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Comparison of fatty acid analysis methods for assessing biorefinery applicability of wastewater cultivated microalgae

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ABSTRACT

In this study, we compared the performances of four different gas chromatography (GC) based microalgal fatty acid analysis methods that are typically applied to biorefinery research using wastewater-adapted microalgae. Compared with the HP-5-type non-polar column, WAX-type polar columns exhibited excellent abilities to quantitatively separate C_{16}-C_{18} polyunsaturated fatty acids (PUFAs) from selected wastewater-adapted microalgae (*Chlorella vulgaris*, *Ankistrodesmus gracilis* and *Scenedesmus quadricauda*) isolates. GC-mass spectroscopy (MS) using the WAX-type polar column provided the strongest detection sensitivity among the tested methods by lowest detection limit, and GC-flame ionized detector (FID) with the same polar column exhibited nearly consistent results to GC-MS analysis. Our statistical comparison of microalgal fatty acid composition profiles generated using various GC methods, microalgal resources and culture media (wastewater, BG11 and nitrogen limitation) suggested that an appropriate GC method and algal resource choice are more important than the optimization of culture conditions to evaluate the applicability of microalgal biorefinery using wastewater resources.

1. Introduction

To address the problems of global warming and fossil fuel exhaustion, bioenergy has been regarded as one of the most attractive alternatives among renewable energy strategies [1]. The first generation of bioenergy strategies achieved biofuel production based on sugar, starch and vegetable or animal oils using conventional technology [2], but these methods have been criticized because they competitively consume food resources [3]. To circumvent this problem, the second generation of bioenergy uses non-edible or waste vegetable oils and agricultural wastes such as lumber, straw and leaves [4]. Recently, algae have been proposed as another charming resource for renewable bioenergy, not only because algae remove carbon dioxide from the atmosphere but also because most microalgae contain a much higher lipid content per biomass than other plants [5–7]. Moreover, since Osward and Golueke [8] proposed the use of microalgae for nutrient removal from wastewater, the nutrient uptake and growth/adaptation of microalgae in wastewater environments have been well established in the literature [9–11]. These features promise a sustainable biofuel production strategy that uses green microalgae in wastewater resources [12,13].

Algal lipid production has been regarded as a key physiological factor in the choice of microalgal resources for biodiesel applications [14]. However, the evaluation of user acceptability of microalgal-based biodiesel has revealed algal fatty acid composition as a critical characteristic because the fatty acid methyl ester (FAME) composition of biodiesel candidates must comply with existing standards such as the American Society for Testing and Materials (ASTM) Biodiesel Standard D6751 and European Union EN standards [5,15]. In addition, the current biodiesel standards have been established mainly for plant-derived fatty acids, but microalgae contain more diverse fatty acids than plants [12]. Some microalgae contain a higher proportion of unsaturated fatty acids with a large number of double bonds than plant oils suitable for biodiesel [5,7,15,16]. Microalgal polyunsaturated fatty acids (PUFAs) such as linolenic acid ($C_{18:3}$), eicosapentaenoic acid (EPA, $C_{20:5}$) and docosahexaenoic acid (DHA, $C_{22:6}$) may be highly valuable materials not only for nutritional or medical purposes [17] but also for various oleochemical applications [18,19] even with tiny amount of compound. Fatty acid profiling is useful for evaluating the applicability of microalgal fatty acids to biorefinery and a useful tool to taxonomically characterize microalgal or

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microbial resources [16,20–22]. Thus, a quantitative and accurate assessment of microalgal fatty acid composition is important to make sound and profitable decisions concerning microalgal fatty acid biorefinery options.

Despite the general acceptance of the importance to accurately assess microalgal fatty acid composition, discrepancies in analysis methodologies exist in the literature. Quantification and composition analyses of algal fatty acids have been performed primarily using GC-flame ionized detection (FID) systems [16,23–25], GC-mass spectroscopy (MS) detection systems [26–28] or both [22,29]. In addition, different GC columns have been used in the literature and are classified as non-polar columns (e.g., phenyl dimethyl-polysiloxane columns such as HP-5, HP ultra-2 and DB-5), polar columns (e.g., polyethylene glycol or cyanoalky polysiloxane columns such as SUPELCOWAX-10, DB-WAX, CP Sil 88, SP2380, SP2560 and BPX-70) or both. Even though there have been some reviews on analytical methods for biodiesel characterization [30,31], to our knowledge, no attempt has been made to explore how different GC methods affect microalgal fatty acid profiling results. Variations in profiling introduce uncertainty for further engineering decisions regarding the feasibility of microalgal biorefinery options, particularly when using wastewater and physiological stress stimuli to produce valuable materials or fuels from microalgae. For instance, some reports claim that microalgal fatty acid composition shifts in response to wastewater [32,33] or to nitrogen limitation as a stress [34], whereas others describe only insignificant effects [15,35]. Because of the current lack of information regarding the potential impacts of fatty acid analysis methods, general conclusions about the feasibility of using wastewater resources with a stress factor cannot be drawn. To address this issue, a methodological exploration is necessary to compare fatty acid profiling performance among the microalgal fatty acid analysis methods typically used in the literature.

In this study, we compared the performance of different GC methods to quantitatively assess the fatty acid composition of wastewater-adapted microalgal isolates. To evaluate the different GC methods typically used for microalgal fatty acid profiling in the literature, multiple FAME peak separation resolutions and quantitative detection sensitivities were examined. Differences in microalgal fatty acid profiling for different GC methods were statistically compared with profiles generated under different culture conditions (wastewater and nitrogen limitation) and using different microalgal organisms (*Scenedesmus quadricauda*, *Chlorella vulgaris* and *Ankistrodesmus gracilis*).

2. Materials and methods

2.1. Algal strains and culture conditions

Three microalgae, *Chlorella vulgaris* AG10032, *Ankistrodesmus gracilis* SAG278-2 and *Scenedesmus quadricauda* AG10308, were selected for study in this work. Algal strains were obtained from the Biological Resource Center of the Korea Research Institute of Bioscience and Biotechnology, South Korea. In aerated batch reactors, strains were cultured for 2 weeks at 25 °C with a continuous illumination and with 120 μmol m^{-2}/s in BG11 medium. The BG11 medium contained 1.5 g of $NaNO_3$, 0.04 g of K_2HPO_4, 0.075 g of $MgSO_4 \cdot 7H_2O$, 0.036 g of $CaCl_2 \cdot 2H_2O$, 0.058 g of $NaSiO_3 \cdot 9H_2O$, 0.006 g of citric acid, 0.006 g of ferric ammonium citrate, 0.001 g of EDTA (disodium salt), 0.02 g of $NaCO_3$ and 1 ml of trace metal mix A_5 in 1 L of distilled water. The trace metal mix A_5 contained 2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4H_2O$, 0.222 g of $ZnSO_4 \cdot 7H_2O$, 0.039 g of $NaMoO_4 \cdot 2H_2O$, 0.079 g of $CuSO_4 \cdot 5H_2O$, and 0.049 g of $Co(NO_3)_2 \cdot 6H_2O$ in 1 L of distilled water. After sterilization using a pressurized autoclave, the pH was adjusted to 8.4. After sufficient growth, algal samples were collected by centrifugation and freeze-dried.

To explore the effects of limiting nutrients, *C. vulgaris* AG10032 cultures were grown for 2 weeks in BG11 and then incubated in the nitrogen-limited condition for 8 day. For this nitrogen limitation, $NaNO_3$ was eliminated from the BG11 medium [36], and the other culture conditions were identical to those described above. To characterize the fatty acids in algae grown in real wastewater, municipal wastewater was collected from the influent point of the Seonam Municipal Wastewater Treatment Plant (Seoul, South Korea). The initial values for the total nitrogen, total phosphorus and pH of the wastewater were 37.5 ± 0.5 mg/L, 3.25 ± 0.05 mg/L and 7.35 ± 0.02 mg/L, respectively. The initial total nitrogen and total phosphorus in the wastewater were measured using a Spectroquant® NOVA 60 (Merck, Germany). The initial pH values were measured using an Orion 3-Star pH Meter (Thermo Scientific, Germany). The three microalgal strains were grown in wastewater with the same temperature, duration and illumination conditions as described above.

2.2. FAME extraction by in situ transesterification

By performing in situ transesterification, lipid extraction and transesterification steps were achieved simultaneously using the methods described by Moore et al. [20]. Fifty milli-grams of each freeze-dried algal sample was saponified with 1 ml of saturated KOH-CH_3OH solution at 100 °C for 30 min and then methylated with 2 ml of 5% HCl in CH_3OH at 80 °C for 10 min. After 1.25 ml of *n*-hexane and methyl-tert butyl ether (1:1) solution was added and mixed gently, samples were positioned until the upper and lower layers were separated. After the lower layer was discarded, each upper layer was washed with 3 ml of 1.2% KOH solution to eliminate any base residue. Finally, saturated NaCl solution was added until the KOH solution was completely separated from the *n*-hexane phase.

2.3. GC analysis

Four different GC methods were tested in this study (Table 1). For GC-FID methods, an Agilent 7890 GC was employed with three different columns: HP-5 (30 m, 0.32 mm i.d., 0.25 μm film thickness), SUPELCOWAX-10 (60 m, 0.32 mm i.d., 0.5 μm film thickness) or DB-WAX (30 m, 0.25 mm i.d., 0.5 μm film thickness). When the HP-5 column (Method 1) was used, the temperature began at 100 °C for 2 min, increased at a rate of 10 °C/min and was finally maintained at 280 °C for 20 min. The total analysis time of Method 1 was 40 min, and the flow rate was 2 ml/min

Table 1
GC methods employed in this study.

Methods	Detector	Identification	Column
Method 1	FID	Standard FAMEs	Non-polar, HP-5 (30 m, 0.32 mm, 0.25 μm)
Method 2	FID	Standard FAMEs	Polar, DB-WAX (30 m, 0.25 mm, 0.25 μm)
Method 3	FID	Standard FAMEs/MS libraries	Polar, SUPELCOWAX-10 (60 m, 0.32 mm, 0.5 μm)
Method 4	MSD	MS Libraries	Polar, SUPELCOWAX-10 (60 m, 0.32 mm, 0.5 μm)

with helium as the carrier gas. when the DB-WAX column was used (Method 2), the temperature began at 50 °C for 1 min, increased at a rate of 25 °C/min up to 200 °C for 5 min and then increased at a rate of 3 °C/min up to 230 °C, at which it was eventually maintained for 18 min. The total analysis time of Method 2 was 40 min, and the gas flow rate was 1.5 ml/min with helium as the carrier gas. When the SUPELCOWAX-10 column was used (Method 3), the temperature started at 100 °C for 5 min and increased at a rate of 10 °C/min up to 250 °C, at which it was maintained for 20 min. The total analysis time of Method 3 was 40 min, and the gas flow rate was 2 ml/min with helium as the carrier gas. In the case of the GC-MS method (Method 4), an Agilent 6890 GC and a 5973 inert Mass Selective Detector were employed with the SUPELCOWAX-10 (60 m, 0.32 mm i.d., 0.5 μm film thickness) column. For this method, the temperature and other conditions were the same as those used for GC-FID with the SUPELCOWAX-10 column (Method 3).

2.4. Evaluation of detection dynamic ranges

To determine the detection dynamic ranges for the detection of the GC area for 5 major FAMEs ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$), high (500 mg/L, 1000 mg/L, 5000 mg/L, 10,000 mg/L, 50,000 mg/L and 100,000 mg/L) and low (10 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L) concentrations of FAMEs were tested using four different GC systems. To prepare the FAME standards at different concentrations in *n*-hexane, FAMEs of $C_{18:1}$ (oleic acid), $C_{18:2}$ (linoleic acid) and $C_{18:3}$ (linolenic acid) of reagent purity $>99\%$ were purchased from Sigma (St. Louis, MO, USA), and FAMEs of $C_{16:0}$ (palmitic acid) and $C_{18:0}$ (stearic acid) were synthesized in our laboratory. $C_{16:0}$ and $C_{18:0}$ fatty acids of reagent purity $>95\%$ were purchased from Samchun Pure Chemical (Seoul, South Korea), and corresponding FAMEs were prepared by methylation as described below. First, 20 g each of $C_{16:0}$ and $C_{18:0}$ fatty acids were added to 400 ml of a $MeOH/H_2SO_4$ solution (230:3, v/v), which was heated at 80 °C for 1 h. After cooling, the pH was adjusted to 7.0 with a saturated NaOH solution. After solidified salts were removed by filtration through No. 41 Whatman filter paper (Brentford, United Kingdom), 500 ml of *n*-hexane was added to the solution to extract the FAMEs. To eliminate any remaining water from the extracted FAMEs, 50 g of Na_2SO_4 was added to the *n*-hexane phase and then mixed by vortexing. After the elimination of any Na_2SO_4 by filtering through No. 41 Whatman filter paper (Brentford, United Kingdom), approximately 18 g and 19 g of FAME was purified by evaporating the *n*-hexane.

2.5. Identification, quantification and evaluation of the lowest detection limit

A mixture of 14 FAME standards ($C_{8:0}$ (8%), $C_{10:0}$ (8%), $C_{12:0}$ (8%), $C_{14:0}$ (8%), $C_{16:0}$ (11%), $C_{16:1}$ (5%), $C_{18:0}$ (8%), $C_{18:1}$ (5%), $C_{18:2}$ (5%), $C_{18:3}$ (5%), $C_{20:0}$ (8%), $C_{22:0}$ (8%), $C_{22:1}$ (5%), and $C_{24:0}$ (8%); F.A.M.E.-Mix C_8-C_{24}, Supelco, Bellefonte, PA, USA) was used to examine the lowest detection limit and the capabilities of various GC methods to identify and quantify FAMEs from algal fatty acids. In the FAME mixture, $C_{16:1}$ (9-hexadecanoic acid), $C_{18:1}$ (9-octadecanoic acid), $C_{18:2}$ (9,12-octadecadienoic acid), $C_{18:3}$ (9,12,15-octadecatrienoic acid) and $C_{22:1}$ (13-docosenoic acid) were unsaturated fatty acids. To generate a stock solution, 100 mg of the FAME standard mixture was dissolved in 2 ml *n*-hexane. From this stock solution, standard solutions of 250 mg/L, 500 mg/L, 1000 mg/L, 2500 mg/L, 5000 mg/L, 7500 mg/L and 10,000 mg/L total FAME were prepared. The lowest detection limit was statistically estimated from the standard data points using the regression method described by Campo et al. [37] and IUPAC (International Union of Pure and Applied Chemistry) [38]. For quantification, standards were used to construct calibration curves from the corresponding GC signals (integrated areas); the *n*-hexane-phase dilution factor was 1:1. Replicate GC runs were performed for each same FAME concentration, and the average and standard error values were estimated. For FAME identification by GC-FID, the retention time and degree of separation between GC peaks were compared with those of individual FAME standards from the FAME mixture. For GC-MS, FAMEs were identified by matching spectra with those from GC-MS libraries. Statistical analyses for constructing calibration curve for quantification and determining the lowest detection limit of each method were performed using Microsoft® Excel 2007 [37].

To validate if a chosen model adequately described the relationship between the responses and the concentrations, analysis of variance (ANOVA) was used to detect a bias (lack-of-fit) in the regression. The lack-of-fit test is a one-sided test which is employed to verify whether the constructed regression model is adequate or not. This was tested by comparing the ratio $F=MS_{LOF}/MS_{PE}$ (mean square due to regression divided by pure error mean square) with the *F*-distribution [39].

2.6. Principal component analysis (PCA) of algal fatty acid composition changes

Principal component analysis (PCA) was performed using SPSS 9.1 (SPSS Inc., Chicago, IL, USA) to examine the changes in algal fatty acid composition in response to different GC-methods and culture conditions. The algal fatty acid composition was calculated by integrating the area representing each molar fraction and then calibrating to the % weight after the quantification of each fatty acid.

3. Results and discussion

3.1. Resolution for the identification of multiple FAMEs

To examine the resolution with which multiple FAMEs could be identified, FAMEs in the standard mixture were analyzed using different GC methods (Fig. 1). When the non-polar HP-5 column was used with GC-FID (Method 1), the peaks of unsaturated FAME standards were poorly separated from the corresponding saturated FAMEs. In particular, the $C_{18:3}$ FAME peak was not fully separated from the $C_{18:1}$ and $C_{18:2}$ peaks. In contrast, when using FID with polar columns (Method 2 with DB-WAX and Method 3 with SUPELCOWAX-10), all the standards were fully separated, including those of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$. GC-MS with the polar SUPELCOWAX-10 column (Method 4) also showed good separation of multiple FAMEs. The enhanced separation of unsaturated FAMEs by the polar columns may have been the result of the higher polarity of materials (polyethylene glycol) in the polar columns than in the nonpolar HP-5 column (5% phenyl-95% dimethyl-polysiloxane) [40]. There was no significant difference in the observed resolution due to the different lengths between the two polar columns, DB-WAX (30 m) and SUPELCOWAX-10 (60 m). The higher resolution obtained for the $C_{18:3}$ FAME using the polar columns is an advantage of this method over the the non-polar column for biodiesel acceptability evaluation; information regarding the $C_{18:3}$ content of total fatty acids is required for the European Union standards for biodiesel [5].

3.2. FAME detection sensitivity and quantification

To compare the sensitivity of the different GC methods to detect FAMEs, varying FAME concentrations in the multiple FAME mixture (Table 2) were used. A linear regression analysis was performed based on GC area signals. According to the correlation coefficient (*R*) values, all of the methods showed good linearity

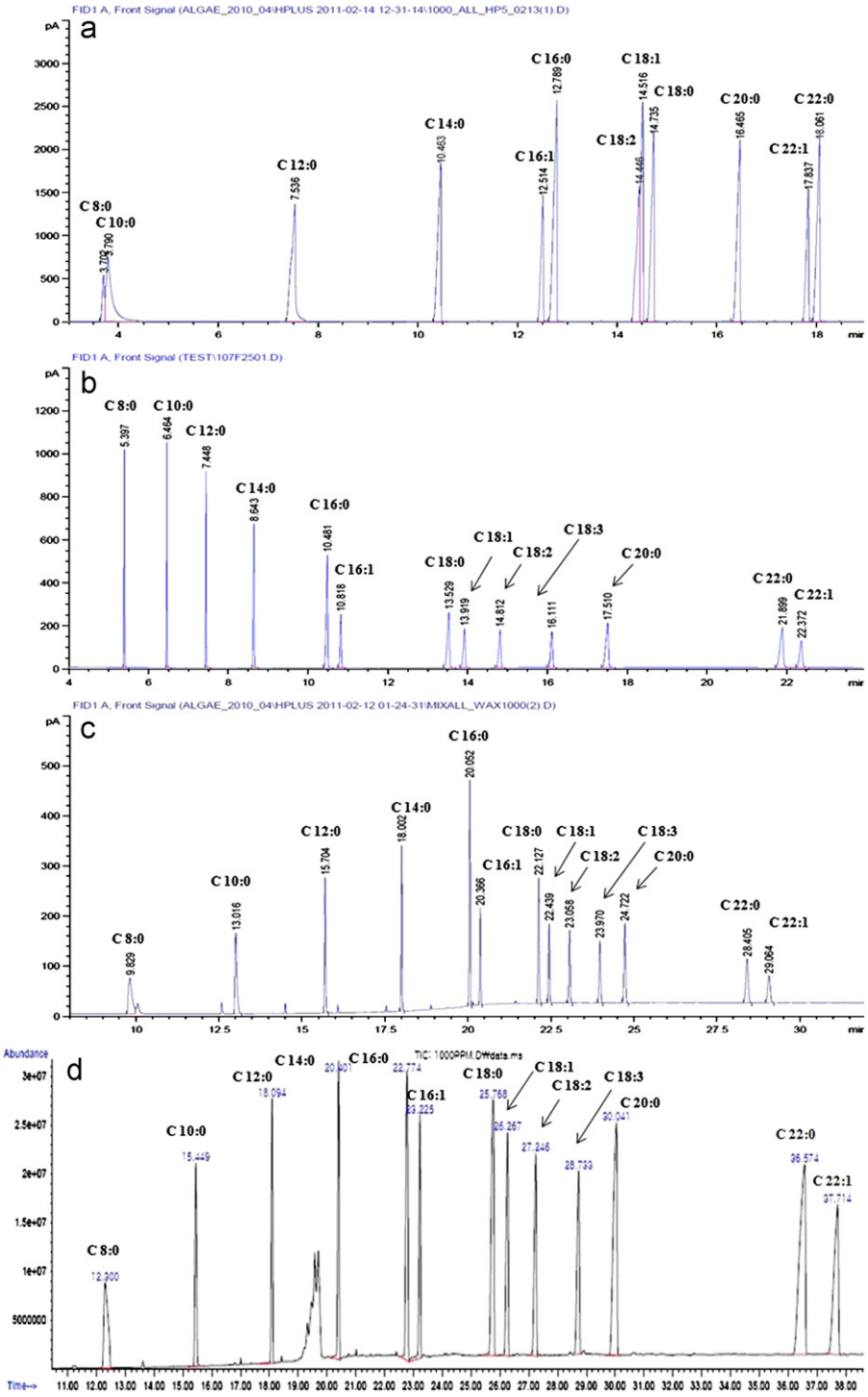

Fig. 1. GC separations of 14 FAME standards using Method 1 (a), Method 2 (b), Method 3 (c) and Method 4 (d).

within the concentration range tested (see "measured linear detection" in the table). When the linear regression slope value (ΔGC area signal/ΔFAME concentration) was evaluated for the FAME standards (Supplementary Table 1), Method 4 showed the highest GC signal change rate, with a slope of 1,978,162 ± 1,079,512 area unit/(mg/L); Methods 1 and 3 exhibited intermediate slopes (10.82 ± 1.30 area unit/(mg/L) and 10.37 ± 2.17 area unit/(mg/L), respectively), and Method 2 showed the lowest slope (1.28 ± 0.28 area unit/(mg/L)). Lowest detection limit (LDL) estimations revealed a similar trend for the slope values. Method 4 showed the strongest sensitivity, as it was able to detect FAMEs with concentrations as low as 0.001 mg/L. Methods 1 and 3 exhibited intermediate LDL levels at 0.1–0.5 mg/L except for the detection of $C_{18:1}/C_{18:3}$ by Method 1. Method 2 showed the lowest sensitivity, as it was only able to detect FAMEs with concentrations higher than 1–5 mg/L. Note that the relatively high LDL values for Method 1 in detecting $C_{18:1}/C_{18:3}$ FAMEs (5.0040 mg/L) were the result of the incomplete separation between the two FAMEs.

According to the *F*-ratio values (Table 2), Method 1, Method 3 and Method 4 passed the lack-of-fit test (*F*-ratio values <4.757 [37,39]), whereas Method 2 failed, which indicates that Method 2 is

Table 2
FAME detection linear ranges, calibration statistical characteristics and lowest detection limits of the tested GC methods[a].

Method	FAME	Measured linear detection range (mg/L)	Slope ± STDEV (AU[b]/(mg/L))	Intercept ± STDEV (AU)	Correlation coefficient	Regression standard deviation	Estimated lowest detection limit (mg/L)	*F*-ratio[c]
Method 1	C16:0	~ 1–100,000	10.78 ± 0.0045	−214.59 ± 0.1044	1.000	6.8583	0.2576	0.8216
	C16:1	~ 1–100,000	10.02 ± 0.0068	−95.97 ± 0.1094	1.000	4.6624	0.3916	0.8483
	C18:0	~ 1–100,000	10.97 ± 0.0054	−158.84 ± 0.1049	1.000	5.9251	0.3007	0.8160
	C18:1/ C18:3[d]	~ 1–100,000	9.30 ± 0.0076	16.89 ± 0.1755	0.997	10.4843	5.0040	0.8202
	C18:2	~ 1–100,000	14.27 ± 0.0239	−318.71 ± 0.3349	0.996	16.4234	0.4154	0.8136
Method 2	C16:0	~ 10–100,000	1.28 ± 0.0131	−51.96 ± 0.7855	0.995	17.2240	2.6744	52.9664
	C16:1	~ 10–100,000	1.17 ± 0.0187	−21.50 ± 0.7922	0.995	11.1098	4.1738	141.8188
	C18:0	~ 10–100,000	1.26 ± 0.0050	−37.65 ± 0.1970	0.995	4.5251	0.9702	58.8542
	C18:1	~ 10–100,000	1.27 ± 0.0063	−23.51 ± 0.2450	0.995	3.5746	1.2283	9.0560
	C18:2	~ 10–100,000	1.27 ± 0.0060	−23.51 ± 0.2588	0.995	3.7911	1.3026	56.9313
	C18:3	~ 10–100,000	1.25 ± 0.0060	−22.53 ± 0.2357	0.995	3.4289	1.2294	63.0534
Method 3	C16:0	~ 1–100,000	10.58 ± 0.0065	−309.33 ± 0.1518	1.000	9.7615	0.2544	0.8498
	C16:1	~ 1–100,000	9.85 ± 0.0096	−134.47 ± 0.1569	0.999	6.5543	0.3930	0.8767
	C18:0	~ 1–100,000	10.67 ± 0.0080	−238.58 ± 0.1602	1.000	8.8018	0.2974	0.8508
	C18:1	~ 1–100,000	10.76 ± 0.0101	−148.97 ± 0.1595	1.000	6.9560	0.3764	0.8474
	C18:2	~ 1–100,000	10.70 ± 0.0101	−148.74 ± 0.1599	1.000	6.9542	0.3769	0.8496
	C18:3	~ 1–100,000	10.40 ± 0.0103	−149.07 ± 0.1653	1.000	7.0813	0.3830	0.8613
Method 4	C16:0	~ 0.01–100,000	1,352,046 ± 7.6621	117,841,988 ± 0.4421	0.976	11581.3238	0.0008	0.4354
	C16:1	~ 0.01–100,000	1,737,800 ± 8.1399	35,129,760 ± 0.2919	0.989	5592.4849	0.0013	0.4807
	C18:0	~ 0.01–100,000	2,055,463 ± 6.9538	75,071,002 ± 0.2882	0.990	7664.0663	0.0008	0.4732
	C18:1	~ 0.01–100,000	2,188,269 ± 8,3717	38,999,153 ± 0.2690	0.990	5751.7259	0.0012	0.4867
	C18:2	~ 0.01–100,000	2,073,656 ± 7.0689	43,189,504 ± 0.2332	0.992	4856.6618	0.0009	0.4937
	C18:3	~ 0.01–100,000	2,072,365 ± 5.7532	14,139,127 ± 0.1952	0.994	3952.7087	0.0023	0.5166

[a] Results for only the FAME standards with C_{16} and C_{18}.
[b] AU stands for "arbitrary unit" which is the unit of integration area.
[c] The lack-of-fit test for a FAME compound fails if its *F*-ratio value is greater than 4.757 at significant level $F_{0.05;4,6}$ [37,39].
[d] In the standard mix, HP-5 column could not separate $C_{18:1}$ and $C_{18:3}$ FAMEs.

the least sensitive. These results led to the following final classifications: Method 4 was "strongly sensitive," Methods 1 and 3 were "intermediately sensitive" and Method 2 was "weakly sensitive".

If a GC signal displays a linear response to varying FAME concentrations (i.e., linear slope values) consistently with different FAME compounds, then fatty acid profiling can be quantitatively assessed based on GC area data using simple calibration with a limited number of FAME standards is typically used for microalgal fatty acid profiling [22,32]. However, it remains unclear whether or not the slope values between FAME concentrations and GC signal magnitudes differ among particular FAME compounds. In this study, FAME compound dependency was compared among the different GC methods. The slope values for Method 1 showed 12.0% relative deviation (i.e., one standard deviation/average) among the different FAME standards with C_8-C_{22} (Supplementary Table 1), which was the lowest variation among the tested GC methods. Methods 2 and 3 showed intermediate variation (21.9% and 20.9%, respectively), and Method 4 showed the highest variation (54.6%). Because C_{16}-C_{18} fatty acids are prevalent in wastewater-adapted microalgae [12,35,41], the same variation analysis was performed with only C_{16}-C_{18} FAME standards (Supplementary Table 1). The FID system with polar columns (Methods 2 and 3) showed remarkably low variation, below 5% (3.3% and 3.4%, respectively), which is at least 5 times lower than the variation recorded for FID with the non-polar column (Method 1, 17.3%) and the MS with polar column (Method 4, 16.5%). The variation among the C_8-C_{22} and C_{16}-C_{18} FAME standards may be attributed to the different characteristics of polar versus non-polar columns in separating unsaturated FAMEs, especially $C_{18:3}$, as shown in Fig. 1. The remarkably constant slope values of the FID-based methods with polar columns in detecting C_{16}-C_{18} FAMEs are useful for FAME calibration to quantify fatty acids from wastewater-adapted algae. The GC signal areas of FAME peaks from uncharacterized wastewater-cultivated microalgae can be easily converted to FAME concentrations using simple calibration with a minimal number of FAME standards.

3.3. Implications for the biorefinery applications of wastewater-cultivated microalgae

The potential impact of using different GC methods to identify fatty acids from wastewater-adapted microalgae was explored with three microalgae species cultivated in the defined medium BG11. FAMEs from the three microalgal species were analyzed using four GC methods (Supplementary Figs. 1–4). The relative abundance of each FAME was estimated based on GC area signals (% area per sample), and the measured abundance was compared among the different methods (Fig. 2). The non-polar column (Method 1) showed inconsistent fatty acid profiles regardless of the organism used, whereas the polar column methods (Methods 2, 3 and 4) provided consistent fatty acid profiles for both detection systems (FID and MS). Polar, but not non-polar, column methods detected $C_{18:3}$ as the dominant fatty acid in the different organisms. This inconsistency among methods was the result of the inability of the non-polar column to completely separate $C_{18:3}$ FAME from $C_{18:1}$ FAME, as shown in Fig. 1. Thus, fatty acid composition profiling using the non-polar column method was not reliable. The polar column methods, however, likely provided reliable fatty acid profiling because the results obtained for *C. vulgaris* were consistent with a previously reported fatty acid composition pattern (rich in $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ fatty acids but lacking C_{22} and longer fatty acids) from the taxonomy literature [16].

The ability of polar columns to separate $C_{18:3}$ may significantly impact the application of wastewater-adapted microalgae to biorefinery because PUFA is a major fatty acid in *Chlorophyceae* [16], which is the class to which most known wastewater-adapted microalgae belong, and in microalgal consortia cultivated

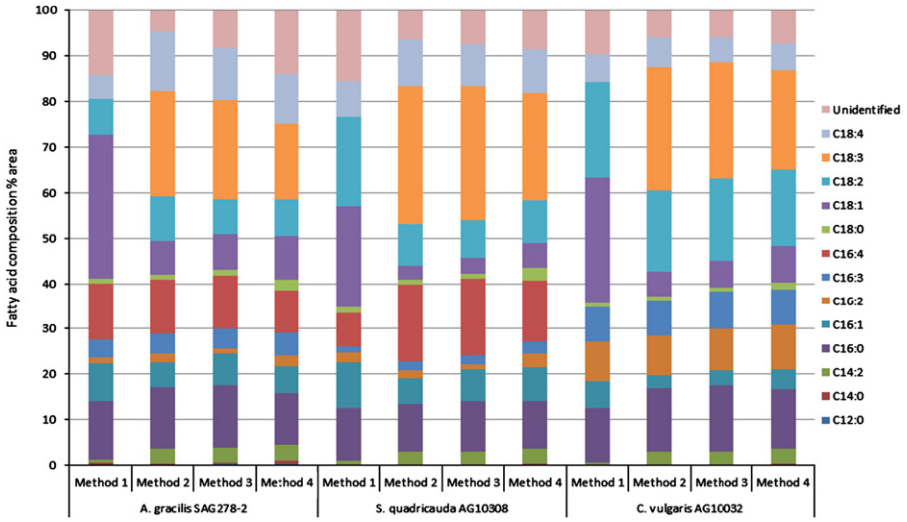

Fig. 2. Fatty acid composition profiles (% area) by different GC methods.

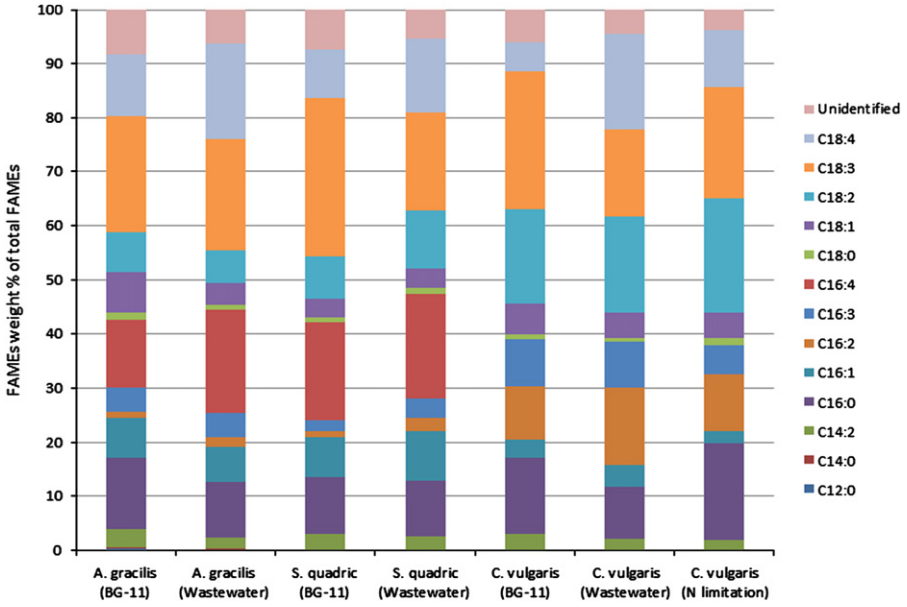

Fig. 3. Calibrated fatty acid composition profiles (% weight FAME) of three algae cultured in wastewater, BG-11 and nitrogen-limited condition using GC-FID with SUPELCOWAX-10 column (Method 3).

using various wastewater resources [12,35]. In addition, $C_{18:3}$ is a high-value PUFA for oleochemical and health food applications even with a small amount rather than biofuel application, whereas oleic acid ($C_{18:1}$) has been regarded as the most appropriate biodiesel source [42]. Quantitative information on the PUFA is critical for evaluating the feasibility of using wastewater-cultivated microalgae for biodiesel applications and other purposes.

To quantitatively evaluate the effects of different microalgae and culture conditions on microalgal fatty acid diversity, FAME measurements were obtained using the FID method with the SUPELCOWAX-10 column (Method 3). For this purpose, the GC method was appropriate because of its ability to separate C_{16}-C_{18} FAME peaks with reasonably high detection sensitivity. Also, this method provided remarkably consistent ratios of detection intensity to FAME concentration (signal-to-concentration ratios) for C_{16}-C_{18} FAMEs (within 5% variation), which permitted FAME peak quantification using relatively simple calibration with the FAME standard mix. For the FAME peaks that were not identified in Method 3 using the standards in the mix, the MS-detected GC results were used for identification, and the GC area signals were calibrated using the signal-to-concentration ratio values averaged from the measured ratio values obtained from structurally similar FAME standards. Fatty acid quantification results showed that most fatty acids were present in different microalgae cultivated in BG11 (Fig. 3) except for the $C_{16:4}$ fatty acid. In the *A. gracilis* and *S. quadricauda* strains, a significant amount of $C_{16:4}$ fatty acid was present, unlike in the *C. vulgaris* strain. A similar trend in fatty acid diversity was observed for cultures with different media (e.g., real wastewater and nitrogen-limited BG11).

The yields of extracted FAME and the portions of saturated and unsaturated fatty acids in response to different algal organisms and culture conditions were also examined (Table 3). The FAME extraction yields decreased by approximately two-fold when microalgae were cultivated in wastewater. A low saturated fatty acid ratio obtained from the extracted FAME (11.29–21.28%) is in agreement with previous reports on wastewater-adapted microalgae [12,15]. Of the unsaturated fatty acids, PUFAs were more

Table 3
Total extracted FAME and the portions of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fatty acids with 4 and more double bonds (FA w/DB >3), and linolenic acid ($C_{18:3}$ FA) determined in this study.

Organism	Culture condition	Extracted FAME % d.w.	SFA % known FAME	MUFA % known FAME	PUFA % known FAME	FA w/DB >3% known FAME	$C_{18:3}$ FA % known FAME
Ankistrodesmus gracilis SAG278-2	BG-11	6.63	17.20	16.14	66.65	25.21	23.69
	Municipal WW	3.09	12.73	10.91	76.36	38.64	22.30
Scenedesmus quandricauda AG10308	BG-11	5.89	13.23	11.22	75.55	28.06	31.85
	Municipal WW	2.32	12.71	13.26	74.02	34.06	19.47
Chlorella vulgaris AG10032	BG-11	5.00	16.65	18.07	65.28	5.74	27.26
	N-limiting BG-11	5.79	21.28	15.22	63.50	11.10	22.12
	Municipal WW	1.83	11.29	20.70	68.01	18.57	17.11

abundant than MUFAs. Because the current European Union EN biodiesel standards require that the content of FAMEs with 4 or more double bonds be less than 1% and the content of $C_{18:3}$ FAME be lower than 15% [5], the microalgal fatty acid composition is not appropriate for biodiesel application. Although the removal of unsaturated fatty acids is technically feasible, additional refinement processes require more energy consumption and toxic chemical use. Nevertheless, the *C. vulgaris* isolate is preferred to *A. gracilis* and *S. quadricauda* for biodiesel application because it contains fewer fatty acids with 4 or more double bonds. However, for high-value PUFA biorefinery application, all three organisms may be equally applicable because of their similar $C_{18:3}$ fatty acid content.

The following PCA results statistically compared the impacts of different microalgal organisms, culture media and GC methods for microalgal fatty acid composition profiling (Fig. 4). When cultured in BG11 and measured by Method 3, the profile of *C. vulgaris* (● of blue color) was significantly different from that of *A. gracilis* and *S. quadricauda* (● of black color for *A. gracilis* and red color for *S. quadricauda*); *A. gracilis* and *S. quadricauda* exhibited similar fatty acid composition profiles. As mentioned above, this difference is due to the existence of $C_{16:4}$ fatty acid in *A. gracilis* and *S. quadricauda*, and its absence in *C. vulgaris*. Although *C. vulgaris* has ω-3 desaturase [43], $C_{16:4}$ fatty acid is absent in *C. vulgaris* [16,43,44]. This is because *C. vulgaris* has $C_{16:3}$ as a $\omega-3$ fatty acid [16,44] as revealed by the GC-MS result, i.e., $C_{16:3}$ (7,10,13-hexadecatrienoic acid) fatty acid. Hence we conclude that *C. vulgaris* does not have an additional desaturase for the fourth cleavage of C-H bond of C_{16} fatty acid, which is assumed to exist in *A. gracilis* and *S. quadricauda*. Compared with the effect of using different organisms, different culture conditions (BG11, wastewater and/or nitrogen limited BG11) did not alter the fatty acid composition profile for each organism when using Method 3 (● and ○ of black color for *A. gracilis*; ● and ○ of red color for *S. quadricauda*; ●, ○ and ◐ of blue color for *C. vulgaris*). When different GC methods were compared, the polar (Methods 2, 3 and 4), but not the non-polar (Method 1), column methods provided similar composition profiles. This difference between methods was expected because of the observed difference in $C_{18:3}$ FAME separations (Fig. 1). The differences among various GC methods (Method 1 versus Methods 2, 3 and 4) were more significant than the differences observed among different organisms and culture media, which suggests that the GC method choice is critical for the evaluation of microalgal fatty acid composition profiles in response to various wastewater and stress conditions.

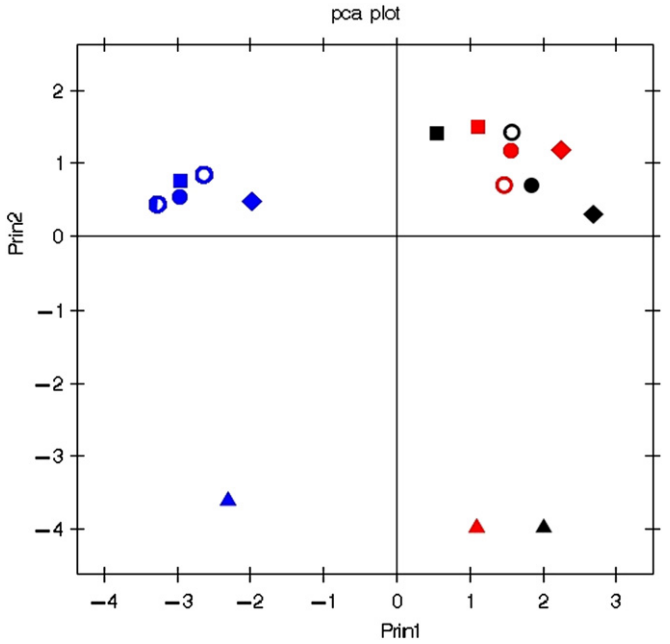

Fig. 4. Principal component analysis (PCA) of fatty acid composition of 3 microalgal strains determined by different analytical methods and culture conditions (Black, *A. gracilis*; Red, *S. quadricauda*; Blue, *C. vulgaris*; ▲, Method 1; ■, Method 2; ●, Method3; ♦, Method 4; solid (●), BG11; vacant (○), wastewater; half-solid (◐), nitrogen-limited condition. (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

4. Conclusions

In this study, we compared the performance of four different GC-based microalgal fatty acid analysis methods. The methods that we employed are typically used biorefinery research that uses wastewater-adapted microalgae. We also discussed the implications for microalgal biorefinery applications using wastewater resources. The resolution of FAME identification, detection sensitivity and quantification were evaluated for each GC method using a defined FAME standard mix as well as previously uncharacterized FAMEs from microalgae cultivated under different conditions. Our findings from GC method comparisons suggest useful criteria in the selection of GC methods for the quantitative analysis of microalgal fatty acids. Polar columns are preferred over non-polar columns because they can better separate and quantify FAMEs from C_{16}-C_{18} PUFAs, which are typically dominant in wastewater-cultivated microalgae. For GC detection system selection, GC-MS with polar columns (especially SUPELCOWAX-type polar columns) are preferred to GC-FID with the same type of polar columns because of their higher quantitative detection sensitivity. Given the costs associated with instrumentation and analysis, GC-FID with a SUPELCOWAX-type polar column is recommended over GC–MS detection because of a lower instrumental cost. GC-FID is also preferable because it has simpler calibration requirements for quantifying multiple fatty acids of previously uncharacterized microalgae. The statistical comparison of the profiles of microalgal fatty acid compositions measured by different GC methods and from different culture conditions illustrates the necessity of choosing an appropriate GC method. A suitable GC method aids in

making sound and profitable decisions regarding microalgal biorefinery using wastewater resources.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.04.002.

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