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Influence of extraction solvent system on the extractability of lipid components from the biomass of *Nannochloropsis gaditana*

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Abstract Microalgae oils are considered to be promising alternative sources of omega-3 LC-PUFA. The aim of this work was therefore to evaluate different solvent (mixtures), currently accepted for use in the food industry, for the extraction of lipids from *Nannochloropsis gaditana*, an omega-3 LC-PUFA-rich microalga. Importantly, not only the total lipid yield but also the lipid class, eicosapentaenoic acid, carotenoid, and sterol yield were investigated. It was shown that the highest yield for each of the components was obtained with dichloromethane/ethanol (1:1). All extracts except the one obtained with dichloromethane/ethanol (1:1) were enriched in neutral lipids and depleted in polar lipids, when compared to the total lipid extract (chloroform/methanol 1:1). Hexane/isopropanol (3:2) seems to be the second best option: it has the advantage of performing better at criteria such as toxicity, but has the disadvantage that almost half of the interesting oil cannot be recovered.

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Introduction

The health benefits of the long-chain omega-3 polyunsaturated fatty acids (omega-3 LC-PUFA), such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are widely recognized (Gogus and Smith 2010). But, despite this rising awareness of their importance, their daily intake is in most countries still below the recommended dose (Meyer 2011). At present, the main commercial source of omega-3 LC-PUFA is fish oil. There are however several issues associated with this source; the most important being the global decline in fish stocks. It is widely accepted that global fisheries cannot sustainably supply the global needs for omega-3 LC-PUFA (Crawfield 2011). There is thus a need for new sources of omega-3 LC-PUFA to answer the growing demand from the market.

Microalgae are the primary producers of EPA and/or DHA in the marine environment and are thus the most promising alternative source for omega-3 LC-PUFA (Khozin-Goldberg et al. 2011). In a previous study (Ryckebosch et al. 2013), we showed that total lipid extracts of several microalgae, such as *Isochrysis* (DHA), *Nannochloropsis* and *Phaeodactylum* (EPA), and *Pavlova* and *Thalassiosira* (EPA and DHA) are sufficiently rich in omega-3 LC-PUFA to serve as a potential alternative for fish oil. The study also showed that an important part of the omega-3 LC-PUFA is present in the glyco- and phospholipid fraction of the microalgae oils. This can be interesting, since there are indications that omega-3 LC-PUFA associated with these polar lipids are on the one hand more efficiently absorbed (Schuchardt et al. 2011) and

more resistant to oxidation (Lyberg et al. 2005; Yamaguchi et al. 2012) than those associated with triglycerides. Furthermore, we showed that microalgae oils also contain phytosterols and carotenoids, components that may provide added value to the microalgae omega-3 LC-PUFA oils because of their nutritional importance and, in the case of carotenoids, also their protective effect against omega-3 LC-PUFA oxidation (Ryckebosch et al. 2013).

Because of this specific composition (omega-3 LC-PUFA as phospho- and glycolipids and presence of phytosterols and carotenoids), extraction technologies commonly used for fish or vegetable oils will probably not be the most suitable for extraction of microalgae oils. Some research on the extraction of lipids from microalgae has already been performed. Unfortunately, most of this has been conducted in the context of biodiesel production, and thus focused on the recovery of the neutral lipids only.

The method of Bligh and Dyer (1959), which uses a monophasic ternary system of chloroform/methanol/water in a ratio 1:2:0.8, is the most commonly used method for the quantitative extraction of lipids from microalgae at analytical level in the last 50 years (Molina Grima et al. 2013). Balasubramanian et al. (2013), Lee et al. (1998) and Ryckebosch et al. (2012) also showed, specifically for microalgae, that a mixture of chloroform and methanol yields the highest extraction efficiency. This solvent mixture however has the disadvantage that chloroform and methanol are toxic solvents. Therefore, large scale and workable lipid extraction using this method is prohibited due to environmental and health risks (Molina Grima et al. 2013) and as such other solvents have been evaluated.

Balasubramanian et al. (2013) showed that dichloromethane/methanol (2:1) performed equally well as chloroform/methanol. When only a nonpolar solvent such as hexane or diethyl ether is used, a much lower lipid yield is obtained (Balasubramanian et al. 2013; Ryckebosch et al. 2012). Addition of polar alcohols to a nonpolar solvent improves its extraction efficiency. Among those mixed-polarity solvent mixtures, Balasubramanian et al. (2013) showed that hexane/methanol (3:2) performed best (when compared to hexane/isopropanol (3:2) and hexane/ethanol (2:1)) although still considerably worse than the chlorinated solvent systems to extract lipids from *Nannochloropsis* sp. using an accelerated solvent system. Long and Abdelkader (2011) selected cyclohexane/1-butanol (9:1) as the best solvent (compared to cyclohexane/isopropanol (2:1) and hexane/isopropanol (3:1)) to extract lipids from *Nannochloropsis* sp. using a Soxhlet extraction. Recent research also shows promising results on "new solvents" such as terpenes (Dejoye Tanzi et al. 2012) and "switchable hydrophilicity solvents" such as tertiary amines (Boyd et al. 2012) for lipid extraction from microalgae.

Next to the extraction yield of the desired component(s), some additional criteria however should be taken into account in solvent selection. First of all, the solvent should be easy to recover, meaning that the boiling point should be sufficiently low. Secondly, the viscosity should be sufficiently low to ensure high mass transfer efficiency. Thirdly, the melting temperature must be below ambient to guarantee ease of handling, and the surface tension should be low to promote wetting of the solids. A suitable solvent should also not be too soluble in water and have a considerably different density than water. For food processing, only nontoxic solvents should be taken into consideration. Corrosive solvents have the disadvantage that they increase equipment cost and might also require expensive pre- and posttreatment of streams. It is also important that the solvent is thermally and chemically stable so it can be recycled. Solvents should also be readily available at a reasonable cost and the environmental impact of the whole extraction procedure should be minimal (Bart 2011; Mercer and Armenta 2011).

The purpose of this study was to evaluate different solvent systems for the extraction of lipids from *Nannochloropsis gaditana*, an EPA-rich microalga, in the context of their use as source of omega-3 LC-PUFA for application in the food industry. Therefore, only extraction solvents allowed in the food industry (dichloromethane, ethanol, hexane, isopropanol, acetone, and ethyl acetate) have been investigated. The choice of solvents to investigate was based on those already suggested in literature for lipid and carotenoid extraction from microalgae (Ryckebosch et al. 2012, Guckert et al., 1988, Lee et al. 1998; Kim et al. 2012). Importantly, not only the lipid yield was considered but also the lipid class, EPA, carotenoid, and sterol yield were investigated. To the best of our knowledge, no studies have so far looked at co-extraction of these other nutritionally interesting components. The extraction efficiency of the different solvent systems was compared to the efficiency of the chloroform/methanol (1:1) extraction, which has proven to extract total lipids from the microalgae biomass (Ryckebosch et al. 2012). This method will therefore be considered as the reference method.

Materials and methods

Batch-cultured biomass of the omega-3 LC-PUFA producing photoautotrophic microalga *N. gaditana* was obtained from LGem (Voorhout, The Netherlands) in the lyophilized state. The composition of the lipid part of the biomass is detailed in Table 1.

Extractions

Six solvent systems often used in literature were tested: dichloromethane (high-performance liquid chromatography (HPLC) grade, Labscan)/ethanol (96 %, Bedalab) (1:1),

Table 1 Composition of the lipid part of the biomass of *N. gaditana*

Total lipid content (g/100 g DW)	35±2
Neutral lipid content (g/100 g DW)	14±1
Glycolipid content (g/100 g DW)	11±1
Phospholipid content (g/100 g DW)	10.3±0.7
EPA content (g/100 g DW)	5±1
Carotene content (mg/100 g DW)	68±18
Diadinoxanthin content (mg/100 g DW)	66±9
Violaxanthin content (mg/100 g DW)	520±53
Zeaxanthin content (mg/100 g DW)	81±25
Cholesterol content (mg/100 g DW)	360±47
Phytosterol content (mg/100 g DW)	405±44

hexane (gas chromatography (GC) grade, Labscan), hexane/isopropanol (HPLC grade, Acros) (3:2), ethanol, acetone (HPLC grade, Labscan), ethyl acetate (HPLC grade, Sigma-Aldrich)/hexane (2:3), and ethyl acetate. Chloroform (HPLC grade, Labscan)/methanol (HPLC grade, Sigma-Aldrich) (1:1) extracts total lipids (Ryckebosch et al. 2012) and will therefore be considered as the reference method. The chloroform/methanol and dichloromethane/ethanol extractions were performed according to the method previously described by Ryckebosch et al. (2012). In summary, 100 mg of biomass was extracted four times with the respective solvent mixture: twice with, and twice without, the addition of water. A cell disruption method was proven not to be necessary (Ryckebosch et al. 2012) for chloroform/methanol extraction and was therefore also not used with the similar dichloromethane/ethanol system. The extractions using the other solvent (mixtures) were performed somewhat differently. The microalgae cells were disrupted with a bead beater (Qiagen Tissuelyser), twice for 60 s at 30 Hz, according to the manual of the apparatus. Subsequently, the biomass (100 mg) was extracted four times with 6 mL of the extraction solvent (mixture). All extractions were performed nine times.

Lipid part of the extract

Some of the solvent (mixtures) may not only extract lipids but also nonlipid polar components such as carbohydrates or proteins. To eliminate these nonlipid polar components, the lipid part of the extracts obtained with the nonhalogenated solvent (mixtures) was determined. To do so, the extracts were dissolved in 8 mL chloroform/methanol (1:1) and washed with 2 mL water. The aqueous layer, containing the nonlipid material such as polysaccharides and proteins, was removed, while the organic layer, containing the lipids, was dried and weighed. This procedure was based on the method for the total lipid content determination developed in our laboratory and described in Ryckebosch et al. (2012). All further analyses were performed on this lipid part of the extract. Elimination of the nonlipid components was already performed during the chloroform/methanol and dichloromethane/ethanol extraction itself and was therefore not repeated.

Analysis of lipid class content

The lipid class content of the extracts was determined using silica solid phase extraction (SPE) (Christie 2003; Chen et al. 2007 with adjustments previously described by Ryckebosch et al. 2012). Quantitative determination of the lipid class content was performed in triplicate.

Analysis of the fatty acid content and composition

To determine the fatty acid content, the lipid extracts were methylated according to Christie (2003) with slight adjustments previously described by Ryckebosch et al. (2012). The fatty acid methyl esters (FAMEs) were separated by gas chromatography with cold on-column injection and flame ionization detection (FID) (Trace GC Ultra, Thermo Scientific, Belgium). An EC Wax column of length 30 m, internal diameter (ID) 0.32 mm, film 0.25 μm (Grace, Belgium) was used with the following time–temperature program: 70–180 °C (5 °C min^{-1}), 180–235 °C (2 °C min^{-1}), 235 °C (9.5 min). Peak areas were quantified with ChromCard for Windows software (Interscience, Belgium). FAME standards (Nu-check, USA) containing a total of 35 different FAMEs were analyzed for provisional peak identification, which was then confirmed by use of GC–mass spectrometry (MS) (Trace GC Ultra, ISQ Single Quadrupole MS, Thermo Scientific) using an Rxi-5 Sil MS column of length 20 m, ID 0.18 mm, film 0.18 μm (Restek, Belgium). For quantification, an internal standard (C12:0) was added to the oil before methylation. A conversion factor based on the difference in molecular weight between the FA and the FAME (0.956) was used to calculate the amount of FA from the amount of FAME. The analysis was performed in triplicate.

Analysis of carotenoid content and composition

For the determination of the carotenoid content and composition, 2 mg of the extract was dissolved in 10 mL methanol. This solution and a 1/10 dilution were analyzed by HPLC coupled to a photodiode array detector (PAD) (Alliance, Waters, Belgium) according to Wright et al. (1991). For quantification, calibration curves were composed for each carotenoid. Alloxanthin, diadinoxanthin, diatoxanthin, lutein, neoxanthin, violaxanthin, and zeaxanthin were purchased from DHI (Denmark). β-carotene was purchased from Sigma-Aldrich. When the area of a carotenoid exceeded the calibration curve, the 1/10 dilution was used to quantify. The analysis was performed in triplicate.

Analysis of cholesterol and phytosterol content

For the determination of the sterol content, 5β-cholestan-3α-ol (200 μg; Sigma-Aldrich) was, for quantification purposes, first added to extracts. Then, saponification was performed according to Abidi (2004), with some modifications. Briefly, 10–20 mg of the oil was stirred overnight with potassium hydroxide (1 M) in ethanol (4 mL). Water (4 mL) was added to the reaction mixture followed by three sequential extractions with diethyl ether (8 mL). The ether extracts were combined, and the solvent was removed using a rotary evaporator, giving the nonsaponifiable fraction. Finally, the sterol components were silylated according to Toivo et al. (2000). For this, anhydrous pyridine (200 μL) and derivatization reagent (200 μL) containing BSTFA (99 %) and TMCS (1 %) were added to the nonsaponifiable fraction. To complete the silylation, solutions were incubated at 60 °C for 1 h. Before GC-analysis, the solution was diluted with 600 μL hexane. The silylated sterols were separated by gas chromatography with cold on-column injection and FID. An Rtx-5 column (length 30 m, ID 0.25 mm, film 0.25 μm) (Restek) was used with the following time–temperature program: 200–340 °C at 15 °C/min, 340 °C (10 min). Peak areas were quantified with ChromCard for Windows software (Interscience). Peak identification was confirmed by use of GC–MS (Trace GC Ultra, ISQ Single Quadrupole MS, Thermo Scientific) using an Rxi-5 Sil MS column of length 20 m, ID 0.18 mm, film 0.18 μm (Restek). Cholesterol was identified separately. All other peaks showing a sterol backbone were summed, resulting in the total phytosterol content. The analysis was performed in triplicate.

Statistics

Multiple results were statistically evaluated using one-way analysis of variance (ANOVA) and a post hoc Tukey test with $\alpha = 0.05$. Single results were statistically compared to 100 using a one-sample t test with $\alpha = 0.05$ (Sigmaplot 11, Systat Software Inc., Chicago).

Results

Extraction yield

The crude extraction yield obtained with the different solvent systems and the division into the lipid and nonlipid part is shown in Fig. 1. The total lipid content of the microalgae biomass, as determined with the reference method, was 35± 2 g/100 g dry weight. Table 2 shows the total lipid recoveries (in percent) obtained in this study as well as the recoveries obtained by other authors. Dichloromethane/ethanol was almost as efficient as chloroform/methanol reaching a 92 % recovery (Table 2). Between 10 and 20 g lipids/100 g dry weight could be extracted using the different nonhalogenated solvent (mixtures), which is equivalent to a recovery of 27 to 58 % (Table 2). Extraction with ethanol gave the highest extraction yield, but only 60 % of the extract consisted of lipids, leading to a lipid recovery of only 52 %. Ethyl acetate/hexane lead to more or less the same recovery, but looked more interesting since a lower nonlipid part (< 5 %) was co-extracted when using this solvent. With hexane, the solvent often used commercially for extraction of food lipids, only

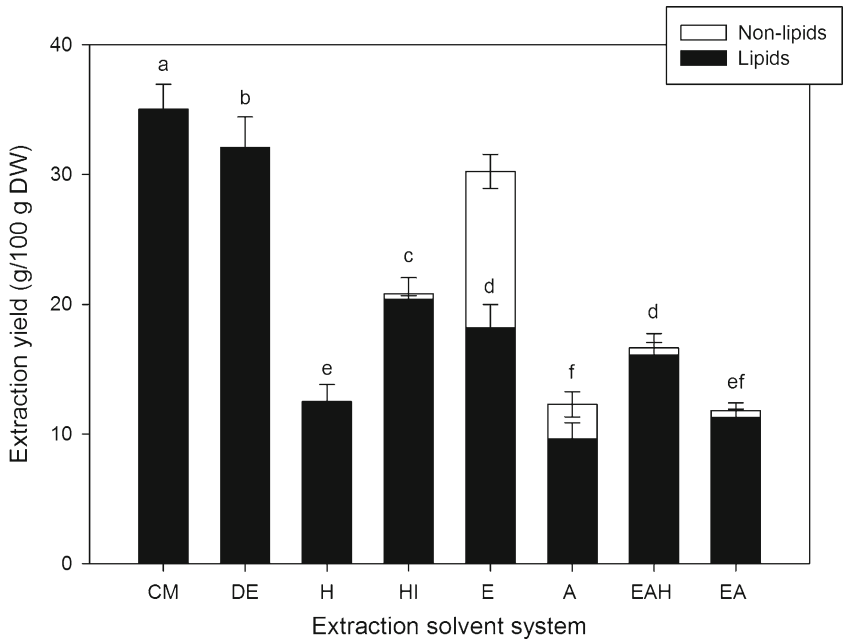

Fig. 1 Extraction yield (in g/100 g DW; mean±SD; $n = 9$) from *N. gaditana* obtained with different solvent systems: *CM* chloroform/methanol, *DE* dichloromethane/ethanol, *H* hexane, *HI* hexane/isopropanol, *E* ethanol, *A* acetone, *EAH* ethyl acetate/hexane, *EA* ethyl acetate. The crude extract was further divided in the lipid and nonlipid part. *Lowercase letters* on top of bars indicate significance of difference ($\alpha = 0.05$)

Table 2 Recovery of total lipids (in percent from total as obtained by CM reference method; mean±SD; $n=3$) from *N. gaditana* using different solvent systems compared with results from literature

Solvent	This study	Balasubramanian et al. (2013)	Lee et al. (1998)	Long and Abdelkader (2011)	Guckert et al. 1988	Ryckebosch et al. (2012)
Dichloromethane/ ethanol	92±8 a	96.5	63			56
Hexane	36±4 d	16		59		
Hexane/Isopropanol	58±3 b	19	70	64	49	43
Ethanol	52±6 bc					
Acetone	27±4 e					47
Ethyl acetate/Hexane	46±4 c					
Ethyl acetate	32±3 de					

Lowercase letters after the values indicate significance of difference ($\alpha=0.05$) between results of different solvents in this study

36 % of the total lipids could be recovered. The highest recovery among the nonhalogenated solvents was obtained with hexane/isopropanol, although still only 58 % of the total lipids could be recovered. An advantage of extraction with hexane/isopropanol is also the limited nonlipid part (<5 %) of this extract. Using acetone and ethyl acetate, the lowest amount (around 30 %) of total lipids could be recovered. The nonlipid part of the acetone extract was rather high (22 %), while it was limited in the ethyl acetate extract.

Lipid class content

The lipids extracted from *N. gaditana* with the different solvent systems were further divided into three lipid classes (neutral lipids (NL), glycolipids (GL), and phospholipids (PL)) and the results are summarized in Fig. 2. The biomass contained 14±1 g NL/100 g dry weight, 11±1 g GL/100 g dry weight, and 10.3±0.7 g PL/100 g dry weight, and using these values, the recoveries for the different lipid classes were calculated and are presented in Table 3.

A very clear distinction between dichloromethane/ethanol and the nonhalogenated solvents could be observed. While dichloromethane/ethanol extracted the three lipid classes with more or less the same efficiency, all other solvent (mixtures) more easily extracted the NL than the polar lipids (GL+PL). All the obtained extracts were thus enriched in NL and depleted in GL and PL, when compared to the total lipid extract.

Within the nonhalogenated solvents, the extraction recovery of NL varied between 44 and 95 % with the highest recovery obtained with hexane/isopropanol and ethyl acetate/hexane and the lowest with hexane, ethyl acetate, and acetone. For the GL and PL, values between 12 and 41 % were obtained. For the GL, hexane/isopropanol showed to be the most efficient solvent, while for the PL ethanol was the most

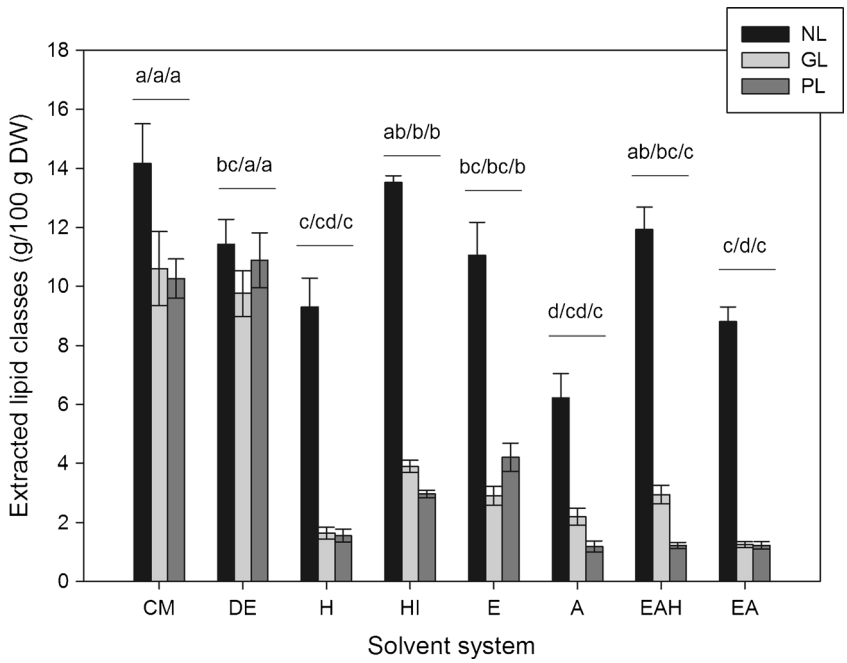

Fig. 2 Amount of the different lipid classes (neutral lipids (*NL*), glycolipids (*GL*) and phospholipids (*PL*)) (in g/100 g DW; mean±SD; $n=3$) extracted from *N. gaditana* with different solvent systems: *CM* - chloroform/methanol, *DE* dichloromethane/ethanol, *H* hexane, *HI* hexane/isopropanol, *E* ethanol, *A* acetone, *EAH* ethyl acetate/hexane, *EA* ethyl acetate. *Lowercase letters* on top of bars indicate significance of difference ($\alpha=0.05$) for NL, GL, and PL

Table 3 Recovery of lipid classes, EPA, carotenoids, and sterols (in percent from total as obtained by CM reference method; mean±SD; $n=3$) from *N. gaditana* using different solvent systems

	Dichloromethane/ethanol	Hexane	Hexane/Isopropanol	Ethanol	Acetone	Ethyl acetate/Hexane	Ethyl acetate
Lipid class recovery							
Neutral Lipids (NL)	81±10 ab*	66±9 bc	95±9 a*	78±11 ab*	44±7 c	84±10 ab*	62±7 bc
Glycolipids (GL)	92±13 a*	15±3 c	37±5 b	27±4 bc	21±4 c	28±4 bc	12±2 c
Phospholipids (PL)	106±11 a*	15±2 c	29±2 b	41±5 b	12±2 c	12±1 c	12±1 c
Fatty acid recovery							
EPA (C20:5 n-3)	74±19 a*	12±3 c	25±5 bc	45±14 b	30±8 bc	22±5 bc	25±5 bc
Carotenoid recovery							
Carotene	75±25 ab*	66±19 ab*	37±12 b	63±18 ab*	47±15 b	48±13 b	43±12 b
Diadinoxanthin	113±25 a*	19±3 c	46±13 bc	56±10 b	31±6 bc	39±8 bc	39±6 bc
Violaxanthin	91±12 a*	8±1 c	37±5 b	40±6 b	31±5 b	25±3 b	30±4 b
Zeaxanthin	117±39 a*	21±7 c	51±17 b	61±20 bc	43±15 c	40±13 c	44±15 c
Sterol recovery							
Cholesterol	106±16 a*	33±5 d	63±11 cd	96±16 ab*	71±14 bc	56±8 cd	58±9 cd
Phytosterols	96±13 a*	40±6 c	62±9 bc	81±13 ab*	59±11 bc	51±6 c	52±7 c

Lowercase letters after the values indicate significance of difference ($\alpha=0.05$) in one row. * indicates that the recovery is not significantly different ($\alpha=0.05$) from 100 %

efficient, followed by hexane/isopropanol. Hexane and ethyl acetate performed worst in extracting GL and PL. Similarly, Balasubramanian et al. (2013) also saw the lowest polar lipid recovery with hexane, while they obtained the highest NL recovery with the hexane/acetone/methanol (3:1:1) mixture.

The results of the lipid class composition can also be used to explain the total lipid recovery. Dichloromethane/ethanol extracts the same amount of GL and PL as chloroform/methanol, but a slightly lower amount of NL, leading to a lipid recovery of 92 %. Hexane/isopropanol and ethyl acetate/hexane extract a similar amount of NL as chloroform/methanol, together with a low amount (~1/3) of GL and PL, leading to a final lipid recovery of only 52–58 %. Hexane, ethyl acetate, and acetone are single solvents only able to extract about 60 % of NL and 15 % of PL and GL leading to a total recovery of around 30 %.

EPA content

The amount of EPA extracted with the different solvent systems is summarized in Fig. 3. The total lipid extract showed

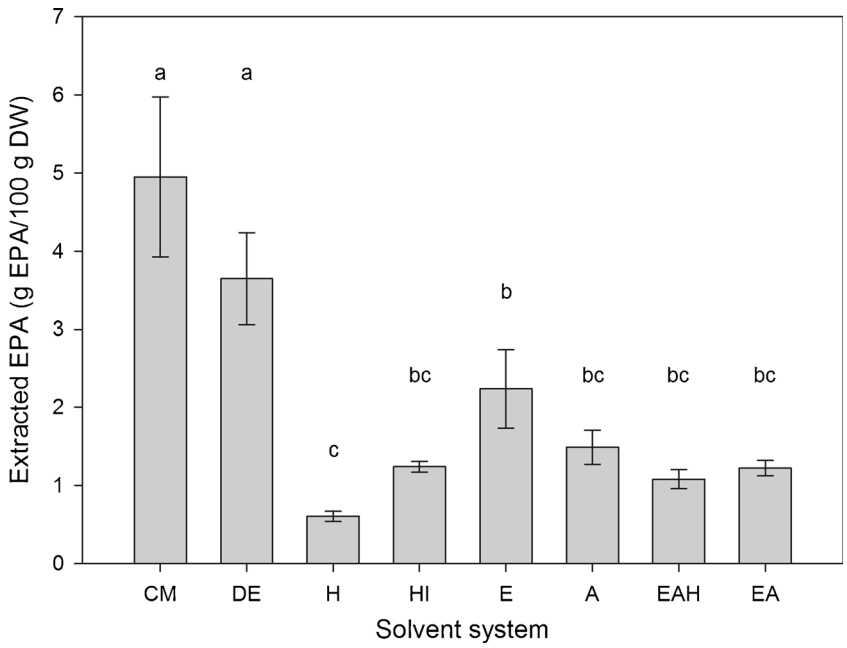

Fig. 3 Amount of EPA (in g/100 g DW; mean±SD; $n=3$) extracted from *N. gaditana* with different solvent systems: *CM* chloroform/methanol, *DE* dichloromethane/ethanol, *H* hexane, *HI* hexane/isopropanol, *E* ethanol, *A* acetone, *EAH* ethyl acetate/hexane, *EA* ethyl acetate. *Lowercase letters* on top of bars indicate significance of difference ($\alpha=0.05$)

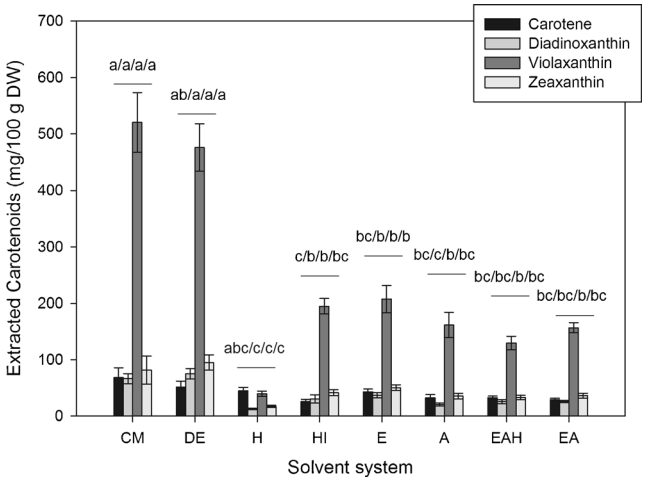

Fig. 4 Amount of carotenoids (in mg/100 g DW; mean±SD; $n=3$) extracted from *N. gaditana* with different solvent systems: *CM* chloroform/methanol, *DE* dichloromethane/ethanol, *H* hexane, *HI* hexane/isopropanol, *E* ethanol, *A* acetone, *EAH* ethyl acetate/hexane, *EA* ethyl acetate. *Lowercase letters* on top of bars indicate significance of difference ($\alpha=0.05$) for carotene, diadinoxanthin, violaxanthin and zeaxanthin respectively

that the biomass contained 5±1 g EPA/100 g dry weight and this value was used to calculate the EPA recoveries presented in Table 3. The highest EPA recovery of 74 % was obtained with dichloromethane/ethanol, while EPA recovery with all other nonhalogenated solvents ranged from 12 to 45 %.

Carotenoid content

The amount of carotenoids extracted with the different solvent systems is summarized in Fig. 4. The extraction with the reference method showed that *N. gaditana* contained 68± 18 mg carotene/100 g dry weight, 66±9 mg diadinoxanthin/ 100 g dry weight, 520±53 mg violaxanthin/100 g dry weight, and 81±25 mg zeaxanthin/100 g dry weight. When extracting with the other tested solvent systems, 37–75 % of carotene, 19–113 % of diadinoxanthin, 8–91 % of violaxanthin, and 21–117 % of zeaxanthin could be recovered (Table 3).

Sterol content

The amount of sterols extracted with the different solvent systems is shown in Fig. 5. *N. gaditana* contained 360± 47 mg cholesterol/100 g DW and 405±44 mg phytosterols/ 100 g DW. Extraction with the other tested solvent systems recovered between 33 and 106 % of cholesterol and between 40 and 96 % of phytosterols (Table 3). The results can be summarized in the following three observations. Firstly, the extraction yield with dichloromethane/ethanol and ethanol was not significantly different than that obtained with the reference method, leading to a recovery not significantly different from 100 %. Secondly, the recovery of the sterols with hexane was clearly lower than with all other solvents. And finally, the recovery of the sterols with all other solvents (except dichloromethane/ethanol, ethanol, and hexane) was more or less the same at around 60 %.

Discussion

The high lipid recovery using dichloromethane/ethanol confirms the results of Balasubramanian et al. (2013). These authors also showed that a higher recovery could be obtained when mixing a polar and a nonpolar solvent, which also coincides with the results presented in this study. The exact recovery values are however somewhat different between different studies (Table 2) which can be explained by the

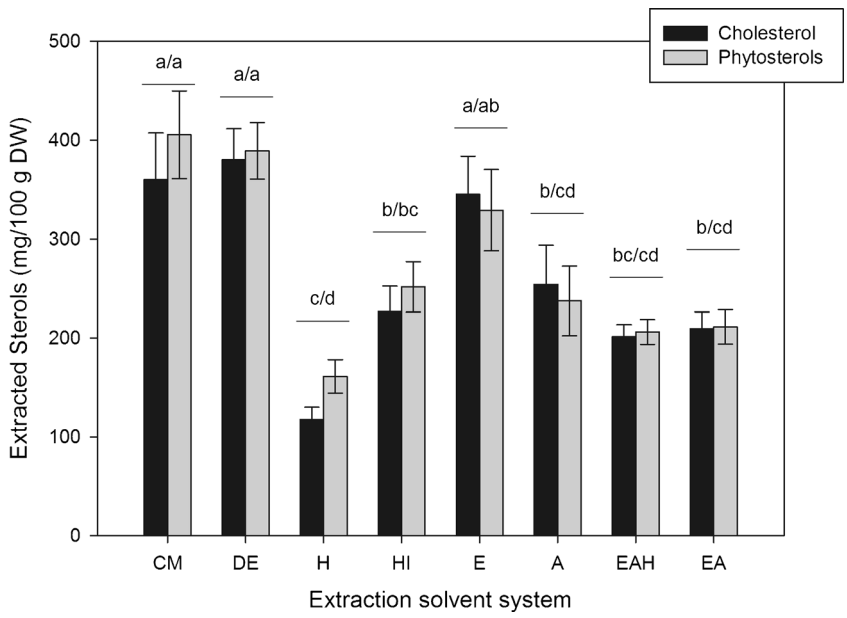

Fig. 5 Amount of sterols (in mg/ 100 g DW; mean±SD; $n=3$) extracted from *N. gaditana* with different solvent systems: *CM* chloroform/methanol, *DE* dichloromethane/ethanol, *H* hexane, *HI* hexane/isopropanol, *E* ethanol, *A* acetone, *EAH* ethyl acetate/hexane, *EA* ethyl acetate. *Lowercase letters* on top of bars indicate significance of difference ($\alpha=0.05$) for cholesterol and phytosterols respectively

difference in composition of the microalgae biomass, the method for total lipid extraction used as a reference, and the cell disruption method used.

The enrichment of extracts obtained by using nonhalogenated solvents with NL accords with the results of Guckert et al. (1988) and Balasubramanian et al. (2013). The former authors explained this NL selectivity by the inability of the solvent system to extract lipids from the chloroplast and membrane, which respectively contain GL and PL. Polar lipids are soluble in, e.g., hexane/isopropanol as it has been shown that they can be extracted very well from prokaryotic plasma membranes using this solvent (Guckert et al. 1988). It is therefore possible that the structural differences between eukaryotic and prokaryotic membranes prevent successful extraction of polar lipids by hexane/isopropanol.

The difference in NL recovery by an apolar (typically hexane) and a mixture of an apolar and polar solvent (typically hexane/isopropanol) can be explained as follows. In (eukaryotic) microalgae cells, NL are known to interact through weak van der Waals forces to form lipid globules, or oil bodies, in the cytoplasm. These "free NL" can be extracted from the cell by using nonpolar solvents such as hexane since similar van der Waals forces are formed between the NL and the nonpolar solvent. However, some NL form complexes with polar lipids, which form hydrogen bonds with proteins in the cell membrane. Nonpolar solvents are not capable of disrupting these hydrogen bonds and therefore cannot extract these "complexed NL" from the cell. In contrast, polar organic solvents, such as methanol or isopropanol, can form hydrogen bonds with the lipids from these complexes and hence break the lipid–protein complexes, leading to extraction of more "complexed NL" from the cells (Halim et al. 2012; Shahidi and Wanasundara 2002). Extraction with a mixture of a polar and a nonpolar organic solvent, such as hexane/isopropanol consequently gives the most complete extraction of NL.

The EPA content in the lipid classes of the total lipid extract showed that EPA was more abundant in the GL (50±1 %) and PL classes (23±1 %) than in the NL class (15.8±0.5 %). This explains the clear correlation (R^2=0.89) between the EPA recovery and the polar lipid recovery (GL+PL). As the extractability of the polar lipid classes in most solvents is not very high, a simultaneous saponification and extraction process, followed by a purification step has been suggested and lead to much higher EPA recoveries than the ones we obtained, e.g., 87–96 % for EPA from *Phaeodactylum tricornutum* (Cartens et al. 1996; Ibáñez González et al. 1998). However, in that case, all fatty acids are free from their glycerol backbone, leading to oil with possible lower oxidative stability and higher rancidity, which may not be applicable for food purposes.

When looking at the results of the carotenoid content of the extracts obtained with the different solvent systems, three summarizing observations could be made. Firstly, only the carotenoid yield obtained with dichloromethane/ethanol was not significantly different from that obtained with the reference method, leading to a recovery not significantly different from 100 %. All nonhalogenated solvent (mixtures) recovered only part of the carotenoids present. Secondly, the recovery of the different carotenoids with hexane showed large differences: the carotene recovery was much higher than that of the xanthophylls diadinoxanthin, violaxanthin, and zeaxanthin. A poor extraction efficiency of xanthophylls (fucoxanthin from *Isochrysis galbana*) with hexane was also observed by Kim et al. (2012). Finally, the recoveries of all carotenoids with all other solvents were more or less the same at around 50 %. While ethanol and acetone seem to be the solvents most widely used in other studies (e.g., Chen et al. 2005; Dunn et al. 2004; Papaioannou et al. 2008; Mendes-Pinto et al. 2001) focusing on extraction of carotenoids, we observed that dichloromethane/ethanol (and chloroform/methanol) performed better.

The observations on extractability of carotenoids can be explained by a combination of the polarity of the carotenoids and the solvent (mixtures) on the one hand and the localization of the carotenoids on the other hand. Firstly, carotenoids can only be extracted with solvent (mixtures) in which they are soluble and which thus have a similar polarity. This can explain the recovery of the carotenoids with the nonpolar solvent hexane: the most polar carotenoid violaxanthin is almost not recovered (8 %), while with decreasing polarity, the carotenoids are more recovered: diadinoxanthin (19 %), zeaxanthin (21 %), and carotene (66 %). Secondly, most carotenoids are naturally occurring in photosynthetic pigment–protein complexes, fitting into or spanning across the lipid bilayer of membranes (Britton 2008). In analogy with lipid extraction, only a polar organic solvent can disrupt these pigment–protein associations by forming hydrogen bonds. This leads to better extraction efficiencies with polar solvent (mixtures) than with hexane. Secondary carotenoids, which are not related to the photosynthetic apparatus, are however harbored in so-called oil bodies, in which also triglycerides are deposited. In *Parietochloris incisa*, the bulk (up to 66 %) of β-carotene was localized in these oil bodies (Solovchenko 2012). If this is also the case in *N. gaditana*, this could help to explain why the overall extractability of carotene with hexane is higher than that of the other carotenoids.

The observations on the sterol content of the different extracts can be explained by a combination of two factors, being the polarity of the sterols and the solvent (mixtures) and their localization. Firstly, as with the carotenoids, sterols can only be extracted with solvent (mixtures) having a similar polarity and in which they are thus soluble. Secondly, sterols are known to play a primary role as architectural components of microalgae membranes (Nes 1974), from which they can only be extracted with a polar solvent able to disrupt the sterol–membrane associations by forming hydrogen bonds.

This explains the low extraction efficiency obtained with hexane.

In the literature (Hrabovski et al. 2012; Abidi 2001; Dunford et al. 2009; Kasim et al. 2010), a multitude of solvent systems have been used and found to be the most effective for sterol extraction from different sources. This may be explained by the study of Tir et al. (2012), who showed that the recovery from sesame seed using different solvents was not the same for the different sterols. In our study, however, the recovery of cholesterol and the sum of all phytosterols was more or less the same for each solvent system. This can probably be explained by the fact that the polarity of cholesterol and 24-ethylcholest-5-en-3β-ol, the main phytosterol present in *N. gaditana* (Méjanelle et al. 2003) is not that different.

When looking at the results presented in this study, it is clear that dichloromethane/ethanol (1:1) is the best solvent to use when lipids are to be extracted from *N. gaditana*. However, based on the solvent properties (International Chemical Safety Cards 2013; Chemicals and Reagents Catalog, Merck 2011-2013) dichloromethane scores particularly bad at the additional criteria. It is known to be (potentially) carcinogenic, and its use is therefore highly regulated: the US Food and Drug Administration has approved its use as an extraction solvent only for spice oleoresins, hops, and caffeine from coffee. Dichloromethane is also highly volatile meaning that large amounts can get lost in the atmosphere. When in contact with water, dichloromethane is also particularly corrosive. The other nonhalogenated solvents investigated in this study are known to be less toxic—although toxicity of solvents is always an issue and therefore guidelines for residual solvents in foods are to be followed—less volatile, and equally or less corrosive, and therefore score better at the additional criteria. When a choice has to be made among these other solvents, hexane/isopropanol (3:2) seems to be the best option. It gives the highest lipid yield and a low nonlipid co-extraction. The recoveries of EPA and the carotenoids are more or less the same for all solvent (mixtures). Only for phytosterols, the recovery was clearly higher when using ethanol. Ryckebosch et al. (2013) however showed that the amount of phytosterols present in microalgae oils is probably too low to provide a significant nutritional benefit. The commercially often used hexane extraction does not perform well when an omega-3 LC-PUFA-rich oil is pursued. Future research on different microalgae species should determine if these results can be extrapolated.

In conclusion, this study showed that the highest lipid, EPA, carotenoid, and sterol yields were obtained with dichloromethane/ethanol (1:1), with which the same yields as with the reference method chloroform/methanol (1:1) could be achieved. This dichloromethane/ethanol (1:1) extract was also the only one which was not enriched in neutral lipids compared to the reference method. Since dichloromethane is (potentially) carcinogenic, its use is highly regulated. Furthermore, it is highly volatile and particularly corrosive in contact with water. When a choice has to be made among the less toxic, nonhalogenated solvents, hexane/isopropanol (3:2) is probably the best alternative because it combines a high lipid yield with a low nonlipid co-extraction. However, it has to be stressed that when nonhalogenated solvents are used, about half of the interesting oil is lost, meaning there is room for improvement to gain a commercially viable extraction process.

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