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# Evaluation of Extraction Techniques for Recovery of Microalgal Lipids under Different Growth Conditions

Goldy De Bhowmick,* Maxence Plouviez, Mariza Gomes Reis, Benoit Guieysse, David W. Everett, Michael P. Agnew, Paul Maclean, and Caroline Thum*

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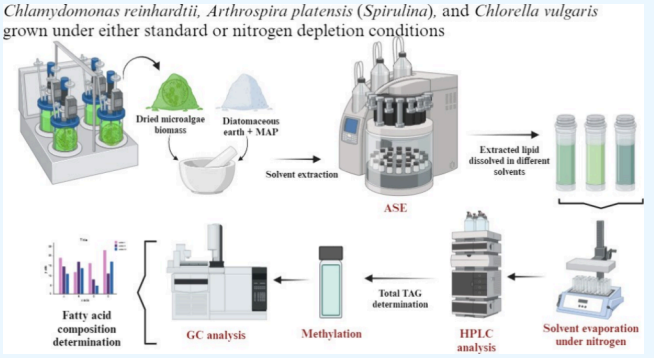
**ABSTRACT:** Microalgal lipids contain a wide array of liposoluble bioactive compounds, but lipid extraction remains a critical limitation for their commercial use. An accelerated solvent extraction (ASE) was used to extract lipids from *Chlamydomonas reinhardtii*, *Arthrospira platensis* (*Spirulina*), and *Chlorella vulgaris* grown under either standard or nitrogen depletion conditions. Under standard growth conditions, ASE using methanol:chloroform (2:1), methyl *tert*-butyl ether (MTBE):methanol:water, and ethanol at 100 °C resulted in the highest recovery of total lipids (352 ± 30, 410 ± 32, and 127 ± 15 mg/g biomass from *C. reinhardtii*, *C. vulgaris*, and *A. platensis*, respectively). Similarly, the highest total lipid and triacylglycerols (TAGs) recovery from biomass cultivated under nitrogen depletion conditions was found at 100 °C using methanol:chloroform, for *C. reinhardtii* (total, 550 ± 21; TAG, 205 ± 2 mg/g biomass) and for *C. vulgaris* (total, 612 ± 29 mg/g; TAG, 253 ± 7 mg/g biomass). ASE with MTBE:methanol:water at 100 °C yielded similar TAG recovery for *C. reinhardtii* (159 ± 6 mg/g) and *C. vulgaris* (200 ± 4 mg/g). Thus, MTBE:methanol:water is suggested as an alternative substitute to replace hazardous solvent mixtures for TAGs extraction with a much lower environmental impact. The extracted microalgal TAGs were rich in palmitic (C16:0), stearic (C18:0), oleic (C18:1,9), linoleic (C18:2n6), and α-linolenic (C18:3n3) acids. Under nitrogen depletion conditions, increased palmitic acid (C16:0) recovery up to 2-fold was recorded from the biomasses of *C. reinhardtii* and *C. vulgaris*. This study demonstrates a clear linkage between the extraction conditions applied and total lipid and TAG recovery.




## 1. INTRODUCTION

Microalgal lipids contain a wide array of liposoluble bioactive compounds with nutraceutical and pharmacological applications.[1,2] Several microalgal species can accumulate lipids up to 20−80% of their dry cell weight.[1,3] Microalgae typically synthesize fatty acids to provide structural and functional roles as charged membrane lipids under optimal growth conditions, but they can also accumulate neutral lipids, such as triacylglycerols (TAGs), under nutrient depletion conditions.[4]

Despite decades of research, lipid extraction remains as a critical limitation to the commercialization of microalgae oils[4] as this downstream process usually accounts for the majority of oil production costs.[5] In addition, limited attention has been given to understanding the effect of lipid extraction conditions, the fatty acid profile of the oil recovered, and key components such as TAGs. Reliable methods for lipid extraction, such as the Bligh and Dyer[6] and Folch methods,[7] have been used over the last half-century but the use of toxic solvents limits subsequent food applications. Moreover, Chen et al. (2020) and Karim et al. (2020) reported a disparity in total lipid yield recovery after different extraction techniques.[8,9] Several other cell wall disruption techniques, such as sonication, osmotic shock, autoclaving, microwave heating, bead-beating, and enzymatic treatment, have been employed to achieve higher lipid extraction efficiency.[1,10,11] Although these techniques increased the extraction efficiency by ∼20%, they were effective only for specific microalgal species.

Accelerated solvent extraction (ASE) is a promising alternative automated lipid extraction technique to traditional methods.[8] Due to the contact efficiency of the solvent-sample matrix coupled with elevated temperatures and pressures, ASE can significantly reduce solvent utilization while shortening the extraction time.[8,10] A 90% reduction in organic solvent usage with a higher lipid recovery rate from cereal, egg yolk, and chicken has been reported using ASE.[10,12] ASE has been used to extract microalgal lipids;[10] however, an optimized method

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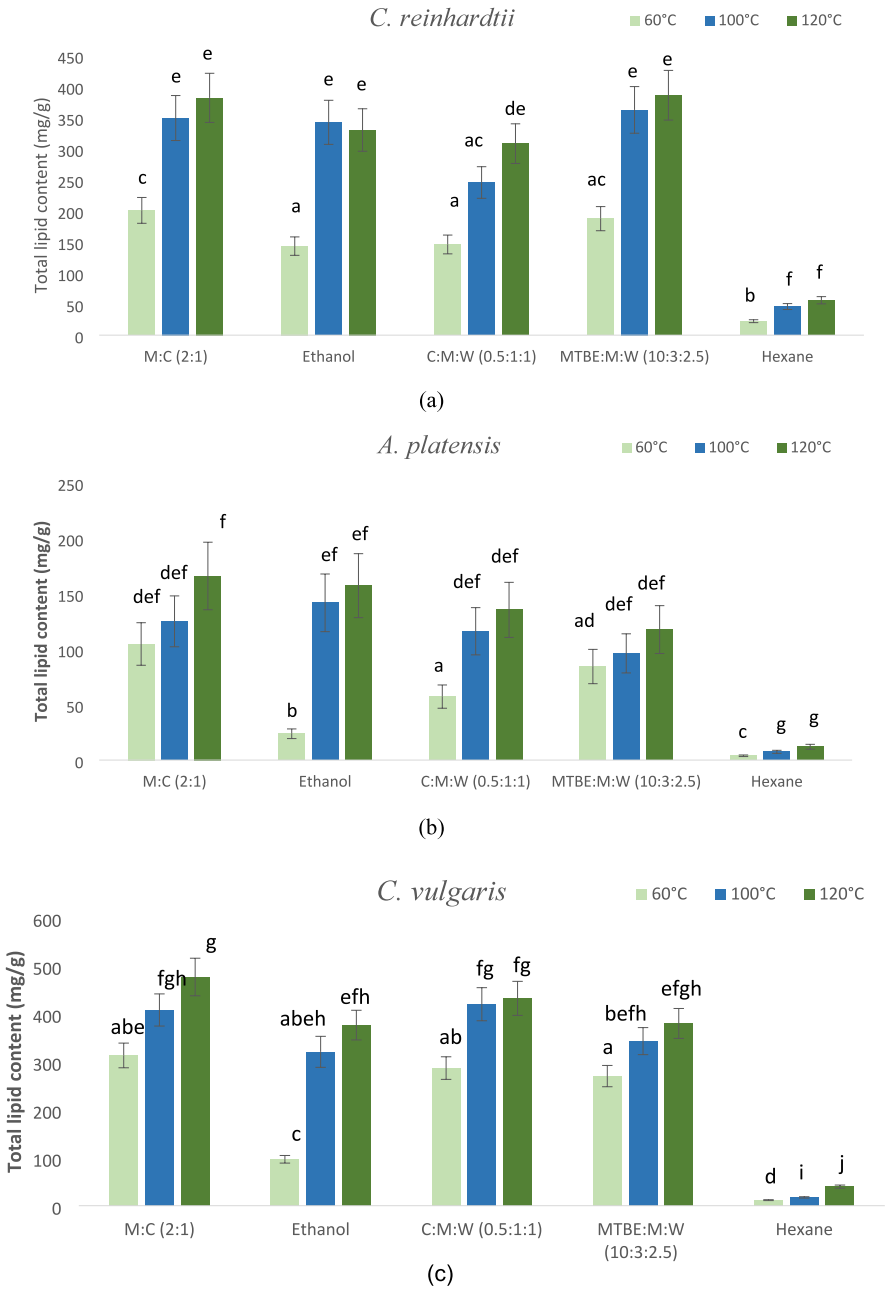


**Figure 1.** Total lipid recovery from *C. reinhardtii* (a), *A. platensis* (b), and *C. vulgaris* (c) grown under standard conditions and extracted at 60, 100, and 120 °C with different solvent mixtures. Superscript letters indicate significant differences at the $p = 0.05$ level. Abbreviations for horizontal axis labels: M: methanol; C: chloroform; W: water.

for TAG recovery from microalgae species with different cell walls remains to be determined.

Microalgae species that accumulate lipids can differ in cell wall structure, thus requiring different lipid extraction methods. *Chlamydomonas reinhardtii*, for example, has a cell wall composed mainly of hydroxyproline-rich glycoproteins, whereas *Chlorella vulgaris* has a rigid structure composed of chitin or chitosan-like polysaccharides, and *Arthrospira platensis* (*Spirulina*—a prokaryotic cyanobacterium) has small amounts of murein but no cellulose.[13] In addition, the conditions used during microalgae cultivation have a profound impact on the yields and compositions of the accumulated lipids. The objectives of this study were therefore (i) to determine the recovery of total lipids and TAGs from *C. reinhardtii*, *C. vulgaris*, and *A. platensis* grown under either standard (i.e., complete nutrient profile in the context of this study) or nitrogen depletion conditions using ASE at various temperatures and with different solvent mixtures and (ii) to evaluate the recovery of individual fatty acids.

## 2. RESULTS AND DISCUSSION

**2.1. Total Lipid Recovery under Conventional Growth Conditions.** The total lipid content of the dry biomass extracted was determined gravimetrically and expressed as milligrams per gram of dry cell weight for all batch extractions (Figure 1). Two-way permutation ANOVA revealed significant differences ($p < 0.05$) in total lipid recovery for *C. reinhardtii* for temperature and solvent factors but not for their interaction ($p = 0.697$). *A. platensis* and *C. vulgaris* lipid recoveries were



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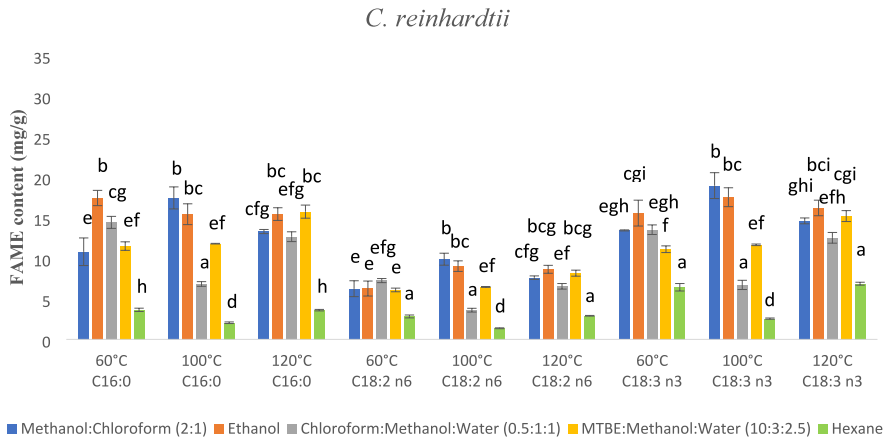
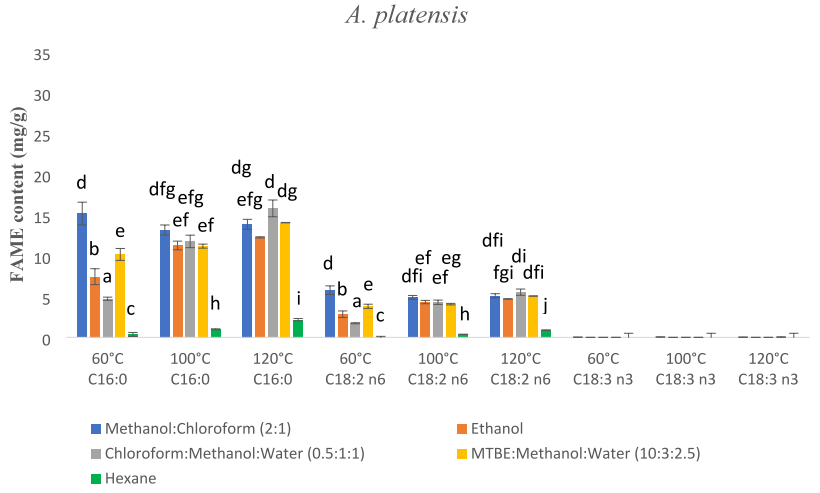



(a)

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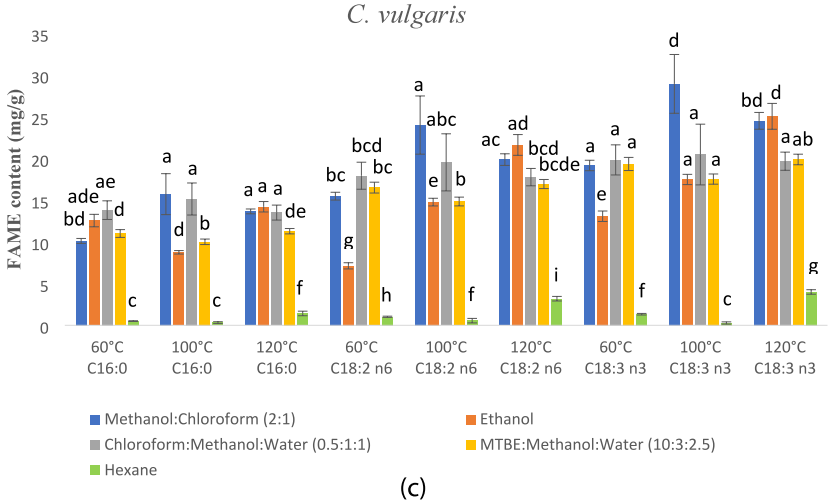


(c)

**Figure 2.** Fatty acid methyl ester (FAME) recovery from *C. reinhardtii* (a), *A. platensis* (b), and *C. vulgaris* (c) grown under standard conditions and extracted at 60, 100, and 120 °C with various solvent mixtures. Data show average ± standard error ($n$ = 3). Superscript letters indicate significant differences at the $p$ = 0.05 level.

significantly affected ($p < 0.05$) by the temperature and solvent used during ASE, as well as their interactions. Notably, our data confirms past studies showing *A. platensis* has a lower total lipid content than *Chlorella* and *Chlamydomonas*.[14]

The highest recoveries of total lipids were recorded in biomass extracted at 100 °C for methanol:chloroform, and these conditions yielded 352 ± 28, 410 ± 32, and 127 ± 15 mg lipid/g biomass from *C. reinhardtii*, *C. vulgaris*, and *A. platensis*, respectively, against 343 ± 13, 322 ± 28, and 144 ± 19 mg/g using ethanol at 100 °C, and 363 ± 14, 344 ± 16, and 100 ± 21 mg/g methyl *tert*-butyl ether (MTBE):methanol:water at the same temperature. These recoveries are consistent with



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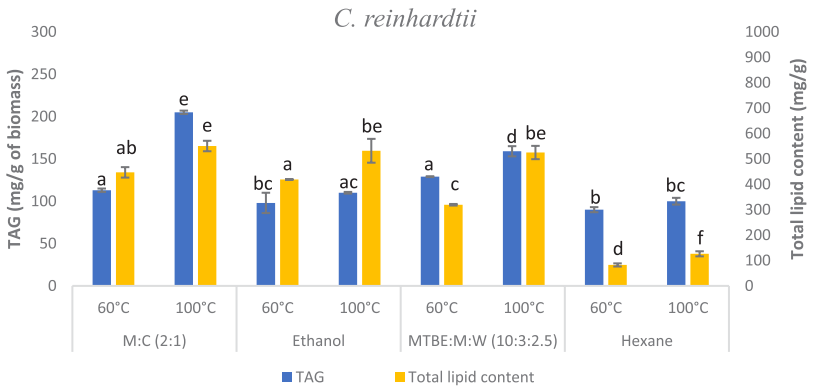
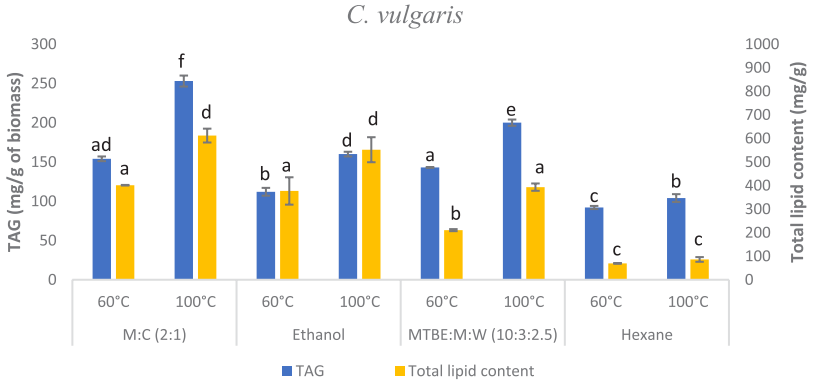



(a)




(b)

**Figure 3.** Triacylglycerol (TAG) and total lipid recovery from (a) *C. reinhardtii* and (b) *C. vulgaris* grown under nitrogen depletion conditions. Average values are represented as standard error ($n$ = 3) with significant differences at the $p$ = 0.05 level. Blue bars represent TAG recovery with abundances on the left axis, while yellow bars represent total lipid recovery with abundances on the right axis. Significance grouping letters above the error bars are for TAG recovery, while significance grouping letters below the error bars are for total lipid recovery. Abbreviations for horizontal axis labels: M: methanol; C: chloroform; W: water.

data from previous studies.[14−16] In agreement with these findings, Gorgich et al. (2020) showed that lipid extraction of *Chlorella zofingiensis*, *Phaeodactylum tricornutum*, and *A. platensis* using MTBE recovered similar or slightly higher recovery than the Bligh and Dyer method solvent mixtures.[6,17] Furthermore, He et al. (2019) also reported higher lipid recovery from the ASE extraction of *Isochrysis galbana* using ethanol compared to chloroform followed by hexane.[18] This may be related to the polarity of the solvent, as the more polar chloroform and ethanol would solubilize more intercellular membrane lipids than hexane. As hexane is a nonpolar solvent and will have a lower ability to extract polar lipids from microalgal cells, this may lead to selective extraction of neutral lipids. Indeed, Balasubramanian et al. (2013) reported an increase in lipid recovery up to 19% when other polar solvents were combined with hexane.[19] The results from our study (Figure 1) and TAG analysis (supplementary Figure S1) suggest that the three microalgae grown under standard growth conditions were not rich in neutral lipids. The highest TAG recoveries from *C. reinhardtii*, *C. vulgaris*, and *A. platensis* grown under standard growth conditions were 1.00 ± 0.03 (MTBE:methanol:water), 1.21 ± 0.03 (methanol:chloroform), and 0.85 ± 0.05 (MTBE:methanol:water) mg/g of dry biomass, respectively.

Temperature significantly impacted lipid recovery irrespective of the solvent mixture and microalgae tested (Figure 1). A significant increase in total lipid recovery was observed when the extraction temperature was increased from 60 to 100 °C, but not from 100 to 120 °C (except for *C. reinhardtii* extraction using chloroform:methanol:water). Thus, the lipid recovery from *C. reinhardtii*, *A. platensis*, and *C. vulgaris* extraction using methanol:chloroform increased by approximately 80%, 10%, and 30%, respectively, by increasing the extraction temperature from 60 to 100 °C. Temperature also significantly impacted the extraction of chlorophyll (Supplementary Figure S2). Following the classical Bligh and Dyer solvent mixture (methanol:chloroform) method, the extracted lipids included pigments[20] at all extraction temperatures. Hexane, however, limited the extraction of pigments in the lipid fractions at lower temperatures for *C. reinhardtii*, *C. vulgaris*, and *A. platensis*.

**2.2. Fatty Acid Concentration in Recovered Lipid Extracts from Microalgae Grown under Standard Conditions.** The concentrations of the main fatty acids detected in *C. reinhardtii* and *C. vulgaris*, palmitic (C16:0), linoleic (C18:2n6), and α-linolenic (C18:3n3) acids, are illustrated in Figure 2. The concentrations of other major fatty acids detected are reported in the Supporting Information (Table S1). The main fatty acids in *A. platensis* were palmitic and linoleic acid (Figure 2 b). Overall, the profile of the fatty



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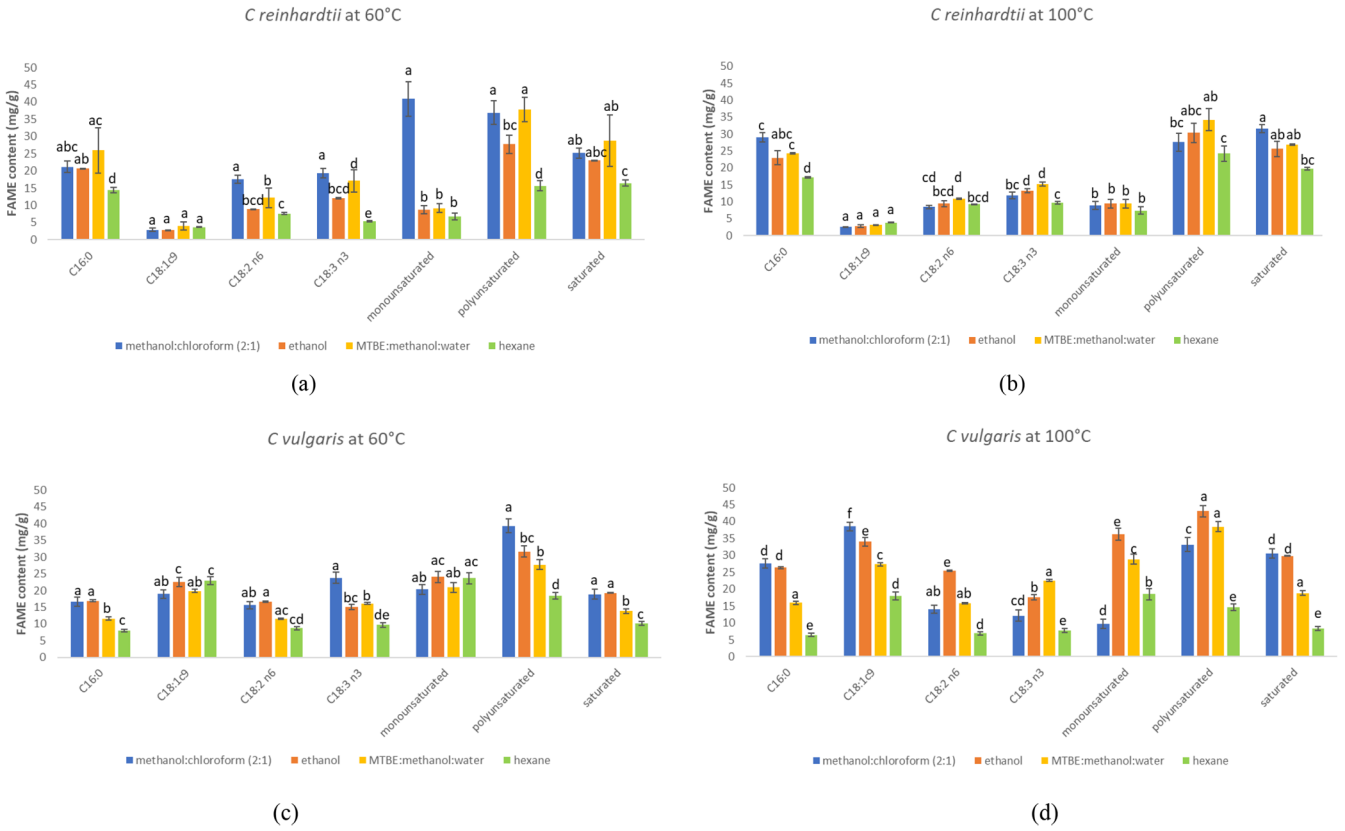


**Figure 4.** Individual and combined fatty acid concentrations for *Chlamydomonas reinhardtii* (a and b) and *Chlorella vulgaris* (c and d) at 60 and 100 °C under nitrogen depletion conditions. Average values are represented with standard errors ($n = 3$) and significant differences at the $p = 0.05$ level.

acids recovered from *C. reinhardtii*, *C. vulgaris*, and *A. platensis* agree with previously reported data for the three microalgae, wherein the fatty acid fractions were mainly composed of 16 and 18 carbon atoms regardless of lipid extraction conditions.[20−24]

Extraction with methanol:chloroform and ethanol at 100 °C yielded the highest C16 fatty acid recovery from *C. reinhardtii*, *C. vulgaris*, and *A. platensis* (Figure 2). These conditions also yielded the highest recovery of C18:2n6 and C18:3n3 from *C. reinhardtii*, but only extraction with methanol:chloroform yielded the best results at 100 °C for *A. platensis* and *C. vulgaris*. Zheng et al. (2012) previously reported optimal recovery of fatty acids from *A. platensis* using methanol:chloroform (2:1) and ASE independent of temperature.[20] In agreement with total lipid recovery data, the use of hexane resulted in poor recovery of lipids containing fatty acids, indicating that the fatty acids from these microalgae grown under standard conditions mainly originate from polar lipids.

**2.3. Total Lipid and TAG Recovery under Nitrogen Depletion Conditions.** Nitrogen depletion is well-known to trigger neutral lipid accumulation in microalgae.[25−27] Moreover, nutrient-depleted microalgae cell walls have been shown to become thicker.[28,29] We therefore carried out another set of extractions for *C. reinhardtii* and *C. vulgaris* grown under nitrogen depletion conditions. *A. platensis* was not further investigated due to its lower total lipid content. ASE extraction was carried out at 60 and 100 °C using methanol:chloroform (2:1), ethanol, MTBE:methanol:water (10:3:2.5), or hexane.

Similar to the results obtained from biomass grown under standard conditions, the highest lipid recoveries from *C. reinhardtii* (550 ± 21 mg/g) and *C. vulgaris* (612 ± 29 mg/g) grown under nitrogen depletion conditions were found at 100 °C using methanol:chloroform. Recovery amounts of 532 ± 47 and 550 ± 53 mg/g were achieved from *C. reinhardtii* and *C. vulgaris*, respectively, using ethanol at 100 °C. Although similar recoveries were recorded from *C. reinhardtii* using MTBE:methanol:water and methanol:chloroform at 100 °C (approximately 525 ± 26), ASE using ethanol yielded a much lower recovery from *C. vulgaris* (390 ± 15 mg/g). Previous studies have shown that changes in intracellular biochemical composition occurred alongside changes in specific morphological appearances in nutrient-depleted *C. vulgaris*.[30] Recently, Canelli et al. (2021) reported that the thickness of the cell wall of *C. vulgaris* varied from 82 to 114 nm under nutrient depletion growth conditions.[31] Therefore, MTBE:methanol:-water may not be as effective as methanol:chloroform and ethanol in penetrating the thick cell wall of nitrogen-depleted *C. vulgaris* cells.

As expected, the lipid yield from microalgae grown under nitrogen depletion conditions (Figure 3) was higher than the yield from biomass grown under standard conditions (Figure 1). Lipid recovery at 100 °C from *C. reinhardtii* and *C. vulgaris* increased by approximately 22% and 50%, respectively, when using methanol:chloroform, 25% and 37% when using ethanol, 57% and 50% when using MTBE, and 25% and 2% when using hexane. These increases in total lipid recoveries were driven by the accumulation of TAGs under nitrogen depletion conditions (Figure 3), as previously reported.[27,32,33]

Two-way ANOVA showed significant differences in TAG recovery from *C. reinhardtii* and *C. vulgaris* (temperature, solvent, and the interaction effect, $p < 0.05$). ASE extraction with methanol:chloroform at 100 °C was most effective at


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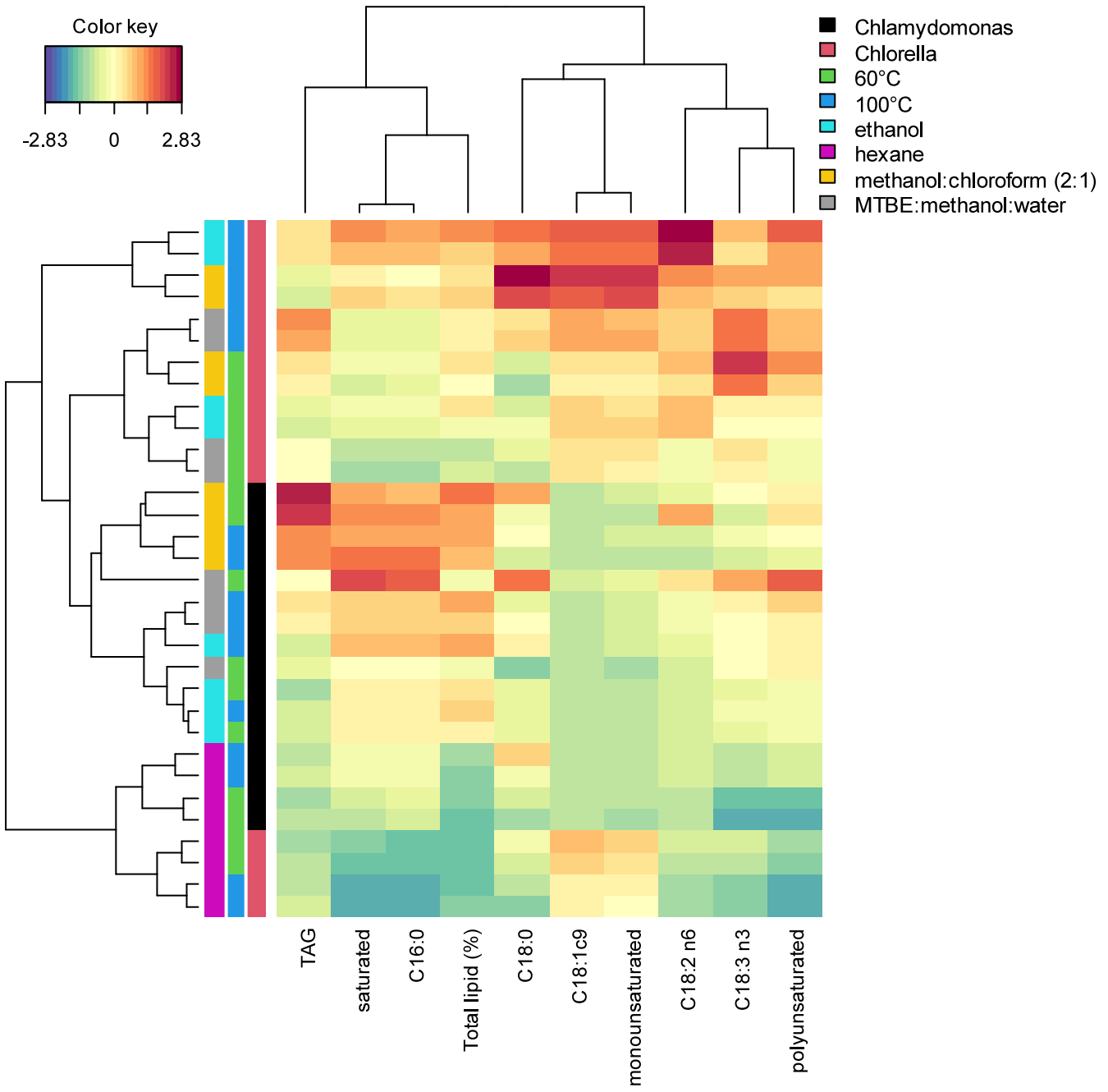


**Figure 5.** Heat map of standardized fatty acid concentrations. Total fatty acids, individual fatty acids, and saturated and unsaturated fatty acid values are given as standardized mg/g. Heat map cells colored orange and red indicate higher values, whereas blue and green indicate lower values relative to each column. The row and column dendrograms show clustering of the samples and measured variables, respectively. The three columns to the left of the dendrogram indicate the sample species, extraction temperature, and extraction batch, respectively.

extracting TAGs from *C. vulgaris* ~ 253 ± 7 mg/g and *C. reinhardtii* ~ 205 ± 2 mg/g, followed by MTBE:methanol:-water (*C. vulgaris* ~ 200 ± 4 mg/g; *C. reinharditi* ~ 159 ± 6 mg/g). TAG recovery increased by 102−126% when *C. reinhardtii* and *C. vulgaris* were nitrogen-depleted compared to the standard growth conditions. The use of hexane alone was more selective at extracting TAGs[34] proportional to total lipids for both the microalgae tested (Figures 3), which could reduce further purifications steps when high TAG yield is the desired outcome.

Nitrogen depletion is typically associated with a decrease in cell growth and a divergence of energy to storage in the form of TAGs in microalgae. This phenomenon is complex, as carbon partitioning depends upon other factors such as nitrogen metabolism and photosynthetic carbon fixation.[26,35] In a previous study, Zhu et al. (2015) reported a multifold increment in TAG content (up to 270.3 mg/g) under nitrogen depletion conditions in *Chlorella zofingiensis*, which was also observed in our findings.[36] Recently, Laraib et al. (2021) reported significant accumulation of neutral lipids from 20% to 40−45% under nitrogen depletion conditions for *C. vulgaris*.[37]

**2.4. Fatty Acid Profiles from Biomass of Microalgae Grown under Nitrogen Depletion.** The fatty acid profiles of extracted lipids from nitrogen-depleted *C. reinhardtii* and *C.*



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*vulgaris* biomasses are shown in Figure 4, with statistical differences listed in the Supporting Information (supplementary Table S2). Similar fatty acids were enriched (e.g., C16:0, C18:1 *cis* 9, C18:2n6, C18:3n3) in *C. reinhardtii* and *C. vulgaris* grown under nitrogen depletion conditions compared to standard conditions (Figure 2 and Supporting Information Table S2). The changes in the fatty acid profile seem to be mainly driven by TAG accumulation, as previously discussed in section 2.3. This observation agrees with past reports of the fatty acid profiles for these microalgae strains grown under standard and nitrogen depletion conditions.[21−24,38,39]

An increase in palmitic acid (C16:0) concentration up to 2-fold was observed from the biomasses of *C. reinhardtii* and *C. vulgaris* cultivated under nitrogen depletion conditions compared to growth under standard conditions. Additionally, an increased in linoleic (C18:2n6) up to 2-fold was observed from *C. reinhardtii*, whereas an increase of $\alpha$-linolenic (C18:3n3) up to 3-fold was recorded from *C. vulgaris*. Earlier, Fidalgo et al. (1998) reported a similar increment in palmitic acid from *I. galbana* grown under severe nitrogen depletion conditions, wherein mostly neutral lipids (TAGs) were intracellularly accumulated.[35] In addition, James et al. (2011) showed that oleosin- and caleosin-like genes were activated in *C. reinhardtii* cultivated under nitrogen depletion conditions and that this activation enhanced TAG intracellular accumulation.[40] Furthermore, Zhu et al. (2015) explained that under nitrogen deprivation, the major saturated fatty acids were mainly derived from neutral lipid fractions,[36] as evident in our findings. Interestingly, in the study from Zhu et al. (2015), the yield of unsaturated fatty acids, including mono- and polyunsaturated, were higher than the yield of unsaturated fatty acids for both *C. vulgaris* and *C. reinhardtii* grown under photoautotrophic cultivation conditions compared to stress conditions.[36] Similarly, Laraib et al. (2021) reported that the yield of unsaturated fatty acids from *C. vulgaris* was higher than the yield of saturated fatty acids under both photoautotrophic cultivation and nitrogen depletion conditions,[37] and the proportion of individual saturated fatty acids increased significantly over time.

Fatty acid yield from *C. reinhardtii* and *C. vulgaris* grown under nitrogen depletion conditions was affected by the temperature and solvent used during ASE. The highest fatty acids recovery was recorded at 100 °C, the highest temperature tested (Figure 4), consistent with TAG and total lipid recoveries (Figures 2 and 3). The highest recoveries of C:16, C18:1 *cis* 9, and C18:2n6 (but not C18:3n3) were obtained from *C. reinhardtii* cultivated under nitrogen depletion conditions using methanol:chloroform, ethanol, and MTBE at 100 °C. Interestingly, for *C. vulgaris*, the extraction conditions yielding the highest recovery of total lipids (methanol:chloroform and ethanol, 100 °C) only yielded the highest recovery for C16:0. Although the use of methanol:-chloroform also yielded a good recovery of C18:1 *cis* 9, this solvent inefficiently recovered C18:2n6 and C18:3n3 from *C. vulgaris* compared to ethanol and MTBE, suggesting degradation of C18:2n6 and C18:3n3 using cholorofom:methanol at 100 °C. High temperature along with MTBE and ethanol might have enhanced the solubility of lipids containing fatty acids, in particular, polyunsaturated fatty acids, for both microalgae tested. Besides, the differences in fatty acid recovery observed for *C. vulgaris* may be due to rigid/thicker cell wall structure resulting from stressed growth.[41]

Overall, the proportion of both saturated and unsaturated fatty acids increased in nitrogen-depleted microalgae. Furthermore, when the fatty acid profile of lipids extracted from *C. vulgaris* and *C. reinhardtii* using ASE was compared with other conventional extraction techniques, palmitic, linoleic, $\alpha$-linolenic, and oleic acid were found to be the main fatty acids. Therefore, our results reveal that lipid extraction is profoundly affected by the type of microalgae, and depending upon the solvent, selective extraction of individual classes of lipids is possible.

**2.5. Heat Map of Interactions.** A heat map of the standardized total lipid, TAGs, and fatty acid concentrations for each sample is presented in Figure 5 to compare the clustering of the samples with the extraction conditions. The vertical dendrogram shows the clustering of the samples for the measured variables with the row side colors indicating the sample matrix, extraction temperature, and extraction batch. Red, orange, and yellow indicate higher abundances of lipid concentrations, whereas blue and green indicate lower lipid concentrations. This study found a clear link between the extraction conditions applied and the total lipid and TAG recovery. Overall, the best yields to extract total lipids, TAGs, and saturated fatty acids (i.e., C16) were obtained by using methanol:chloroform as a solvent at 100 °C to extract lipids from *Chlorella*. If polyunsaturated fatty acids (i.e., C18:2n6) are of interest, the best conditions were obtained from the same algae using ethanol at 100 °C or methanol:chloroform as a solvent at 60 °C (i.e., C18:3n3).

## 3. CONCLUSIONS

The ASE extraction results clearly demonstrate that the extraction solvent has a significant effect on the extraction efficiency for the total lipids and TAGs for all three microalgae (*Chlamydomonas reinhardtii*, *Arthrospira platensis* (*Spirulina*), and *Chlorella vulgaris*) tested. Under standard growth conditions and among all the solvents tested, methanol:chloroform (2:1), methyl *tert*-butyl ether (MTBE):methanol:water, and ethanol at 100 °C resulted in the highest recoveries of total lipids (352 ± 30, 410 ± 32, and 127 ± 15 mg/g biomass from *C. reinhardtii*, *C. vulgaris*, and *A. platensis*, respectively). Furthermore, under nitrogen depletion conditions, the highest lipid recoveries were observed for *C. reinhardtii* and *C. vulgaris* (550 ± 21 and 612 ± 29 mg/g biomass) at 100 °C using methanol:chloroform and ethanol, respectively.

Nitrogen depletion was highly effective at elevating the TAG content for all three microalgae extractions. Again, methanol:-chloroform at 100 °C resulted in the highest recovery of TAGs from *C. reinhardtii* (205 ± 2 mg/g) and *C. vulgaris* (253 ± 7 mg/g), respectively. Additionally, MTBE:methanol:water also resulted in higher recovery of TAGs for *C. reinhardtii* (159 ± 6 mg/g) and *C. vulgaris* (200 ± 4 mg/g). Therefore, MTBE:methanol:water is suggested as an alternative substitute to replace hazardous solvent mixtures for lipid extraction with a much lower environmental impact. Furthermore, the fatty acid analysis revealed that the microalgae oil was rich in palmitic (C16:0), stearic (C18:0), oleic (C18:1 *cis* 9), linoleic (C18:2n6), and $\alpha$-linolenic (C18:3n3) acid. Under nitrogen depletion conditions, increased palmitic acid (C16:0) recovery up to 2-fold was recorded from the biomasses of *C. reinhardtii* and *C. vulgaris*. Similarly, an increase in linoleic acid (C18:2n6) up to 2-fold was recorded for *C. reinhardtii*, whereas an increase of $\alpha$-linolenic acid (C18:3n3) up to 3-fold was recorded for *C. vulgaris*.


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The research findings from this study suggest that there is a clear link between the extraction conditions applied and the total lipid and TAG recovery. Overall, the best yields to extract total lipids, TAGs, and saturated fatty acids (i.e., C16) from *C. vulgaris* and *C. reinhardtii* were obtained by using methanol:-chloroform as a solvent at 100 °C.

Future studies will investigate the total lipidomic profile of microalgae oil produced under different growth conditions and extracted using an optimized ASE methodology. It is expected that a more in-depth understanding of the effects of starvation and extraction conditions on total lipid profile will be observed.

## 4. MATERIALS AND METHODS

**4.1. Chemicals.** Analytical grade methanol, chloroform, ethanol, methyl *tert*-butyl ether (MTBE), HPLC grade isooctane, ethyl acetate, isopropyl alcohol, acetic acid, and hexane were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Triundecanoin (tri C11:0), tripalmitin, and fatty acid methyl ester (FAME) standards were purchased from Sigma-Aldrich (Auckland, New Zealand).

**4.2. Microalgal Species.** *C. reinhardtii*, *A. platensis*, and *C. vulgaris* were selected as commercially relevant microalgae. *C. reinhardtii* 6145 was purchased from the Chlamydomonas Resource Center (St. Paul, MN, USA), *C. vulgaris* UTEX 259 was purchased from the Culture Collection of Algae at the University of Texas (Austin, TX, USA), and *A. platensis* (a prokaryotic cyanobacterium) biomass was donated by NZ Algae Innovation (Mapua, New Zealand).

The terms nutrient-limitation, nutrient-depletion, nutrient-starvation, or nutrient-deprivation are used in the literature to describe conditions ranging from near-normal growth in the absence of detectable extracellular nutrient to severe growth inhibition. In this study, the term nitrogen depletion is used to describe photosynthetic cultivation in the absence of an exogenous nitrogen supply (none of the microalgae tested can fix $N_2$), and standard growth is used to describe photosynthetic cultivation in a medium supplied with nitrogen.

- *Standard growth*: *C. reinhardtii* and *C. vulgaris* were grown in tris acetate phosphate buffer[42] and BG11 medium,[43] respectively. All cultures were inoculated from 500 mL stock cultures to 4.5 L of media in 5 L Duran bottles mounted with caps equipped with tubing and 0.22 $\mu$m filters to sparge the cultures with 2% $CO_2$ at 1 L/min. The cultures were grown under constant temperature (22 °C) and illumination (200 $\mu$mol/cm$^2$/s provided by three Phillips TLD 58W/865 cool fluorescent daylight tubes) conditions for 7 days before harvesting.
- *Nitrogen depletion*: *C. reinhardtii* and *C. vulgaris* were first grown under standard conditions for 7 days as described above. The cultures were then harvested by centrifugation, resuspended in their respective nitrogen-free media, and photosynthetically grown under nitrogen depletion conditions for another 7 days.

Harvesting was carried out in batches by centrifugation (10,000 × *g* for 3.5 min). The harvested biomass was frozen at −80 °C before freeze-drying in a Buchi Lyovapor L-300 for 24 h at 0.2 mbar with a temperature profile starting at −30 °C and finishing at 20 °C. All the glassware and media were autoclaved prior to use.

**4.3. Lipid Extraction, Experimental Design, and Sample Preparation.** A Dionex ASE 350 equipped with a dionium extraction cell instrument (Thermo Fisher Scientific) was used to extract oil from microalgal biomass. Two variable factors of solvent mixture ratio and temperature and two constant factors of static time and cycle of extraction were tested in this study. A full factorial experimental layout was, therefore, used for five separate batch extractions. The solvent and solvent mixtures used were methanol:chloroform (2:1, v/v), ethanol, chloroform:methanol:water (0.5:1:1, v/v/v), MTBE:methanol:water (10:3:2.5, v/v/v), and hexane. Three temperatures of 60 °C, 100 °C, and 120 °C were tested for all three microalgae. Altogether, a total of 135 individual extractions were carried out with a static time of 5 min and four extraction cycles. Based on the results obtained from each microalgal biomass extraction, further extractions at 60 and 100 °C with methanol:chloroform (2:1 v/v), ethanol, MTBE:methanol:water (10:3:2.5 v/v), and hexane were carried out on *C. reinhardtii* and *C. vulgaris* grown under nitrogen depletion conditions. All extractions were carried out in triplicate.

Prior to each extraction, 200 mg of freeze-dried biomass was ground with an ASE adsorbing agent (dry powdered mixture of diatomaceous earth and moisture adsorbing powder at a 10:1 ratio) by using a mortar and pestle to form a uniform mixture. The mixed powder was then poured into 22 mL stainless-steel ASE extraction cells, and the headspace was filled with white sand. A 0.2 $\mu$m cellulose fiber filter (Dionex, Sunnyvale, CA, USA), specific for ASE, was used to prevent the blockage of the frit in the bottom cap. Cells containing the samples were loaded into the ASE instrument, and the instrument software was set to follow the extraction protocol and experimental layout. The extraction steps were as follows: (i) the extraction solvent was used to rinse the cell; (ii) the extraction cells containing biomass samples were loaded into the ASE; (iii) the cells were filled with solvents and pressurized to 10.3 MPa; (iv) the cells were automatically preheated for 5 min; (v) extractions were carried out for 40 min with a flush volume ∼100% of cell volume and purge time ∼60 s; (vi) the cells were again rinsed using the extraction solvent. The extracted lipid samples were collected in 30 mL of ASE glass tubes for analysis.

**4.4. Processing of Extracted Lipids.** The lipid extracts were dried at 36−37 °C (REACTI-THERM III, heating module TS-18824) under nitrogen evaporation to prevent lipid oxidation. The total lipid content was determined gravimetrically. The dried extracts were then resuspended in chloroform (10 mL) and divided into two aliquots for further analysis. One aliquot was used for analyzing the TAG content by normal phase HPLC and the other aliquot was used for fatty acid derivatization following the one-step procedure developed by Agnew et al. (2019).[44]

*4.4.1. Total Lipid Content and HPLC-ELSD Analysis.* The total lipid content was determined gravimetrically and expressed in milligrams per gram of dry cell biomass. The resuspended crude lipid extracts were further reduced to 1 mL for TAG quantification using a Shimadzu low-temperature HPLC with an evaporative light scattering detector (ELSD) LT III system (Kyoto, Japan) equipped with two LC-10 Advp pumps, an SCL-10 Advp gradient system, a DGU-14 Advp module degasser, and an automatic injector. The analytical column was a YMC-pack PVA-SIL-NP column (250 × 4.6 mm, 5 $\mu$m) protected by a Waters Guard Pak guard column containing Nova-Pak silica inserts. Chromatographic separation was carried out by modifying the method developed by


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Jones et al. (2012).[45] The flow rate was kept at 1 mL/min, and the total run time was 81 min. Tripalmitin was used as the standard for determining the TAG content in the samples. Approximately 20 μL of samples were injected into the system. All analyses were carried out in triplicate.

*4.4.2. Fatty Acid Derivatization and Gas Chromatographic Analysis.* FAME-derivatized extracted lipids were analyzed according to the AOCS (1992) with modifications and using fatty acid methyl ester tri C11 as the internal standard.[46] Methanolic sodium methoxide ($CH_3ONa$, 0.5 N) and 1.5 M methanolic boron trifluoride were used for methylation instead of toluene/methanol 5% sulfuric acid as the methylating reagent. FAME mixtures were extracted using isooctane (2,2,4-trimethylpentane) and saturated NaCl solution. Briefly, tri-C11 (2 mg, internal standard) was added to each sample in glass tubes. Subsequently, a methanolic sodium methoxide solution (1.5 mL) was added to each tube and heated at 80 °C in a heating block (REACTI-THERM III heating module) for 10 min. After the incubation period, methanolic boron trifluoride (3 mL) was added to each tube and further heated for 30 min at 80 °C. Isooctane (1 mL) and saturated NaCl solution (5 mL) were added to each tube and either shaken vigorously or vortexed for 1 min and then centrifuged at 1000 × *g* for 4 min. An aliquot of the top layer was transferred to a 1.5 mL gas chromatography (GC) vial for GC-flame ionization detector (FID) analysis. Upon collection of the top layer of the FAME mixture, the samples were re-extracted by adding additional isooctane (1 mL). The samples were vigorously mixed, and the top layer was collected in the same GC vial that corresponded to the sample set.

FAME samples were analyzed by GC-FID (Shimadzu) equipped with a split injector.[44] An RTX 2330 (90% bis-cyanopropyl) column (105 nm × 0.25 mm inner diameter; 0.2 μm film thickness) from Restek (Bellefonte, PA, USA) was used for GC analysis. The column temperature was kept at 175 °C for 17 min, then raised to 220 °C at a rate of 6 °C/min, and held for 10 min. Hydrogen was used as the carrier gas at a constant velocity of 50 cm/s with a split ratio of 50. The injector temperature was kept at 260 °C, and the detector temperature was at 300 °C. Aliquots of FAME samples (∼1 μL), prepared as described, were injected into the GC. Individual FAME isomers were identified by comparison with standards.

**4.5. Statistical Analysis.** All the experiments were carried out in triplicate, and the results shown represent the mean ± standard error of the three replicates. Two-way permutation ANOVA was carried out using R (version 4.1.1, R Core Team, 2021) and the aovp function of the lmPerm R package, version 2.1.0,[47] with 1 million permutations to determine significant differences between recoveries recorded under different extraction conditions. Posthoc tests and the resulting significance groupings were calculated using the PredictMeans R package, version 1.0.8.[48] Statistical difference was assumed at $p < 0.05$. A heatmap was generated using the mixOmics package, version 6.16.3.[49]

## ■ ASSOCIATED CONTENT

### Data Availability Statement

The data sets generated for this study are available upon request to the corresponding author.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00221.

Figure S1 showing the triacylglycerol content of five extraction batches using four different solvent mixtures and two temperatures; Figure S2 showing pigment extractability; Table S1 showing the fatty profile of *Chlamydomonas*, *Spirulina*, and *Chlorella* from the five extraction batches at the three temperatures tested; and Table S2 showing the fatty acid profile of *Chlamydomonas* and *Chlorella* grown under nitrogen starvation and four extraction batches and two temperatures (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Authors

**Caroline Thum** − *AgResearch Ltd., Palmerston North 4474, New Zealand*; orcid.org/0000-0002-9823-1012; Email: caroline.thum@agresearch.co.nz

**Goldy De Bhowmick** − *AgResearch Ltd., Palmerston North 4474, New Zealand*; Email: goldy.debhowmick@agresearch.co.nz

### Authors

**Maxence Plouviez** − *School of Engineering and Advanced Technology, Massey University, Palmerston North 4442, New Zealand*; orcid.org/0000-0001-6824-697X

**Mariza Gomes Reis** − *AgResearch Ltd., Palmerston North 4474, New Zealand*

**Benoit Guieysse** − *School of Engineering and Advanced Technology, Massey University, Palmerston North 4442, New Zealand*; orcid.org/0000-0002-8711-3045

**David W. Everett** − *AgResearch Ltd., Palmerston North 4474, New Zealand; Riddet Institute, Palmerston North 4442, New Zealand*; orcid.org/0000-0001-5094-5123

**Michael P. Agnew** − *AgResearch Ltd., Palmerston North 4474, New Zealand*

**Paul Maclean** − *AgResearch Ltd., Palmerston North 4442, New Zealand*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c00221


### Author Contributions

G.D.B. − conceptualization; investigation; methodology; data curation; formal analysis; resources; original draft preparation; review and editing. M.R.G. − conceptualization; methodology; validation; supervision; review and editing. M.P. − conceptualization; investigation; formal analysis; review and editing. B.G. and D.W.E. − critical revision of the manuscript. M.A. − methodology. C.T. − funding acquisition; project management; review and editing. P.M. − formal analysis; review and editing. All authors have read and agreed to the published version of the manuscript.


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### Notes

The authors declare no competing financial interest.

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