Received: 5 May 2018 | Revised: 11 July 2018 | Accepted: 28 August 2018

DOI: 10.1111/gcbb.12563

TECHNICAL ADVANCE

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

Pressurized extraction of unsaturated fatty acids and carotenoids from wet *Chlorella vulgaris* and *Phaeodactylum tricornutum* biomass using subcritical liquids

Felix Derwenskus[1] | Felix Metz[2] | Andrea Gille[3] | Ulrike Schmid-Staiger[1] | Karlis Briviba[3] | Ursula Schließmann[1,2] | Thomas Hirth[4]

[1]Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Stuttgart, Germany

[2]Institute of Interfacial Process Engineering and Plasma Technology IGVP, University of Stuttgart, Stuttgart, Germany

[3]Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany

[4]Karlsruhe Institute of Technology, Karlsruhe, Germany

Correspondence
Felix Derwenskus, Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Stuttgart, Germany.
Email: Felix.Derwenskus@igb.fraunhofer.de

Funding information
Ministry of Science, Research and the Arts of Baden-Wuerttemberg, Grant/Award Number: 7533-10-5/91/2

Abstract

The objective of this study was to investigate the extraction of lipids, for example, mono- and polyunsaturated fatty acids (PUFA) as well as carotenoids, from wet microalgae biomass using pressurized subcritical extraction solvents, which meet the requirements of food and feed applications. To demonstrate the effect of the solvent and temperature on the lipid yield, we chose two microalgae species, viz. *Chlorella vulgaris* and *Phaeodactylum tricornutum*, differing in their biochemical composition fundamentally. In case of *P. tricornutum,* ethanol showed the highest fatty acid yield of 85.9% w/w. In addition to eicosapentaenoic acid (EPA), the ethanolic extracts contained exceptional amounts of fucoxanthin (up to 26.1 mg/g d. w.), which can be beneficial to protect unsaturated fatty acids from oxidation processes and in terms of human nutrition. For *C. vulgaris*, a fatty acid yield of 76.5% w/w was achieved from wet biomass using ethyl acetate at 150°C. In general, an increase in the extraction temperature up to 150°C was found to be important in terms of fatty acid yield when extracting wet microalgae biomass. The results suggest that it is possible to efficiently extract both fatty acids and carotenoids from wet microalgae by selecting suitable solvents and thus circumvent energy-intensive drying of the biomass.

KEYWORDS

diatom, eicosapentaenoic acid, fatty acids, fucoxanthin, lipids, microalgae, subcritical solvent extraction

1 | INTRODUCTION

Microalgae are unicellular, eukaryotic, or prokaryotic organisms. Similar to plants, they are able to convert light, CO_2, and water into valuable biomass, which can be used in human nutrition, feed in aquaculture, or as an alternative energy source (Posten & Feng Chen, 2016). Compared to terrestrial plants, microalgae have several advantages, for example, an up to five times higher biomass productivity, they can be grown on non-arable land and reduce water consumption when cultivated in closed bioreactor systems like flat-panel bioreactors (Meiser, Schmid-Staiger, & Trösch, 2004; Silva Benavides, Torzillo, Kopecký, & Masojídek, 2013).

Depending on the species, various valuable compounds can be present in microalgae biomass. Green microalgae

like *Chlorella vulgaris* are well-known for a high protein content and their capability to accumulate high amounts of triacylglycerides (TAG) with mainly monounsaturated fatty acids (Ursu et al., 2014). In contrast, diatoms like *Phaeodactylum tricornutum* can provide a high amount of polyunsaturated fatty acids (PUFA), for example, eicosapentaenoic acid (EPA, 20:5 ω-3) as well as carotenoids (Gille, Trautmann, Posten, & Briviba, 2016; Ryckebosch et al., 2013; Zhang, Wang, Gao, Huang, & Zhang, 2018).

However, not only the origin of the strain but also the cultivation conditions (e.g., light availability, nutrient supply, temperature, salinity) have a crucial impact on the amount and composition of potential products present in microalgae (Pal, Khozin-Goldberg, Cohen, & Boussiba, 2011). Several cultivation conditions are described to modify the amount of specific ingredients, for example, nutrient starvation (nitrogen and phosphorus starvation) to increase the fatty acid content (Morales-Sánchez, Tinoco-Valencia, Caro-Bermúdez, & Martinez, 2014; Mulders, Janssen, Martens, Wijffels, & Lamers, 2014; Münkel, Schmid-Staiger, Werner, & Hirth, 2013). This capability provides a huge opportunity to produce tailor-made biomass by applying appropriate cultivation conditions, but it is also a tough challenge concerning downstream processing, since parameters have to be adapted not only to each specific species but also to changeable biomass compositions.

In particular, extraction methods have to be adjusted in order to increase the added value for each species and product. When applying a biorefinery concept in order to get a holistic utilization of microalgae biomass, it is furthermore necessary to extract multiple products (e.g., lipids and proteins), which have to be efficiently recovered and separated.

Extraction techniques like supercritical fluid extraction (SFE) with CO_2 are described for the extraction of lipids from microalgae biomass (Liau et al., 2010; Mendes, Reis, & Palavra, 2006). Main advantages of this extraction technique are the low environmental impact since no organic solvent is used, as well as low extraction temperatures. Due to their low viscosity and relatively high diffusivity, supercritical fluids provide better transport properties than liquids and can penetrate solid materials (Herrero, Cifuentes, & Ibanez, 2006). Due to the low polarity of supercritical CO_2, this technique is especially suitable for the extraction of nonpolar lipids like TAGs and nonpolar carotenoids (e.g., beta-carotene) from algae biomass (Macías-Sánchez, 2005). In order to extract more polar compounds, for example, astaxanthin from *Haematococcus pluvialis*, co-solvents have to be added to get satisfying yields (Fujii, 2012; Krichnavaruk, Shotipruk, Goto, & Pavasant, 2008). Furthermore, this method is restricted to dry biomass, since CO_2 cannot penetrate water layers surrounding the microalgae cells. Hence, a previous energy-intensive drying step is mandatory (Goto, Kanda, & Wahyudiono, & Machmudah, 2015).

Nowadays, several further extraction techniques for microalgae biomass are in focus of research. Examples are pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), pulsed electric field-assisted extraction (PEF), and microwave-assisted extraction (MAE) (Parniakov et al., 2015; Pasquet et al., 2011; Plaza et al., 2012).

However, a promising alternative to SFE is pressurized liquid extraction since it might offer the possibility to avoid cost-intensive drying of the biomass and a wide range of solvents providing different dielectric constants (polarity) can be used for extraction. During the PLE process, the solvent is kept in a liquid, subcritical state even when applying temperatures above the boiling point of the solvent. Organic solvents like methanol, ethanol, 2-propanol, acetone, chloroform, and hexane as well as mixtures of these solvents can be used for the extraction of microalgae biomass (Koo, Cha, Song, Chung, & Pan, 2012; Ryckebosch, Muylaert, & Foubert, 2012; Santoyo et al., 2006). Thus, the solvent can be selected according to different microalgae species and specific products. Due to high pressure and elevated temperature, in general around 100 bar and between 50 and 200°C, the reaction kinetics as well as the interaction between the solvent and the biological sample are increased compared to common solvent extraction methods like Soxhlet (Saini & Keum, 2018). Hence, less solvent is necessary for extraction. PLE is already described for the extraction of carotenoids from freeze-dried microalgae as well as macroalgae biomass (Denery, Dragull, Tang, & Li, 2004; Jaime et al., 2010; Shang, Kim, Lee, & Um, 2011).

The objective of this study was to evaluate parameters for the extraction of lipids from different types of wet microalgae biomass, to demonstrate the possibility to avoid an energy-intensive drying step prior to PLE. For this purpose, we investigated the extraction of dry and wet biomass (30% w/w of dry weight) at different extraction temperatures to generate lipid-rich fractions of two different microalgae species, viz. *Chlorella vulgaris* and *Phaeodactylum tricornutum.*

These species were chosen since they fundamentally differ in their cell wall properties, cell size as well as composition of ingredients and related industrial applications. *C. vulgaris* was grown in flat-panel airlift photobioreactors (FPA) in a two-stage process with nitrogen limitation for the accumulation of TAG (Münkel et al., 2013). TAGs, including mainly mono- and unsaturated fatty acids, are especially interesting as a renewable energy source, for example, as a green fuel. Unlike *P. tricornutum*, *C. vulgaris* is not able to synthesize polyunsaturated fatty acids (PUFA), usually occurring in the form of glycolipids

(MGDG), as part of the chloroplast membranes in various microalgae species (Pieber, Schober, & Mittelbach, 2012). Due to its high PUFA content, especially eicosapentaenoic acid, the diatom *P. tricornutum* is widely used as a feed stuff in aquacultures (Sørensen, Berge, Reitan, & Ruyter, 2016) and it provides a good source of EPA for the application in human nutrition.

For the extraction of lipids from wet biomass, a dry biomass content of 30% w/w was chosen, since it is a common concentration after cell disruption and the removal of water-soluble proteins, for example, by centrifugation, which is very common in microalgae downstream processing (Günerken et al., 2015; Schwenzfeier, Wierenga, & Gruppen, 2011). To extract the lipids, we focused on organic solvents which are suitable for the utilization of foodstuff and feedstuffs and varied the extraction temperature from 50 to 150°C.

2 | MATERIALS AND METHODS

2.1 | Microalgae production

Phaeodactylum tricornutum UTEX 640 was produced in an outdoor plant at the Fraunhofer Center for Chemical-Biotechnological Processes CBP, Leuna, Germany, using flat-panel airlift bioreactors (FPA) with a volume of 180 L each, operating as a fed-batch process. The FPA bioreactor has been previously described in detail in *Bergmann et al.* (Bergmann & Trösch, 2016). Modified Mann and Myers medium (Mann & Myers, 1968) was used as culture media as described in Meiser et al. (Meiser et al., 2004). The cultivation was conducted from March to September 2015. During a time period of 113 days, the biomass was harvested by centrifugation, combined, and stored at −20°C. *Chlorella vulgaris* was cultivated in FPA bioreactors with a volume of 30 L in an outdoor plant at the Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Stuttgart, Germany. An automated two-stage process based on nitrogen starvation using modified DSN culture media was conducted to increase the fatty acid content of the biomass. The process as well as the modified culture media is described in detail in Münkel et al. (Münkel et al., 2013). The biomass was immediately frozen after harvesting by centrifugation and stored at −20°C.

2.2 | Cell disruption

Prior to cell disruption, the microalgae cells were washed with distilled water twice, followed by centrifugation to remove salt and impurities from the culture media. For cell disruption, a stirred ball mill (PML-2, Buhler, Germany) with process unit Centrex S1 with a grinding volume of 0.22 dm^3 and stabilized ceramic grinding media (Draison Yttrium Ultra Power, Buhler, Germany) with a density of 5.95 g cm^{-3} were used. An external pump (PSF3, Ragazzini, Italy) was attached to the ball mill in order to continuously pump biomass from the collection container with a volume of 5 L through the grinding chamber. For each batch, 0.5 kg of dry biomass was diluted with 5 L of water to get a dry matter concentration of 10% w/w. The disruption time was 2.5 hr, and the rotor speed was set to 3,500 rpm. After cell disruption, the cells were either freeze-dried (VaCo 5, Zirbus, Germany) or concentrated to a dry matter content of 30% w/w for the extraction.

2.3 | Pressurized liquid extraction

After cell disruption, pressurized liquid extraction (PLE) was performed using an accelerated solvent extractor (ASE 350, Thermo-Fisher Scientific, USA). Extraction cells consisting of stainless steel with a volume of 100 ml were used for extraction. Cellulose filters were placed in the cells to prevent any clogging of the outlets. Depending on the experimental set-up, either 5 g freeze-dried or 16.7 ml water containing 5 g of dry biomass (30% w/w) was mixed with 10 g of diatomic earth and transferred to the extraction cells. This corresponds to a biomass/solvent/water ratio (g/ml/ml) of 1/14/0 for dry biomass and 1/12/3 for wet biomass during batch extraction. All experiments were conducted at a constant pressure of 103.4 bar (1,500 psi) to keep the solvents in a subcritical state. Extraction temperatures were chosen between 50 and 150°C. Static extraction time was set to 20 min with a rinse volume of 60% and a nitrogen purge time of 300 s. Immediately after extraction, 0.25 mg/ml of butylated hydroxytoluene (BHT) was added to the extracts to prevent oxidative degradation of lipid substances. The extracts were stored at −20°C in light-protected flasks until analysis.

2.4 | Gravimetric determination of extracted lipids

In order to determine the amount of total lipids (extracted oil), 2 ml of each extract was transferred to an Eppendorf tube immediately after extraction. The solvent was evaporated (Concentrator plus, Eppendorf, Germany) at a pressure of 20 mbar for 3 hr at 1,400 rpm and 30°C. Afterward, the sample was weighed.

2.5 | Determination of the fatty acid profile

The fatty acid content and profile of extracts were analyzed based on the method of Lepage and Roy (Lepage & Roy, 1984) with slight modifications as already described in Meiser et al. (Meiser et al., 2004). Prior to GC analysis, the extracts were mixed with methanol/acetyl chloride

(20:1 v/v) for transesterification. Fatty acid methyl esters (FAME) were analyzed using a Supelco SPB-PUFA 30 m × 0.32 mm × 0.2 µm column (Sigma-Aldrich, USA). An Agilent 7890A (Agilent, USA) was used as a gas chromatograph, equipped with a FID detector. Results were compared to a certificated C_4 – C_{24} FAME-Mix (Supelco-18919–1AMP, Sigma-Aldrich, USA). Reference values of the total fatty acid content of both microalgae species were determined according to the direct transesterification method described by Lepage and Roy (1984) using freeze-dried biomass.

2.6 | Determination of carotenoids by HPLC

The carotenoid and chlorophyll contents were determined by applying the method previously described by Gille et al. (Gille et al., 2016) with slight changes. In brief, samples were analyzed with reversed-phase HPLC using a Suplex pKb 100 (5 µm, 250 × 4.6 mm) column (Supelco, Bellefonte, PA) and a Waters photodiode array detector. For separation of carotenoids, a binary gradient with a flow rate of 1 ml/min was applied. The samples were diluted five times and injected with a volume of 20 µl. The mobile phase A consisted of methanol/acetonitrile/2-propanol (54/44/2, v/v/v) and mobile phase B of mobile phase A/water (85/15, v/v). The gradient cycle started with a segment of 40% A, followed by increasing A to 80% at 10 min and to 100% from 20 min to 28 min. From 29 to 36 min, the gradient was changed to 40% A and 60% B. The carotenoids and chlorophyll a were detected at 450 nm or 430 nm, respectively. The concentration of ß-carotene, zeaxanthin, fucoxanthin, and chlorophyll a were calculated from calibration curves prepared with standard solutions purchased from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (USA).

2.7 | Determination of the total fucoxanthin content in P. tricornutum

The total amount of fucoxanthin in the biomass was determined in order to calculate the fucoxanthin extraction yield. Since there are no reference methods available, multiple subsequent pressurized liquid extractions were conducted on a single sample of freeze-dried biomass at 100°C (biomass/solvent ratio: 1/14 g/ml). Ethanol was used for extraction at a static extraction time of 20 min for each cycle. All other parameters as well as the PLE set-up were chosen as already described above. All fractions were collected separately and analyzed by HPLC.

2.8 | Statistical analysis

All extractions and analyses were performed at least as triplicates for each microalgae species and at each extraction parameter, if not denoted differently. Results were evaluated using one-way analysis of variance (ANOVA) and were considered as significant when passing the Turkey test with $p = 0.05$ (Design-Expert, Stat-Ease, USA).

3 | RESULTS AND DISCUSSION

3.1 | Fatty acid profile

Prior to extraction experiments, the fatty acid profile and the amount of total fatty acids for both microalgae species were determined (Table 2). The main fatty acids present in *C. vulgaris* biomass were C16:0, C16:3, C18:1, and C18:3, constituting about 88.5% of the total amount of fatty acids. 43.36% ± 1.08% w/w of the total dry matter was found to be fatty acids. This is not unusual since the biomass was cultivated using nitrogen depletion, which causes the accumulation of triacylglycerides (TAG) with C16 and C18 fatty acids, as described before (Münkel et al., 2013).

The main fatty acid in *P. tricornutum* was found to be eicosapentaenoic acid (EPA, 20:5n3), as already described for *P. tricornutum* and several strains of *Nannochloropsis* in the literature (Meiser et al., 2004; Zittelli et al., 1999). Nevertheless, the amount of EPA in *P. tricornutum* was remarkably high with 5.14% ± 0.11% w/w of total dry matter.

TABLE 1 Fatty acid profile of *Chlorella vulgaris* (cultivated at nitrogen depletion) and *Phaeodactylum tricornutum* (growth phase) biomass used in this study. Amounts as mass % of total dry weight (mean ±SD; $n = 4$; n.d. = not detected)

Fatty acid	*Chlorella vulgaris*	*Phaeodactylum tricornutum*
C14:0	0.06 ± 0.01	0.63 ± 0.02
C14:1	0.08 ± 0.01	0.15 ± 0.01
C16:0	6.41 ± 0.15	0.92 ± 0.02
C16:1	0.21 ± 0.01	1.48 ± 0.04
C16:3	5.60 ± 0.16	n.d.
C18:0	0.53 ± 0.01	n.d.
C18:1n9	16.30 ± 0.35	0.04 ± 0.01
C18:2n6	4.09 ± 0.12	0.24 ± 0.01
C18:3n3	10.08 ± 0.26	n.d.
C20:3n3	n.d.	0.22 ± 0.01
C20:5n3 (EPA)	n.d.	5.14 ± 0.11
C24	n.d.	0.15 ± 0.01
SUM (C14, C16)	12.37 ± 0.34	3.33 ± 0.10
SUM (C18)	31.00 ± 0.74	0.28 ± 0.02
SUM (C20)	n.d.	5.36 ± 0.12
SUM (FA)	43.36 ± 1.08	8.97 ± 0.24

3.2 | Total lipid yield

Following mechanical cell disruption, ethanol, ethyl acetate, and *n*-hexane were used as solvents to compare the lipid yield of freeze-dried and wet *C. vulgaris* as well as *P. tricornutum* biomass at different extraction temperatures. These solvents differ strongly in polarity, since the dielectric constants range from 1.9 to 25.3 (see Table 1). Hence, it was possible to evaluate the dependency of the extraction yield on the solvent polarity. Additionally, these solvents provide a different water solubility which can be crucial when extracting wet biomass since a water layer surrounding the cells might prevent the access of hydrophobic solvents to the biomass even at high pressure (Islam, Brown, O'Hara, Kent, & Heimann, 2014).

In case of freeze-dried biomass, the results (Figure 1a,c) indicate that the overall lipid yield is highly dependent on both the microalgae strain and the extraction solvent. For dry *C. vulgaris* biomass, ethyl acetate and *n*-hexane extracted 52.6% ± 0.8% w/w and 51.6% ± 1.5% w/w of the total dry matter at 150°C, respectively. The yield decreased when using the more polar solvent ethanol. This is presumably due to the high amount of nonpolar TAG present in *C. vulgaris*, constituting a major part of the crude dry matter. However, for *P. tricornutum* the extraction of freeze-dried biomass with ethanol resulted in an increased yield of 35.1% ± 2.3% w/w compared to ethyl acetate and *n*-hexane, indicating that most of the microalgae components exhibit a more polar character. Similar results were already reported for *N. oculata* using PLE (Pieber et al., 2012) and for *N. granulata* as well as *P. tricornutum* using Soxhlet extraction (McNichol, MacDougall, Melanson, & McGinn, 2012). When using freeze-dried biomass, the extraction temperature showed less impact on the extraction yield than in case of wet biomass.

In case of wet biomass (Figure 1b,d), an increase in temperature from 50 to 150°C almost doubled the total lipid yield for both strains using ethanol. In general, the best yields were observed using ethanol. This is presumably due to the very good miscibility of ethanol with water. Hence, ethanol provides a better penetration of the water layer and biomass during extraction. The overall yields using wet biomass and ethanol were even higher than with dry biomass. However, an increase in temperature from 50°C to 150°C also increases the solubility of ethyl acetate and *n*-hexane in water since water distinctively changes its dielectric constant at high pressure and temperature (Reddy et al., 2014). This might be the reason for the 2.1-fold increase in yield when using ethyl acetate with wet *C. vulgaris* biomass. These findings correlate with the study of Islam et al. (Islam et al., 2014), who also observed a significant impact of the temperature when using hexane for the extraction of fatty acids from microalgae polycultures.

TABLE 2 Physical constants of extraction solvents used in this study (Rumble, 2017)

Solvent	Dielectric constant Ɛ (at 20°C)	Boiling point in °C	Critical point P_c (T, p)
Ethanol	25.3	78.2	241°C, 63 bar
Ethyl acetate	6.1	77.1	250°C, 39 bar
n-Hexane	1.9	69.0	234°C, 30 bar

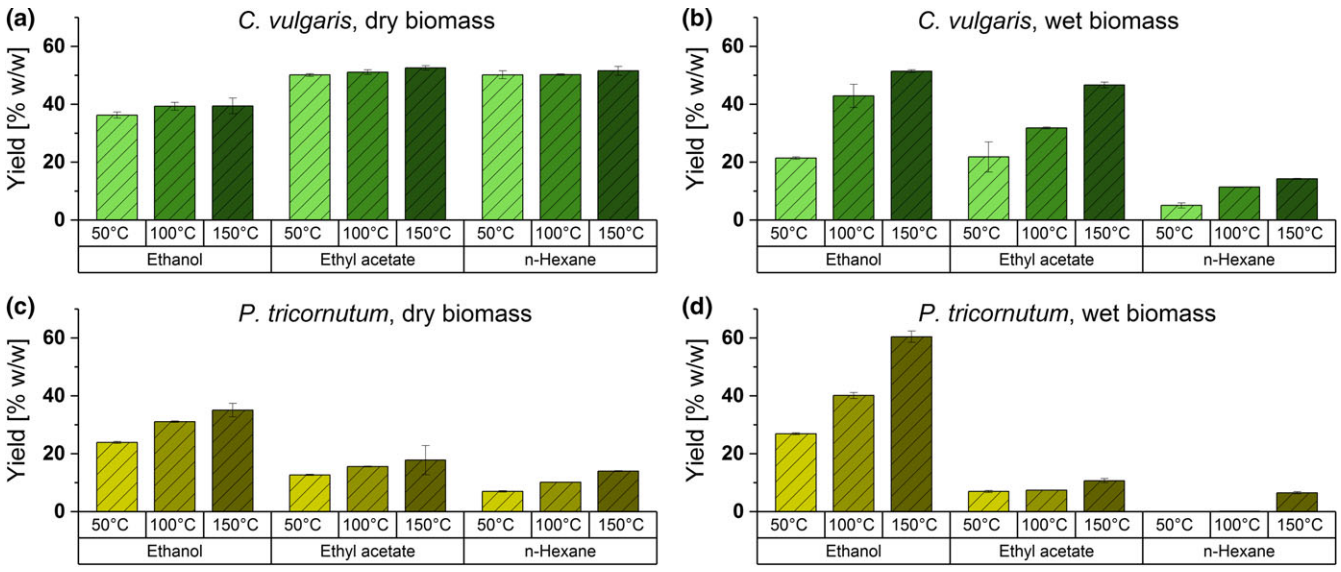

FIGURE 1 Total lipid yield (in % w/w of dry weight) by PLE (100 bar, t = 20 min) using different extraction solvents and temperatures for *C. vulgaris* (green) and *P. tricornutum* (yellow). Extraction was conducted with (a) dry *C. vulgaris* biomass, (b) wet *C. vulgaris* biomass (70% w/w H_2O), (c) dry *P. tricornutum biomass* and (d) wet *P. tricornutum* biomass (70% w/w H_2O). The error bars indicate the mean standard derivation (n = 3)

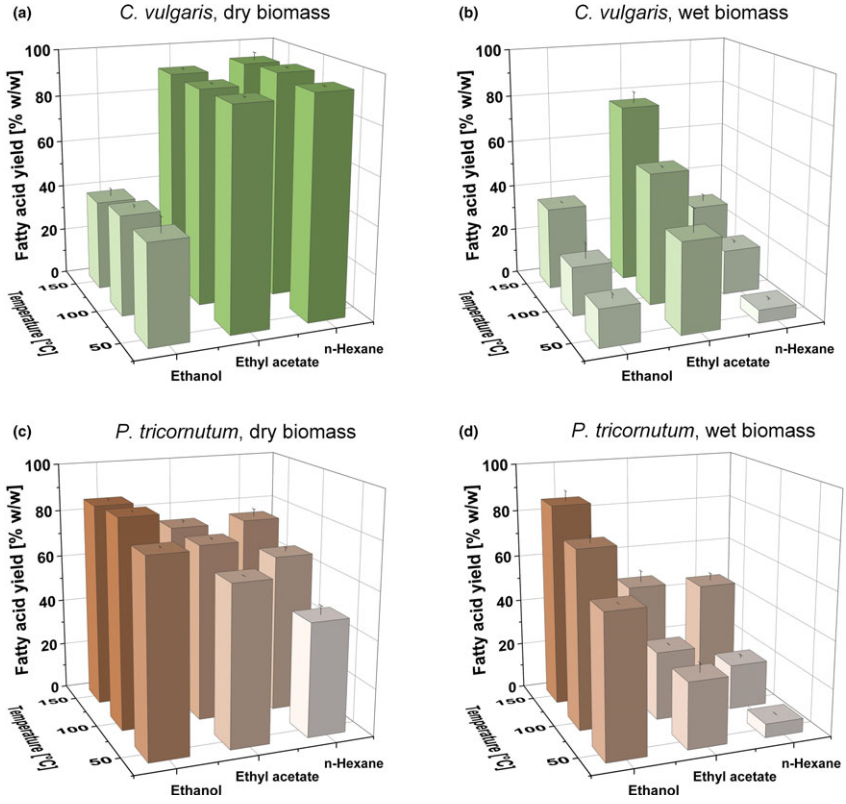

FIGURE 2 Fatty acid yield (in % w/w of dry weight) by PLE (100 bar, $t = 20$ min) in fractions using different solvents and temperatures for the extraction of (a) dry biomass of *C. vulgaris* (b) wet biomass of *C. vulgaris,* (c) dry biomass of *P. tricornutum* (d) and wet biomass of *P. tricornutum*. The yield was calculated based on the amount present in each fraction and in relation to the total amount of fatty acids available in the biomass (Table 1). The error bars indicate the mean standard derivation ($n = 3$)

3.3 | Fatty acid yield

Extraction with *n*-hexane and ethyl acetate resulted in the highest fatty acid yield in case of *C. vulgaris*, when using freeze-dried biomass (Figure 2a). The fatty acid yield with hexane (93.2% ± 0.5% w/w) was slightly higher than with ethyl acetate (91.2% ± 0.7% w/w) when extracting at the lowest temperature of 50°C, respectively. In case of all solvents, an increase in the extraction temperature to 150°C did not significantly affect the fatty acid yield.

For dry *P. tricornutum* biomass, solvents with higher dielectric constants such as ethanol and ethyl acetate resulted in the highest fatty acid yield. The extraction with ethanol at 50°C already yielded 80.3% ± 0.7% (Figure 2c) with only slight increase at higher temperature. Nevertheless, all solvents led to an adequate fatty acid recovery above 70% w/w at increased extraction temperatures. In case of hexane, an increase in temperature from 50 to 150°C led to an increase in fatty acid recovery from 47.8 ± 3.8 to 74.8% ± 3.2%.

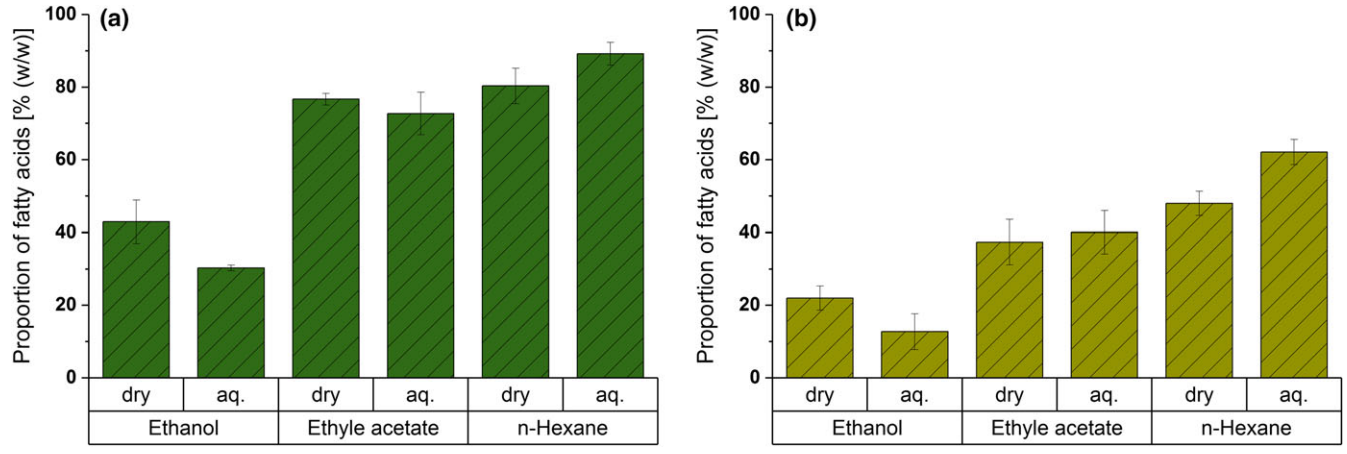

FIGURE 3 Proportion of fatty acids (as % w/w of dry weight) in the extracted oil fraction derived from dry and wet (30% w/w dry weight) biomass of (a) *C. vulgaris* and (b) *P. tricornutum* by PLE (100 bar, 150°C, $t = 20$ min) using different solvents. The error bars indicate the mean standard derivation ($n = 3$)

When using wet biomass of *C. vulgaris,* only ethyl acetate provided a reasonable extraction yield of 76.5% ± 4.9% at 150°C (Figure 2b) and the extraction temperature showed an increasing impact compared to the extraction of dry biomass. The increased yield is presumably due to the improved water solubility of ethyl acetate in water at high pressure and high temperature. The miscibility of both liquids might be beneficially influenced due to the shift in the dielectric constant of water at high temperature. When using wet *P. tricornutum* biomass, ethanol extracted the largest amount of fatty acids for all temperature settings tested (Figure 2d). This is not surprising since ethanol is miscible with water even at room temperature and atmospheric pressure. Nevertheless, an increase in the extraction temperature increased the fatty acid yield from 59.4 ± 0.1 to 85.9% ± 4.4%. Although the fatty acid yield varied for both species depending on the solvent and temperature, we could not observe a significant change in the overall fatty acid profile of each fraction compared to the initial biomass, neither for extracts derived from freeze-dried nor wet biomass.

3.4 | Proportion of fatty acids in each fraction

By comparing the total lipid yield and the fatty acid content (shown above), we had a look at the proportion of fatty acids to the dry matter in each corresponding fraction (see Figure 3). The results show that with increasing polarity of a solvent, the share of fatty acids in the extracts tends to decrease. These findings correlate with the data published by Pieber et al. (2012), who observed an inverse relationship between the gravimetric extraction yield and the fatty acid content of fractions from *Nannochloropsis oculata,* since the crude extraction yield strongly increased with the polarity of the solvent (Pieber et al., 2012).

Thus, for dry and wet *C. vulgaris* biomass, the highest share of fatty acid of 80.3 ± 4.9 and 89.2% ± 3.1% w/w was received using *n*-hexane at 150°C, respectively. In comparison, the proportion of fatty acids in the ethanolic fractions was 42.9% ± 5.9 and 30.0% ± 0.8% w/w. In general, these results indicate that the share of fatty acids in the extracts tend to increase with decreasing solvent polarity.

3.5 | Carotenoid and chlorophyll content

To get an impression of the carotenoid- and chlorophyll content in fractions extracted with different solvents, selected fractions were analyzed by HPLC. The main pigments in the fractions of *C. vulgaris* were chlorophyll a and b, lutein as well as trace amounts of violaxanthin and beta-carotene. Ethanol extracted 4.4 ± 0.4 mg/g of chlorophyll a, 1.8 ± 0.2 mg/g of chlorophyll b, and 1.6 ± 0.1 mg/g of lutein. In general, for all solvents, the total amount of pigments in the extracts of *C. vulgaris* was found to be much lower compared to the extracts derived from *P. tricornutum*.

Fucoxanthin was the most abundant pigment in all fractions of *P. tricornutum* (Figures 4 and 5). The highest yield of fucoxanthin was 18.6 ± 0.8 mg/g using ethanol. As has already been reported, ethanol appeared to be one of the best solvents to extract fucoxanthin from *P. tricornutum* (Kim, Jung, et al., 2012) as well as macroalgae (Shang

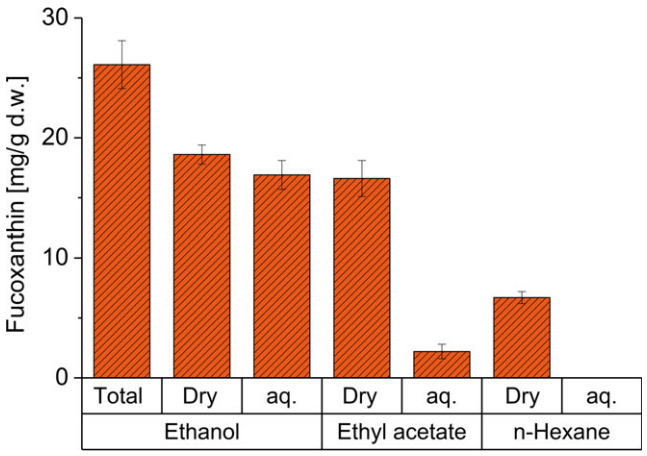

FIGURE 5 Fucoxanthin yield (in mg/g of dry weight) in fractions derived from freeze-dried and wet (30% w/w dry weight) *P. tricornutum* biomass by PLE (100 bar, 100°C, t = 20 min) using different extraction solvents. The error bars indicate the mean standard derivation (n = 3)

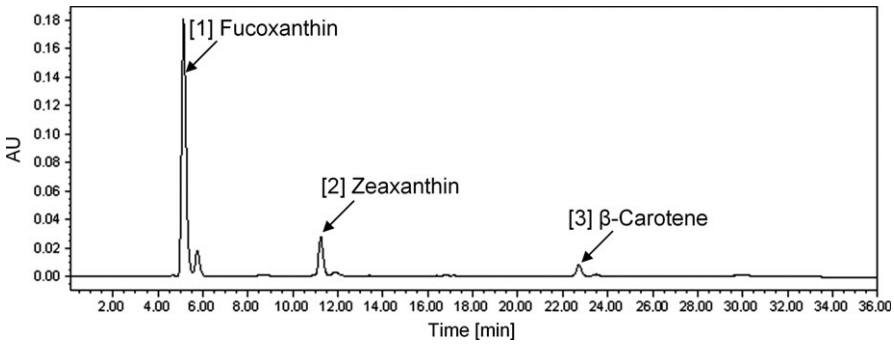

FIGURE 4 HPLC chromatogram of a lipid fraction from *P. tricornutum* (freeze-dried) extracted by PLE at 100 bar and 100°C using ethanol. Main peaks were identified as [1] fucoxanthin, [2] zeaxanthin, and [3] beta-carotene

et al., 2011). Additionally, minor amounts of zeaxanthin (1.4 ± 0.1 mg/g) as well as traces of beta-carotene were detected in the ethanolic fractions of *P. tricornutum*.

To get an impression of the total fucoxanthin content available in the *P. tricornutum* biomass, the PLE extraction was repeated on single samples until no fucoxanthin was detected in the last corresponding fraction. Hence, a total fucoxanthin yield of 26.1 ± 2.0 mg/g was obtained. Fucoxanthin levels of 7.73 to 16.33 mg/g have already been reported for *P. tricornutum* (Gilbert-López, Barranco, Herrero, Cifuentes, & Ibáñez, 2016; Kim, Jung, et al., 2012) as well as other microalgae species like *Isochrysis aff. galbana* and *Odontella aurita* with 18.23 mg/g and 21.67 mg/g, respectively (Kim, Kang, Kwon, Chung, & Pan, 2012; Xia et al., 2013). Nevertheless, to the knowledge of the authors, this is the highest fucoxanthin content reported for *P. tricornutum* biomass produced outdoors yet. Since constituents, especially carotenoids, of diatoms like *P. tricornutum* can change readily according to the cultivation conditions (Herbstová et al., 2015), we assume that the high overall fucoxanthin content is presumably due to the optimized cultivation conditions and superior light distribution provided by flat-panel airlift reactor systems (Bergmann, Ripplinger, Beyer, & Trösch, 2013).

In general, a high content of antioxidants such as carotenoids in *n*-PUFA-rich extracts might be advantageous to protect unsaturated lipids from undergoing oxidation processes (Goiris et al., 2012). For *P. tricornutum,* we observed a higher antioxidant capacity for the ethanolic than for the *n*-hexane extract measured by FRAP (ferric reducing antioxidant power) assay (data not shown). This might be due to the higher carotenoid content as well as the improved extraction of polyphenols with ethanol.

4 | CONCLUSION

The results show that an adjustment of the extraction solvent to each microalgae species is mandatory to efficiently extract lipids from both wet *C. vulgaris* and *P. tricornutum* biomass by PLE. Based on the data introduced in this study, we conclude that a drying step prior to extraction might be unnecessary to yield fatty acids and fucoxanthin from *P. tricornutum* biomass, when using polar, water-miscible extraction solvents like ethanol. These findings and related data can be of importance considering scale-up and the economic analysis of microalgae downstream processing.

In case of TAG-rich *C. vulgaris*, medium-polar solvents like ethyl acetate seem to be more suitable for subcritical extraction of fatty acids from wet biomass while both very polar solvents like ethanol and nonpolar solvents like hexane either do not efficiently extract TAGs due to polarity or are not able to penetrate the water layer surrounding the cells.

In general, fatty acid yields of above 75% w/w where achieved for both microalgae species in a single extraction step at increased temperatures of up to 150°C using wet biomass. Temperatures above the boiling point of the extraction solvents showed to drastically increase the fatty acid yield, enabling an efficient extraction at a very low fixed extraction time. The high amounts of pigments, for example, fucoxanthin, in the extracts furthermore indicate that the low extraction time can be also beneficial to extract thermosensitive components, which can protect unsaturated lipids from undergoing oxidation processes during further processing.

ACKNOWLEDGEMENTS

This work by Felix Derwenskus was supported by a grant (7533-10-5/91/2) from the Ministry of Science, Research and the Arts of Baden-Wuerttemberg (MWK), Germany, as part of the BBW ForWerts Graduate Program. The authors wish to thank all project partners involved in the research area "Microalgae—Integrated use for food and feed" of the Bioeconomy Research Program Baden-Württemberg funded by the MWK.

ORCID

Felix Derwenskus http://orcid.org/0000-0002-6465-8990

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How to cite this article: Derwenskus F, Metz F, Gille A, et al. Pressurized extraction of unsaturated fatty acids and carotenoids from wet *Chlorella vulgaris* and *Phaeodactylum tricornutum* biomass using subcritical liquids. *GCB Bioenergy*. 2019;11:335–344. https://doi.org/10.1111/gcbb.12563