complexing fraction was 12:0 (0.5–5.7 % of total fatty acids). In fact, shorter chain saturated fatty acids may not complex with urea during the crystallization process (*35*). The fractions of 14:0, 16:0, 16:1ω9 and 22:5ω3 in the non-urea complexing fraction were less than 2 % of total fatty acids; 18:0 was absent. As a result, non-urea complexing fractions with high proportions of DHA were obtained (89.4–99.2 % of total fatty acids).

A two factor analysis of variance (ANOVA) was performed on the data in order to find out which of the tested variables (urea/fatty acid ratio or temperature) had the most significant effect on the results. The ANOVA table decomposes the variability of data into contributions due to factors (*21*). Therefore, the variable that showed the most significant effect on the data was the temperature (F-ratio=22.32; p=0.005), when compared with the urea/fatty acid ratio (F-ratio=3.89; p=0.12), α=0.05.

Wanasundara and Shahidi (*28*) used an experimental design to optimize the conditions that led to the maximum concentration of EPA and DHA from seal blubber oil, using the urea method concentration. The authors obtained 88.2 % of total ω3 fatty acids at an urea/fatty acid ratio of 4.5, a crystallization time of 24 h and a

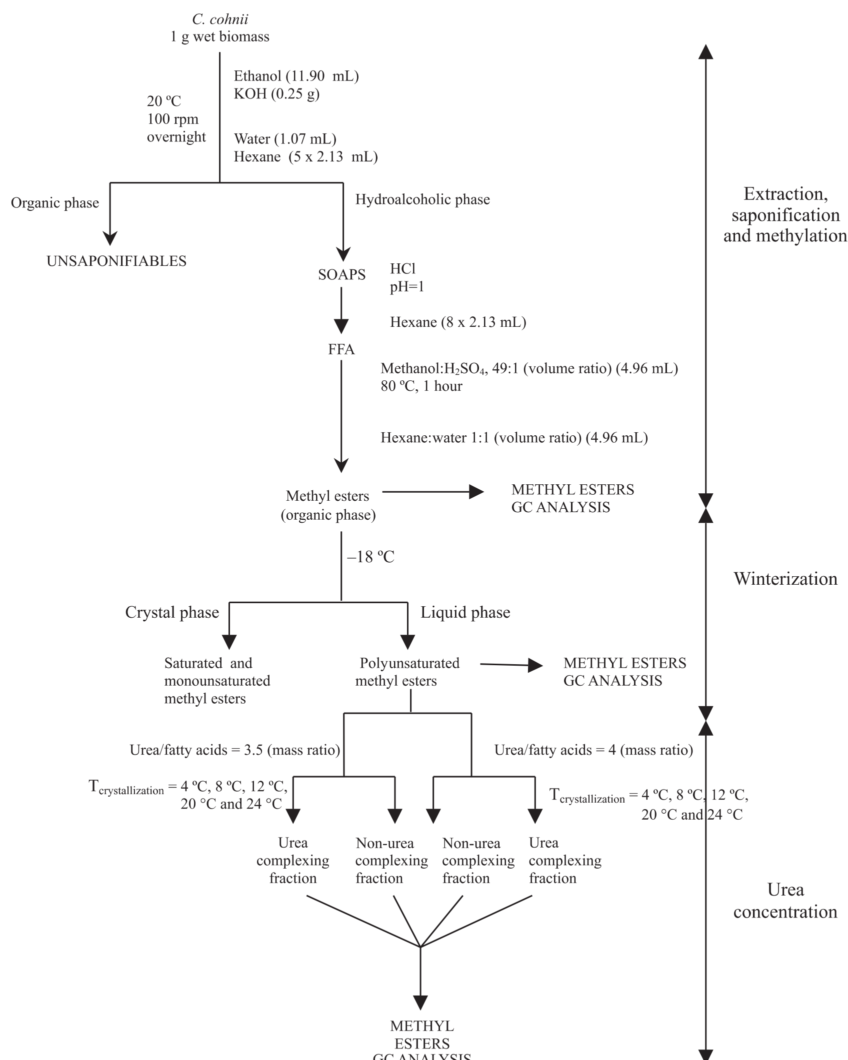

Fig. 1. Scheme for the DHA concentration and purification from *C. cohnii*

Table 1. *C. cohnii* fatty acid composition (total fatty acids/%) before winterization and in liquid fraction after winterization

	12:0	14:0	16:0	16:1ω9	18:0	18:1ω9	22:5ω3	22:6ω3
Before winterization	15.7±0.8	26.3±0.8	18.1±0.3	0.5±0.0	1.2±0.0	5.6±0.1	0.1±0.0	32.6±1.8
After winterization (in liquid fraction)	10.8±0.64	20.78±0.28	17.0±0.06	0.4±0.2	1.3±0.0	6.1±0.1	0.2±0.1	41.0±0.5

Data are represented as the average± standard deviation of two GC injections

crystallization temperature of –10 °C. However, the DHA purification usually requires a further preparative HPLC step. Grima *et al*. (*36*) used the urea complexation method to concentrate PUFAs from the marine microalga *Isochrysis galbana* biomass fatty acids, at the urea/fatty acid ratio of 4 and 4 °C. The stearidonic acid (SA), EPA and DHA separation was then obtained by HPLC and the SA, EPA and DHA fraction purities were 94.8, 96.0 and 94.9 %, respectively. Such purification steps are not easily implemented at a large scale. In the present work, the fractions with the highest DHA fraction (99.2 % of total fatty acids) were obtained at the urea/fatty acid ratio of 3.5, and the temperatures of 4 and 8 °C (Table 2, Fig. 2a). Fractions with such proportions of DHA may not require further purification steps, which are of major concern in an industrial bioprocess, since they are usually the most expensive. After this maximum, the DHA fraction steadily decreased, reaching a minimum at 24 °C (93.0 % at the urea/fatty acid ratio of 3.5 and 89.4 % at the urea/fatty acid ratio of 4).

As expected, the DHA fractions of total fatty acids in the urea complexing fractions (18.2–35.8 %) were much lower than those found in the non-urea complexing fractions. However, these fractions decreased with the increase of temperature (from 4 to 8 °C), remaining almost unchanged at higher temperatures (Fig. 2b).

As stated before (*36*), purity is susceptible to improvement only at the expense of a decrease in yield. This is exemplified in Fig. 3, where DHA recoveries in both

Table 2. Fatty acid composition (total fatty acids/%) of both urea complexing and non-urea complexing fractions as a function of the urea/fatty acid ratio and crystallization temperature

Fraction	Urea/Fatty acids (by mass ratio)	Crystallization temperature/°C	12:0	14:0	16:0	16:1ω9	18:0	18:1ω9	22:5ω3	22:6ω3
Urea complexing fraction	3.5	4	15.3±1.4	23.5+2.1	16.8+1.4	0.5+0.1	1.2+0.1	6.4+0.6	0.3+0.1	35.8+5.0
		8	18.8±1.6	29.8±2.6	21.1±1.7	0.4±0.2	1.5±0.1	7.7±0.4	0.1±0.0	20.5±4.8
		12	18.6±1.6	30.8±0.6	22.4±1.2	0.5±0.0	1.6±0.2	7.8±0.6	0.1±0.0	18.2±0.8
		20	16.8±1.5	30.8±1.3	23.2±0.3	0.5±0.1	1.7±0.0	8.6±0.2	0.1±0.0	18.4±2.6
		24	15.9±1.1	31.0±0.4	22.5±1.2	0.4±0.0	1.6±0.2	8.5±0.5	0.1±0.0	19.9±0.6
	4	4	17.6±1.1	26.9±2.1	19.7±1.2	0.6±0.0	1.4±0.1	6.8±0.4	0.2±0.1	32.8±4.5
		8	18.5±1.7	29.6±1.6	21.7±2.1	0.6±0.4	1.6±0.2	7.8±0.3	0.1±0.0	20.2±4.4
		12	17.6±1.5	29.0±0.6	21.4±1.0	0.5±0.0	1.6±0.2	8.3±0.6	0.1±0.0	21.42±0.5
		20	15.8±1.5	29.8±1.6	24.4±1.4	0.4±0.0	1.8±0.1	8.0±0.3	0.1±0.0	19.6±4.5
		24	11.4±2.8	29.0±2.0	27.1±2.4	0.3±0.0	2.3±0.4	8.7±1.1	0.1±0.1	21.1±1.4
Non-urea complexing fraction	3.5	4	0.5±0.2	0.1±0.2	0.1±0.1	0	0	0	0.1±0.1	99.2±0.3
		8	0.5±0.1	0	0	0	0	0	0.3±0.0	99.2±0.2
		12	1.3±0.5	0	0	0	0	0.1±0.0	0.3±0.0	98.3±0.6
		20	3.3±1.3	0.3±0.1	0	0.2±0.1	0	0.6±0.4	0.3±0.0	95.2±1.9
		24	4.4±0.6	0.6±0.1	0	0.3±0.0	0	1.4±0.2	0.2±0.2	93.0±1.1
	4	4	0.6±0.2	0.1±0.1	0	0	0	0	0.2±0.0	99.0±0.2
		8	1.0±0.1	0.1±0.1	0	0	0	0	0.3±0.1	98.5±0.2
		12	1.4±0.6	0	0	0	0	0.2±0.1	0.3±0.1	98.1±0.7
		20	4.1±0.8	0.6±0.4	0.1±0.1	0.3±0.1	0	1.2±0.7	0.4±0.0	93.3±2.0
		24	5.7±0.3	1.7±1.5	0.1±0.0	0.4±0.0	0	2.6±0.1	0.4±0.0	89.4±0.4

Data are represented as the average±standard deviation of two independent replications and four GC injections

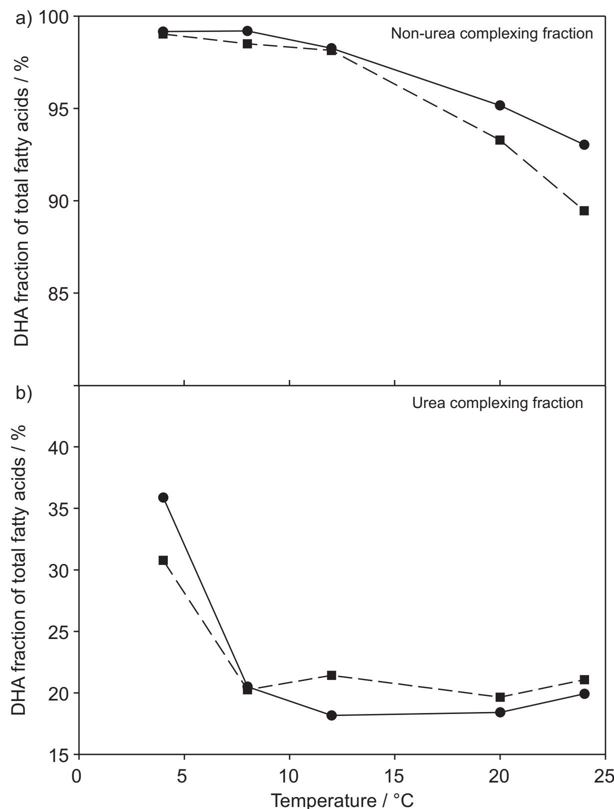

Fig. 2. DHA percentage of total fatty acids in the non-urea complexing (a) and urea complexing (b) fractions, at different crystallization temperatures and urea/fatty acid ratios
—●— U/FA = 3.5 (mass ratio)
—■— U/FA = 4.0 (mass ratio)

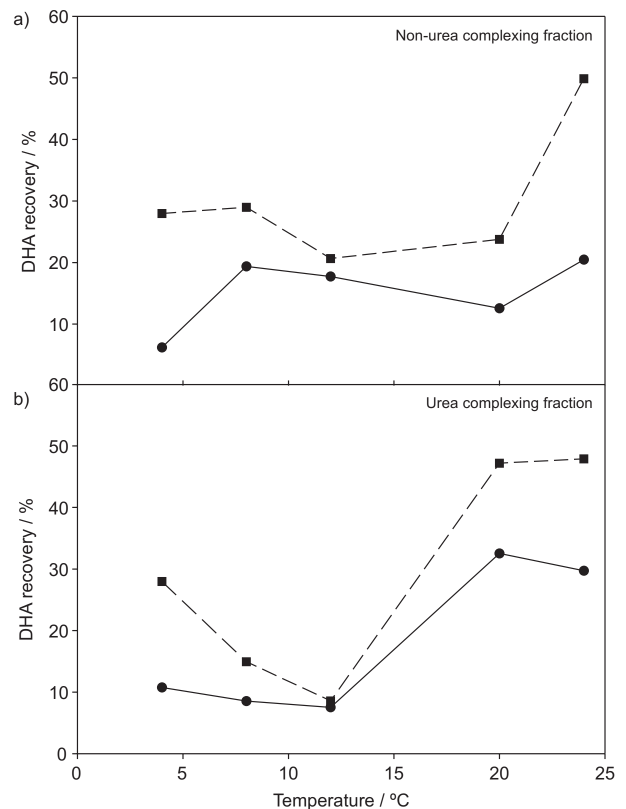

Fig. 3. DHA recoveries in the non-urea complexing (a) and urea complexing (b) fractions at different crystallization temperatures and urea/fatty acid ratios
—●— U/FA = 3.5 (mass ratio)
—■— U/FA = 4.0 (mass ratio)

non-urea complexing and urea complexing fractions are plotted against temperature, at different urea/fatty acid ratios. Lower DHA recoveries in the non-urea complexing fractions (4–12 °C) corresponded to higher DHA fractions (of total fatty acids), whereas higher DHA recoveries (24 °C) were obtained at lower DHA fractions (Figs. 2 and 3). The highest DHA recovery (49.9 %) was obtained at 24 °C, at the urea/ratio of 4.0, corresponding to 89.4 % of DHA of total fatty acids. Bearing this in mind, a new serial urea complexation recycling steps could be applied to the urea complexing fraction in order to recover the DHA mass in this fraction (47 % of the initial DHA mass), increasing the DHA proportion in the non-urea complexing fraction. In this way, the urea could be recovered and used in further purification cycles and the overall DHA recovery of the urea complexation step could be increased.

The fractions with the highest proportion of DHA (99.2 % of total fatty acids) corresponded to DHA recoveries of 6.2 % (4 °C) and 19.4 % (8 °C) (Figs. 2 and 3). Obviously, the best conditions to produce DHA depend on the end use of the product. Pharmaceutical applications require DHA purity above 95 %, as already mentioned, thus the DHA fractions obtained in this work with purity higher than 95 % may be suitable for this purpose.

Senanayake and Shahidi (*27*) concentrated DHA from the oil extracted from the microalga *C. cohnii* and reported a DHA enrichment from 47.4 to 97.1 % with a process yield of 32.5 % of the mass of the original algal oil. However, the present work presents an alternative fatty acid extraction, saponification and methylation process carried out in wet biomass that may represent a significant economical benefit when compared with the traditional procedures (usually carried out in extracted oils or lyophilized cells), which are expensive and time consuming. Moreover, this inexpensive and simple DHA purification method can be easily scaled-up from the marine heterotrophic microalga *Crypthecodinium cohnii*.

Conclusions

The current procedure (saponification and methylation performed in wet biomass, winterization and urea complexation in a sequential way) is an appropriate way for concentration and purification of DHA from *C. cohnii*, considering the high proportions of DHA obtained in non-urea complexing fractions.

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