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Satya Girish Chandra Avula
Cleveland State University

Joanne M. Belovich
Cleveland State University, j.belovich@csuohio.edu

Yan Xu
Cleveland State University, y.xu@csuohio.edu

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Determination of fatty acid methyl esters derived from algae *Scenedesmus dimorphus* biomass by GC–MS with one-step esterification of free fatty acids and transesterification of glycerolipids

Satya Girish Chandra Avula | Joanne M. Belovich | Yan Xu

Algae can synthesize, accumulate and store large amounts of lipids in its cells, which holds immense potential as a renewable source of biodiesel. In this work, we have developed and validated a GC–MS method for quantitation of fatty acids and glycerolipids in forms of fatty acid methyl esters derived from algae biomass. Algae *Scenedesmus dimorphus* dry mass was pulverized by mortar and pestle, then extracted by the modified Folch method and fractionated into free fatty acids and glycerolipids on aminopropyl solid-phase extraction cartridges. Fatty acid methyl esters were produced by an optimized one-step esterification of fatty acids and transesterification of glycerolipids with boron trichloride/methanol. The matrix effect, recoveries and stability of fatty acids and glycerolipids in algal matrix were first evaluated by spiking stable isotopes of pentadecanoic-2,2-d_2 acid and glyceryl tri(hexadecanoate-2,2-d_2) as surrogate analytes and tridecanoic-2,2-d_2 acid as internal standard into algal matrix prior to sample extraction. Later, the method was validated in terms of lower limits of quantitation, linear calibration ranges, intra- and inter-assay precision and accuracy using tridecanoic-2,2-d_2 acid as internal standard. The method developed has been applied to the quantitation of fatty acid methyl esters from free fatty acid and glycerolipid fractions of algae *Scenedesmus dimorphus*.

KEYWORDS

algae, esterification, fatty acid methyl esters, gas chromatography-mass spectrometry, *Scenedesmus dimorphus*

1 | INTRODUCTION

The interest in biofuels as an alternative renewable source of energy has increased over the recent years due to the concerns of consumption of fossil fuels and maintenance of

ecosystems. Biodiesel is often produced from agricultural biomasses such as vegetables, animals or algae, and comprised of mono-alkyl esters of long-chain fatty acids (e.g., C_{12} to C_{24}) with over 90% of them being between C_{16} and C_{18} [1]. Among the biomasses, algae stands out because its oil yield is nearly an order of magnitude higher than the others [2], and unicellular aquatic green algae *Chlorophyceae* is the most desirable class since it can double its biomass in less than 24 h. *Scenedesmus dimorphus*, an algal species under *Chlorophyceae* class, is one of the best for oil production [3].

Algae is a simple autotrophic organism that is made up of proteins, carbohydrates, lipids (biodiesel precursors) and

other bioactive compounds. While the percent content of lipids may vary from species to species, *Scenedesmus dimorphus* can accumulate lipids up to 40% of their dry masses [4, 5]. It is these lipids that can be converted to fatty acid methyl esters (FAMEs).

Increasing lipid content in algal biomass would be one of the effective ways to overcome the high cost of biodiesel production. Hence, a substantial effort has been made by the researchers over the past few years. It was found that factors such as nitrogen [6], light intensity [7], temperature [8], nutrients [9,10], carbon dioxide, growth stages, organic carbon content [11] and salt stress [12] were found to affect the accumulation and profile of lipids in algae.

It is worth noting that among all classes of lipids in algal cells (e.g., glycerolipids, glycerophospholipids, cholesterol, etc.), only glycerolipids (monoglycerides, diglycerides and triglycerides) can be converted to FAMEs for use in the traditional unmodified diesel engines, due to lack of phosphorus and sulfur groups in their structures [13]. While glycerophospholipids hinders the transesterification process, sulfur content is regulated in vehicle emissions [14]. It is known that the chemical nature of fatty-acid chains of glycerolipid determines the quality of biodiesel (FAMEs). Generally, cetane number, viscosity, density, flash point, heat of combustion and lubricity are determined by the structure and length of fatty acids [15]. For example, the polyunsaturated fatty acids affect cold flow properties of biodiesel, whereas the saturated fatty acids provide oxidative stability and ignition properties [14]. Hence, it is important to know the chemical structures, and the quantity of fatty acids and glycerolipids in algae biomass, particularly, the saturated and unsaturated fatty acids with carbon numbers of 16 and 18 [i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3)] which are viable indicators for the assessment of biodiesel quality [16].

Over the past few years, much effort has been devoted to the quantitative determination of glycerolipids and fatty acids in algae by various analytical techniques [17,18]. Chen and his colleagues [19] developed and used a two-step microwave assisted Nile red staining method for the quantification of neutral lipids in green algae. In another study, Soh and Zimmerman used LC–MS to quantify the concentration of fatty acid methyl esters from lipids, phospholipids and pigments extracted under various supercritical temperatures and pressures [20]. McNichol and his associates [21] used 18 different algal strains to investigate the effectiveness of using automated Soxhlet extraction. They carried out the identification and quantification using GC with flame ionization detection (FID) and HPLC with charged aerosol detection (CAD). In another study, Sakthivel, Elumalai and Santhiya [22] studied the changes in lipid production of the algal strains, *Scenedesmus dimorphus* and *Chlorococcum infusionum* under the influence of light and dark conditions

by GC–MS. Recently, Shah and his group [23] analyzed and characterized the algal oil from *Scenedesmus dimorphus* by thin layer paper and GC techniques for the high production of biodiesel. Although most of the published articles were on the measurement of total content of fatty acids, Paik and coworkers [24] did successfully quantify triacylglycerols and free fatty acids by GC–MS upon their separation using SPE. The triacylglycerols were saponified, acidified, extracted and esterified with *tert*-butyldimethylsilyl, while the FFA was directly acidified, extracted and esterified for GC analysis. Although several methods were used over the previous years for the analysis of lipids, these methods were not cost effective, time-consuming and are not validated.

Hence, in this work, we developed and optimized a simple and rapid GC–MS method for quantitation of FAMEs by one-step esterification of free fatty acids and transesterification of glycerolipids. The method developed was validated in terms of matrix effect, recovery, lower limits of quantitation (LLOQ), linear calibration ranges, intra- and inter-assay precision and accuracy, as well as stability. It was applied to the composition and content analyses of fatty acids and glycerolipids in algae *Scenedesmus dimorphus* dry mass.

2 | MATERIALS AND METHODS

2.1 | Chemicals

HPLC-grade acetonitrile, chloroform and hexane were purchased from Pharmco-AAPER (Louisville, KY, USA). Boron trichloride/methanol (BCl_3-MeOH, 12% w/w) and 2,2-dimethoxypropane were from Sigma–Aldrich (St. Louis, MO, USA). Fatty acid standards [i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3)] were from MP Biomedicals (Solon, OH, USA). Deuterated fatty acid and glycerolipid standards [i.e., pentadecanoic-2,2-d_2 acid, tridecanoic-2,2-d_2 acid, and glyceryl tri(hexadecanoate-2,2-d_2)] were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). FAME mixed standards (AOCS Mix 6, 50.0 mg FAMEs/50.0 μL hexane) (containing methyl myristate (C14:0), 2.01% (w/v); methyl palmitate (C16:0), 29.81% (w/v); methyl palmitoleate (C16:1, *cis*), 3.17% (w/v); methyl stearate (C18:0), 13.91% (w/v); methyl oleate (C18:1, *cis*-9), 40.95% (w/v); methyl linoleate (C18:2, *cis*, *cis*), 6.98% (w/v); and methyl linolenate (C18:3, all *cis*), 3.17% (w/v)) (Catalog #: 625030) were obtained from Grace Davison Discovery Sciences (Waltham, MA, USA). All other chemicals were ordered from Sigma–Aldrich (St. Louis, MO, USA). Type 1 deionized water (with a resistance reading of 18.2 MΩ·cm) was made freshly by a Barnstead NANOpure®

water purification system (Thermo Scientific, Waltham, MA, USA).

2.2 | Algal culture and sample preparation

Algae *Scenedesmus dimorphus* (strain #: UTEX 1237) stock (in agar) was obtained from the UTEX The Culture Collection of Algae (Austin, TX, USA). Algal cultures used in the growth experiments were prepared by inoculating algal cells in agar medium into a 1L bottle containing 750 mL of 3N-BB medium [25] and kept in a sterile hood. The bottles were sparged with 5% CO_2 in air at a flow rate of 0.1 L/min, and continuously agitated on a stir plate at room temperature. Illumination was provided by a Bulbrite F14T5/830 14-watt linear fluorescent T5 bulb (14 W, 24") (Bulbrite, Moonachie, NJ, USA) on a 12 h on/12 h off cycle with a measured intensity of 150–200 ft candles at the liquid surface.

The algal cells were harvested after 12 days of growth using a common procedure followed by the algal research community [8,20,25]. In this procedure, 50 mL of algal culture was centrifuged by a Sorvall ST 40R centrifuge of Thermo Scientific (Waltham, MA, USA) at $872 \times g$ for 20 min. The supernatant was decanted, and the cell pellets were re-suspended in 50 mL of deionized water and centrifuged for another 20 min at $872 \times g$ to remove the remaining salts and other unwanted soluble species. The supernatant was poured off to the waste and the remaining cell pellets were dried for 2 days at 45°C in a Precision Scientific (Chicago, Illinois, USA) Model 6EG Gravity Convection Oven. After drying, the algal cells were pulverized to a fine powder by a mortar and pestle, and kept at −20°C before use.

2.3 | GC–MS system

FAME compounds were analyzed by a Varian 3900 GC with a Saturn 2100T ion-trap mass spectrometer using a TRACE TR-FAME GC column (30 m × 0.25 mm × 0.2 μm) of Thermo Scientific (Grand Island, NY, USA) with helium as a carrier gas at a constant flow rate of 1.0 mL/min. For GC separation, sample injector was maintained at 250°C and sample injection volume was kept at 1.00 μL with a split ratio of 1:10. The elution of FAMEs was accomplished within 19.14 min by a temperature program where the oven temperature was initially set at 40°C and kept for 1 min, increased to 180°C at 20°C/min and held for 1 min, then increased to 210°C at 5°C/min and held for 1 min, and finally increased to 250°C at 35°C/min and held for 2 min before returning to the initial temperature. For MS detection, electron impact (EI) ionization energy was determined by automatic gain control (AGC) ranging 50–150 eV (depending on prescan segment and maximum ionization time). The optimized ionization parameters were as follows: target TIC, 11 000 counts; maximum ionization time, 25 000 μs; prescan ionization time, 100 μs;

background mass, 40 *m/z*; and RF dump, 650 *m/z*. Mass spectra were obtained in the mass range of 50 to 410 amu. Quantitation of FAMEs was carried out by extracted ion chromatogram (EIC) mode with a solvent delay of 3 min for MS data acquisition. The quantifier and qualifier ions used in the GC–MS analyses are listed in Table 1. The resultant chromatograms and mass spectra were viewed and processed by Varian MS workstation with service pack 6.8.

2.4 | Preparation of standard solutions

The mix standard stock solutions (10.0 mm) of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were prepared in methanol. The standard stock solutions (10.0 mm) of pentadecanoic-2,2-d_2 acid (as surrogate analyte for fatty acids in the studies of matrix effect, recovery and stability in algal matrix) and glyceryl tri(hexadecanoate-2,2-d_2) (as surrogate analyte for glycerolipids in the studies of matrix effect, recovery and stability in algal matrix or as the secondary internal standard (IS) for the analysis of glycerolipids from algal dry mass) were prepared individually in methanol and chloroform. The standard stock solution (10.0 mm) of tridecanoic-2,2-d_2 acid (as the primary IS in all the experiments) was prepared in methanol. All the above stock solutions were kept in glass vials and stored at −20°C before use.

The mix standard working solutions (1.00 and 5.00 mm) of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were prepared by diluting its mix standard stock solution in methanol/chloroform (4:1, v/v). The standard working solutions (1.00 and 5.00 mm) of pentadecanoic-2,2-d_2 acid and glyceryl tri(hexadecanoate-2,2-d_2) were prepared separately by diluting their standard stock solutions in methanol/chloroform (4:1, v/v). The IS working solutions (1.00 mm) of tridecanoic-2,2-d_2 acid and glyceryl tri(hexadecanoate-2,2-d_2) were prepared separately by diluting their standard stock solutions in methanol/chloroform (4:1, v/v).

For the optimization of GC separation conditions, FAME mix standard solution (50.0 ng FAMEs/mL hexane) was prepared by serial dilutions of the AOCS Mix 6 in hexane.

For the optimization of esterification of free fatty acids and transesterification of glycerolipids, and the conversion/yield studies, the solvent mix QCs of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (1.50, 200, 400 and 500 μM) were prepared freshly by mixing appropriate amounts of each standard working solutions in known volumes of methanol/chloroform (4:1, v/v). The solvent QCs glyceryl tri(hexadecanoate-2,2-d_2) (0.600, 80.0, 160 and 200 μM) were prepared freshly by serial dilutions of its standard working solution in methanol/chloroform (4:1, v/v). The standard FAME calibrators (0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 μM) of methyl myristate (C14:0),

TABLE 1 The quantifier and qualifier ions used in GC-MS analyses of FAMEs from algae *Scenedesmus dimorphus* lipid extract

FAME	Fatty acid chain	Retention time (min)	FAME ion (*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)
Methyl palmitate	C16:0	10.57	270.2	270.2	219.1
Methyl palmitoleate	C16:1	10.90	268.2	55.2	236.2
Methyl stearate	C18:0	12.12	298.3	74.1	255.3
Methyl oleate	C18:1	12.46	296.2	55.2	264.3
Methyl linoleate	C18:2	13.03	294.2	67.1	263.2
Methyl linolenate	C18:3	13.76	292.1	79.1	236.0
Methyl tridecanoate-2,2-d_2	C13:0-d_2	8.72	231.3	231.2	187.1
Methyl pentadecanoate-2,2-d_2	C15:0-d_2	9.87	258.2	187.1	202.0
Methyl hexadecanoate-2,2-d_2	C16:0-d_2	10.57	272.3	274.2	243.2

methyl palmitate (C16:0), methyl palmitoleate (C16:1, cis), methyl stearate (C18:0), methyl oleate (C18:1, *cis*-9), methyl linoleate (C18:2, *cis*, *cis*) and methyl linolenate (C18:3, all *cis*) were prepared individually by serial dilutions of the AOCS Mix 6 in hexane.

For the studies of LLOQ, intra- and inter-run precision and accuracy, and linear calibration ranges, the solvent mix standard calibrators of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 µM) and glyceryl tri(hexadecanoate-2,2-d_2) (0.200, 1.00, 5.00, 10.0, 50.0, 100 and 200 µM) with a fixed concentration (50.0 µM) of IS (tridecanoic-2,2-d_2 acid), and the solvent mix QCs of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid fatty acids (0.500, 1.50, 200 and 400 µM) and glyceryl tri(hexadecanoate-2,2-d_2) (0.200, 0.600, 80.0 and 160 µM) with a fixed concentration (50.0 µM) of IS (tridecanoic-2,2-d_2 acid) were prepared freshly by mixing appropriate amounts of each standard working solutions in known volumes of methanol/chloroform (4:1, v/v).

For the studies of matrix effect, recovery and stability in algal matrix, the solvent mix QCs of pentadecanoic-2,2-d_2 acid (1.50, 200 and 400 µM) and glyceryl tri(hexadecanoate-2,2-d_2) (0.600, 80.0 and 160 µM) containing a fixed concentration (50.0 µM) of IS (tridecanoic-2,2-d_2 acid) were prepared freshly by diluting each standard working solutions in known volumes of methanol/chloroform (4:1, v/v).

For the composition and content analyses of fatty acids and glycerolipids in algae *Scenedesmus dimorphus* dry mass, the solvent mix calibrators of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 µM) with dual ISs (tridecanoic-2,2-d_2 acid, 50.0 µM; and glyceryl tri(hexadecanoate-2,2-d_2), 20.0 µM), and the solvent mix QCs of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid fatty acids (1.50, 200 and 400 µM) with dual ISs (tridecanoic-2,2-d_2 acid, and 50.0 µM; glyceryl tri(hexadecanoate-2,2-d_2), 20.0 µM) were prepared

freshly by mixing appropriate amounts of each standard working solutions in known volumes of methanol/chloroform (4:1, v/v).

2.5 | Preparation of QCs in algal matrix before and after extraction and fractionation

The algal matrix mix QCs of pentadecanoic-2,2-d_2 acid (1.50, 200 and 400 µM) and glyceryl tri(hexadecanoate-2,2-d_2) (0.600, 80.0 and 160 µM) with a fixed concentration (50.0 µM) of IS (tridecanoic-2,2-d_2 acid) before the extraction and fractionation of lipids were prepared by transferring 300 µL of each corresponding solvent mix QCs into individual borosilicate glass tubes (12 × 75 mm) from Fisher Scientific (Hanover Park, IL, USA), then drying under nitrogen before adding 3.00 mg of algal dry mass to each tube, whereas the corresponding algal matrix QCs after the extraction and fractionation of lipids were prepared by spiking 300 µL of each corresponding solvent mix QCs into each tube containing the dried residuals of after extraction and fractionation of 3.00 mg algal dry mass (see Sections 2.6 and 2.7).
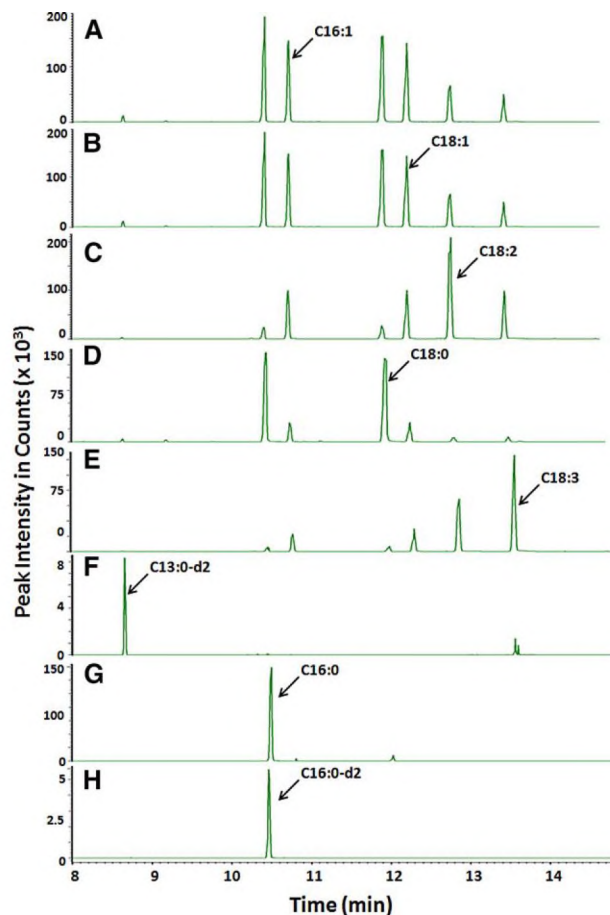
2.6 | Algal lipid extraction

The total lipids in algal dry mass were extracted by the modified Folch method which has high recoveries towards fatty acids, polar and neutral lipids [26]. Since there were different usages of algal dry masses (i.e., one for fatty acids content and composition analyses, and others for preparation of algal matrix mix QCs (see Section 2.5)), the details of extraction procedures were as follows: For content and composition analyses of fatty acids, 3.00 mg of algal dry mass in a borosilicate glass tube (12 × 75 mm) was first wetted by 300 µL of methanol/chloroform (2:1 v/v), then followed by addition of 25.0 µL of 1.00 mm tridecanoic-2,2-d_2 acid (the IS for analysis of FAMEs from the fraction of free fatty acids) and 10.0 µL of 1.00 mm glyceryl trihexadecanoate-2,2-d_2 (the IS for analysis of FAMEs from the fraction of glycerolipids). For the preparation of algal matrix mix QCs, 3.00 mg of algal dry
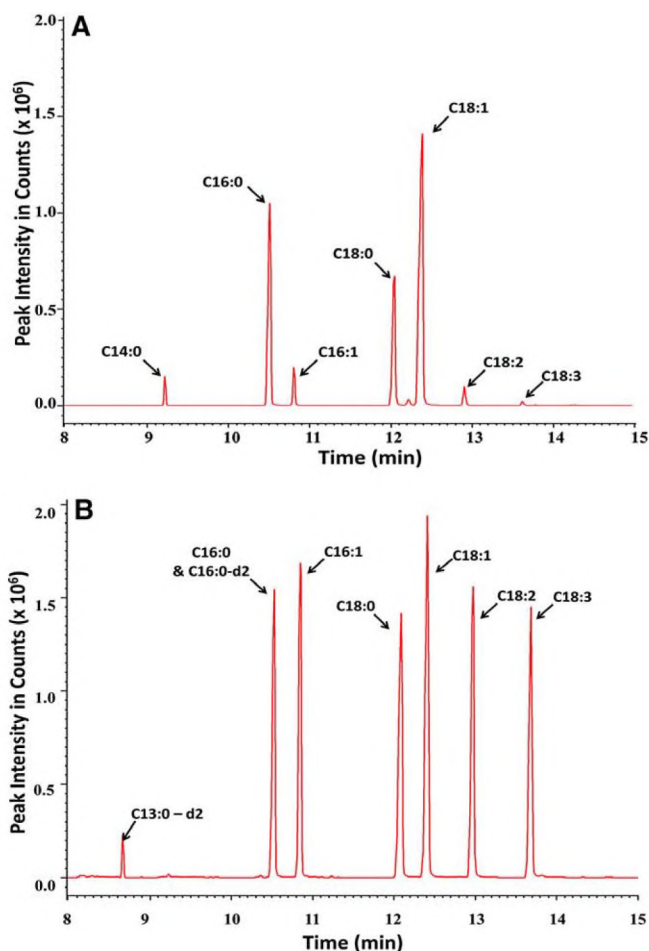
FIGURE 1 Total ion chromatograms: (A) diluted AOCS mix 6 standard solution (100 μg total FAMEs/mL); and (B) products of esterification of tridecanoic-2,2-d₂ acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, and product of transesterification of glyceryl tri(hexadecanoate-2,2-d₂), and the concentrations of fatty acids and glycerolipid were 200 μM and 5.00 μM respectively. The GC–MS conditions were the same as described in Section 2.3

FIGURE 2 Representative extracted ion chromatograms of FAME products from esterification of fatty acids and transesterification of glycerolipid. (A) methyl palmitoleate (C16:1) at m/z 55.2; (B) methyl oleate (C18:1) at m/z 55.2; (C) methyl linoleate (C18:2) at m/z 67.1; (D) methyl stearate (C18:0) at m/z 74.1; (E) methyl linolenate (C18:3) at m/z 79.1; (F) methyl tridecanoate-d2 (C13:0-d2) at m/z 231.2; (G) methyl palmitate (C16:0) at m/z 270.2; (H) methyl hexadecanoate-d2 (C16:0-d2) at m/z 274.2. Experimental conditions were the same as those in Figure 1b

mass in a borosilicate glass tube (12 × 75 mm) was first wetted by 300 μL of methanol/chloroform (2:1 v/v), then followed by addition of 35.0 μL of methanol/chloroform (4:1, v/v). After acidifying each sample with 15.0 μL of 0.1 N HCl, each tube was vortexed for 5 min before adding 300 μL of chloroform; then, each tube was vortexed for 5 min, and followed by addition of 200 μL of deionized water, and vortexing for 2 min. At the end, the solution was centrifuged at 872 × g for 5 min, the lower organic phase was transferred to a clean borosilicate glass tube. The upper aqueous phase was re-extracted with 300 μL of chloroform by vortexing and centrifuging for 5 min each. The organic phase of the second extraction was transferred and combined with the organic phase from the first extraction. The combined organic extract (i.e., total algal lipid extract) was used for lipid fractionation.

2.7 | Fractionation of free fatty acids and glycerolipids by SPE

The total algal lipid extract was fractionated into fractions of glycerolipids and free fatty acids by the SPE method developed by Kaluzny et al. [27]. In this method, the total lipid extract was loaded onto a 500 mg aminopropyl cartridge from Agilent technologies (Wilmington, Delaware, USA), which was pre-conditioned with 3 mL of hexane. Then, 6 mL of chloroform/isopropanol (2:1, v/v) was added to the cartridge to elute the neutral lipid fraction (i.e., cholesterols, cholesteryl esters, triglycerides, diglycerides and monoglycerides). After washing the cartridge with 3 mL of 2% acetic acid in diethyl ether, another 6 mL of 2% acetic acid in diethyl ether was added to elute the free fatty acids fraction. It was noted that chlorophyll (a major interferent to the analysis of neutral lipids

and free fatty acids) remained on the cartridge together with polar lipid fraction.

The neutral lipid fraction was then dried in a TurboVap® LV evaporator (Biotage, Charlotte, NC, USA) at room temperature under nitrogen (15 psi) for 50 min, and reconstituted in 1 mL of hexane. To separate glycerolipids from other neutral lipids, a cascade of two aminopropyl SPE cartridges connected sequentially with Agilent Bond Elut adapter was used, which was pre-conditioned with 3 mL of hexane. The neutral lipid fraction in hexane was loaded on the first cartridge of the cascade. Then, 3 mL of 1% diethyl ether and 10% dichloromethane in hexane was added and the eluate containing triglyercides was collected at the end of second cartridge. After disposal of the second cartridge from the cascade, which retained cholesteryl ester escaped from the first one, the first cartridge was washed with 4 mL of 5% ethyl acetate in hexane to remove cholesterol. Following that, 3 mL of 15% ethyl acetate in hexane was added to the cartridge to elute diglycerides. Finally, 3 mL of chloroform/methanol (2:1, v/v) was added to the cartridge to elute monoglycerides. The tri-, di- and monoglycerides were collected into the same borosilicate glass tube, which is known as the glycerolipid fraction. A schematic illustration of the fractionations of free fatty acids and glycerolipids can be found in the reference [27].

For algal fatty acids content and composition analyses (see Section 2.6), the fractions of free fatty acids and glycerolipids were dried individually in the TurboVap® LV evaporator at room temperature under nitrogen (15 psi) for 20 and 50 min, respectively, and each dried residual was reconstituted in 300 μL of methanol/chloroform (4:1, v/v). For algal matrix mix QCs spiked in before extraction, both fatty acids and glycerolipid fractions were mixed and dried, and the dried residual was reconstituted in 300 μL of methanol/chloroform (4:1, v/v) (see Section 2.5). For algal matrix mix QCs spiked in after extraction, both fatty acids and glycerolipid fractions were mixed and dried, and the dried residual was spiked in 300 μL of solvent mix QCs in methanol/chloroform (4:1, v/v) (see Section 2.5).

2.8 | Optimized conditions for esterification of fatty acids

300 μL of the solvent mix QCs of fatty acids (see Section 2.4) (or the reconstituted free-fatty-acid fraction from the total algal lipid extract (Section 2.7)) were mixed with 500 μL of BCl$_3$-methanol (12% w/w) and 15.0 μL of 2,2-dimethoxypropane in a 3 mL clear micro-reaction vessel (Type I) from Sigma–Aldrich (St. Louis, MO, USA). After tightening the vial cap to prevent evaporation of solvent and entry of moisture from the atmosphere, the esterification reaction was allowed to proceed at 100°C for 15 min in the convection oven by placing the micro-reaction vessel inside a sand bath containing sea sand from Fisher

Scientific (Hanover Park, IL, USA). After cooling the micro-reaction vessel to room temperature, 500 μL of deionized water and 300 μl of hexane were added. The extraction of the reaction products (FAMEs) by hexane was achieved by vortexing the liquids inside the micro-reaction vessel for 5 min. Then, 100 μL of the upper hexane layer containing FAMEs were transferred into a 150 μL glass inserted in an autosampler vial from Fisher Scientific (Hanover Park, IL, USA) which was capped afterward for GC–MS analysis.

2.9 | Optimized conditions for transesterification of glycerolipids

300 μL of the solvent QCs glyceryl tri(hexadecanoate-2,2-d$_2$) (Section 2.4) (or reconstituted glycerolipid fraction from the total algal lipid extract (Section 2.7)) was mixed with 500 μL of BCl$_3$-methanol (12% w/w) and 15.0 μL of 2,2-dimethoxypropane in a 3 mL clear micro-reaction vessel. After tightening the vial cap, the transesterification reaction was allowed to proceed at 100°C for 50 min in the convection oven by placing the micro-reaction vessel inside a sand bath. After cooling the micro-reaction vessel to room temperature, 500 μL of deionized water and 300 μL of hexane were added. The extraction of the reaction products (FAMEs) by hexane was achieved by vortexing the liquids inside the micro-reaction vessel for 5 min. Then, 100 μL of the upper hexane layer containing FAMEs were transferred into a 150 μL glass inserted in an autosampler vial which was capped afterward for GC–MS analysis.

2.10 | Method validation

The GC–MS method developed was validated in reference to the US-FDA guidance for industry (2001) on bioanalytical method validation [28] in terms of matrix effect, recovery, LLOQ, linear calibration range, accuracy and precision, as well as stability.

2.10.1 | Recovery and matrix factor

The absolute recovery of fatty acid or glycerolipid was determined by the mean peak area of fatty acid or glycerolipid at a specific concentration in algal matrix over the mean peak area of fatty acid or glycerolipid at the concentration in the extracted/fractionated algal matrix multiplying by 100%, and the IS normalized recovery was determined by the absolute recovery of fatty acid or glycerolipid over that of the IS multiplying by 100%; whereas the absolute matrix factor (MF) of fatty acid or glycerolipid was determined by the mean peak area of fatty acid or glycerolipid at a specified concentration in the extracted/fractionated algal matrix over that of fatty acid or glycerolipid at the concentration in the solvent, and the IS normalized MF was determined by the absolute MF of fatty acid or glycerolipid over that of the IS.

In this study, algal matrix mix QCs of pentadecanoic-2,2-d$_2$ acid (1.50, 200 and 400 μM) and glyceryl tri(hexadecanoate-2,2-d$_2$) (0.600, 80.0 and 160 μM) with a fixed concentration (50.0 μM) of IS (tridecanoic-2,2-d$_2$ acid) before the extraction and fractionation (Section 2.5) were subjected to sample extraction and fractionation (Sections 2.6 and 2.7). The reconstituted solutions (300 μL each) together with equal volume of the corresponding algal matrix mix QC after the extraction and fractionation and equal volume of the corresponding solvent mix QC were undergone esterification/transesterification reactions (Sections 2.8 and 2.9), and the resulting FAME compounds were measured by GC–MS. Three parallel measurements of three identical QC samples at each QC concentration were performed, and the data were used to calculate the mean recoveries and MFs.

2.10.2 | LLOQ

The LLOQ of the method for an analyte was defined as the concentration of standard calibrator at the lowest concentration of the analyte in a calibration plot, which had accuracy and precision of ≤ ±20% and 20% by five replicate measurements in the solvent matrix.

2.10.3 | Calibration curves

For the composition and content analyses of FAME compounds generated by the free-fatty-acid fraction and glycerolipid fraction of algae dry mass, calibration curves of FAME compounds were prepared by the reactions of BCl$_3$-MeOH with the solvent mix calibrators of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, together with dual ISs (i.e., tridecanoic-2,2-d$_2$ acid for free fatty acids, and glyceryl tri(hexadecanoate-2,2-d$_2$) for glycerolipids). Each calibration curve consisted of a double blank (with neither analytes nor ISs), a single blank (without analytes but ISs: tridecanoic-2,2-d$_2$ acid, 50.0 μM; and glyceryl tri(hexadecanoate-2,2-d$_2$), 20.0 μM), and seven non-zero standard calibrators (0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 μM with the ISs). The calibration curve of a FAME compound from the fraction of free fatty acids was constructed using the peak area ratios of an analyte to tridecanoic-2,2-d$_2$ acid (y) versus the analyte concentrations (x) with 1/x weighting, whereas the calibration curve of a FAME compound from the fraction of glycerolipids was constructed using the peak area ratios of an analyte to glyceryl tri(hexadecanoate-2,2-d$_2$) (y) versus the analyte concentrations (x) with 1/x weighting.

2.10.4 | Accuracy and precision

Accuracy was expressed as percentage relative error (RE) and precision as the coefficient of variation (CV). In this work, the inter-assay accuracy and precision were assessed by five parallel analyses of five identical solvent QC samples at each of the three QC concentrations (for mix fatty acids: 1.50, 200 and 400 μM, and for lipid (glyceryl tri(hexadecanoate-2,2-d$_2$)): 0.600, 80.0 and 160 μM) over five separate days. The intra-assay accuracy and precision were assessed by five replicate analyses of each QC samples in the same day.

2.10.5 | Stability

The stabilities of fatty acids and glycerolipids were investigated using standard stock solutions of pentadecanoic-2,2-d$_2$ acid (10.0 mm) and glyceryl tri(hexadecanoate-2,2-d$_2$) (10.0 mm), low- and high-QCs in algal matrix of pentadecanoic-2,2-d$_2$ acid (1.50 and 400 μM) and glyceryl tri(hexadecanoate-2,2-d$_2$) (0.600 and 160 μM) with tridecanoic-2,2-d$_2$ acid (50.0 μM) as the internal standard.

The stabilities of the stock standards and QCs were assessed for short-term storage (6 and 24 h; 23°C) on bench top (pre-preparative) and in autosampler (post-preparative); three freeze–thaw cycles (where the samples were subjected to freezing temperature at −20°C for at least 24 h and thawed at 23°C (room temperature) unassisted. The stabilities expressed as percent recoveries were determined by comparing the mean-peak-area ratios of the analyte to the IS in the test samples to those of freshly prepared samples. For this study, all measurements were done in five replicates.

2.11 | Method application

The validated GC–MS method was applied to the composition and content analyses of fatty acids from the fractions of free fatty acids and glycerolipids in algae *Scenedesmus dimorphus* dry mass. Algal cells were grown and prepared by the procedures described in Section 2.2. During the analysis, algal samples together with nine solvent mix standard calibrators (i.e., double- and single-blank, and seven non-zero), and a set of solvent mix QCs (at low, medium and high concentrations) were processed by the procedures described in Sections 2.6–2.9 and analyzed by the method described in Section 2.3.

3 | RESULTS AND DISCUSSION

3.1 | Method development

3.1.1 | GC–MS

A Thermo Scientific™ TRACE™ TR-FAME capillary GC column was used for this study because of its high-polarity cyanopropylphenyl-based phase for separation of FAMEs and *cis/trans* isomers with various degrees of unsaturation. The TR-FAME column provided not only higher separation efficiency of FAMEs, but also provided better resolution, reproducibility and more symmetrical peaks. GC separation of FAMEs was initially tested on this column using FAME mixed standard solution prepared from the AOCS Mix 6. The
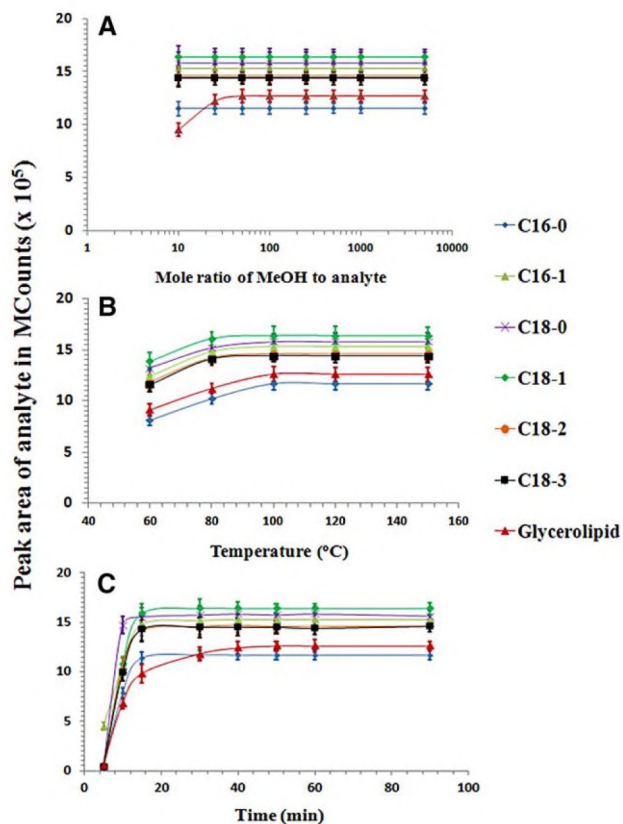
FIGURE 3 Optimization of reaction conditions of one-step esterification/transesterification of fatty acids and glycerolipid. Experimental conditions were as follows: (A) fatty acids, 500 µM; glyceryl tri(hexadecanoate-2,2-d_2), 200 µM; MeOH with 12% BCl$_3$ w/w; reaction temperature, 100°C; reaction time, 15 min for fatty acids and 50 min for glyceryl tri(hexadecanoate-2,2-d_2); (B) same as (A) except the mole ratio of MeOH to analyte was at 162:1; and (C) same as (B) except the reaction temperature was at 100°C

temperature program (Section 2.3) was chosen because its ramping rate at 5°C/min was particularly effective to resolve the isomeric analytes of methyl stearate and methyl linolenate between 180°C and 210°C. For this work, the injector temperature was maintained at 250°C to ensure complete vaporization of analytes and 1.00 µL injection with a split ratio of 1:10 was chosen to minimize sample zone broadening. The FAMEs of interest for this work were resolved in less than 14 min with a total program run time of 19.14 min (Fig. 1a). These optimized GC conditions were later applied to the separation of the products of fatty acid esterification and glycerolipid transesterification (Fig. 1b).

Quantitation of the FAMEs were carried out by the extracted ion chromatogram (EIC) mode of the ion-trap mass spectrometer with the quantifier and qualifier ions listed in Table 1. Although some FAME compounds shared the same quantifier ions (i.e., m/z 55.2 for methyl palmitoleate and methyl oleate), these compounds could be resolved by GC and identified by their unique qualifier ions; whereas the coeluted FAME compounds (i.e., methyl hexadecanoate-2,2-d_2 and

methyl palmitate) could be quantified by their unique quantifier and qualifier ions. The representative EIC mass chromatograms for various FAME compounds are given in Fig. 2, which demonstrated the unparalleled selectivity of the GC–MS method.

3.1.2 | Use of stable heavy isotopes as surrogate analytes and internal standards

Due to the endogenous nature of fatty acids and glycerolipids in algae, stable heavy isotopes pentadecanoic-2,2-d_2 acid and glyceryl tri(hexadecanoate-2,2-d_2) were employed as surrogate analytes for the assessment of matrix effect, recovery, and stability of fatty acids and glycerolipids in algal matrix. Since the matrix effect was negligible (Section 3.2.1), the method developed was then using solvent mix standard calibrators of fatty acids and glycerolipid validated in terms of lower limits of quantitation, linear calibration ranges, intra- and inter-assay precision and accuracy. In these experiments, a single stable heavy isotope (tridecanoic-2,2-d_2 acid) was used as the IS because neither fatty acids nor glycerolipids was fractionated. However, when the method developed was applied to the quantitation of FAME compounds from free fatty acid and glycerolipid fractions of algae *Scenedesmus dimorphus* dry mass, dual stable isotopes (tridecanoic-2,2-d_2 acid for free fatty acids, and glyceryl tri(hexadecanoate-2,2-d_2) for glycerolipids) were used for both calibration plots and algal sample preparation because fractionations of fatty acids and glycerolipids were performed.

3.1.3 | One-step esterification of free fatty acids and transesterification of glycerolipids

Fatty acids and glycerolipids are not very volatile by nature; therefore, when measuring them by GC–MS, it is common to convert these compounds into the more volatile FAMEs. In this work, BCl$_3$-MeOH (12% w/w) was used as the esterifying reagent for one-step esterification of fatty acids or transesterification of glycerolipids. Compared to BF$_3$-MeOH, another frequently used esterifying reagent, BCl$_3$-MeOH was found not only to be more stable when stored properly and to produce less artifacts [29,30], but also is suited for both esterification of fatty acids and transesterification of glycerolipids without prior hydrolysis [31,32].

The strategy on the use of BCl$_3$-MeOH as an esterifying reagent for one-step esterification of fatty acids and transesterification of lipids was developed in the 1960s [31] and modified in the 1970s [32] using chemical standards. This strategy and its reaction conditions derived from chemical standards in the early works were adopted to various sample analyses, such as musk melon leaves [33], vegetable oils [34] and corn grains [35] without further optimization.

In our work, we not only applied the strategy of using BCl$_3$-MeOH for one-step transesterification of lipids for the first

TABLE 2 Matrix effect and recovery of fatty acid and glycerolipid from algal matrix

Analyte	Pentadecanoic-2,2-d_2 acid			Glyceryl tri(hexadecanoate-2,2-d_2)		
QC (µM)	1.50	200	400	0.600	80.0	160
PA_{Ana}[a] in matrix ± SD (×10⁴)	0.094 ± 0.004	7.3 ± 0.4	15.7 ± 0.1	0.074 ± 0.002	3.6 ± 0.1	6.2 ± 0.2
PA_{Ana} in extracted matrix ± SD (×10⁴)	0.107 ± 0.005	7.9 ± 0.1	17.8 ± 0.1	0.089 ± 0.004	4.2 ± 0.1	7.6 ± 0.3
PA_{Ana} in solvent ± SD (×10⁴)	0.110 ± 0.002	8.2 ± 0.1	17.4 ± 0.3	0.091 ± 0.003	4.2 ± 0.1	7.8 ± 0.2
MF_{Ana}[b] ± SD	0.97 ± 0.05	0.96 ± 0.02	1.02 ± 0.02	0.98 ± 0.05	1.00 ± 0.03	0.97 ± 0.05
$Recovery_{Ana}$[c] ± SD (%)	88 ± 6	92 ± 5	88 ± 1	83 ± 4	86 ± 3	82 ± 4
PA_{IS}[d] in matrix ± SD (×10⁴)	2.66 ± 0.08	2.63 ± 0.09	2.45 ± 0.06	2.66 ± 0.08	2.63 ± 0.09	2.45 ± 0.06
PA_{IS} in extracted matrix ± SD (×10⁴)	2.77 ± 0.07	2.72 ± 0.05	2.63 ± 0.06	2.77 ± 0.07	2.72 ± 0.05	2.63 ± 0.06
PA_{IS} in solvent ± SD (×10⁴)	2.93 ± 0.05	2.72 ± 0.08	2.67 ± 0.06	2.93 ± 0.05	2.72 ± 0.08	2.67 ± 0.06
MF_{IS}[e] ± SD	0.95 ± 0.03	1.00 ± 0.03	0.99 ± 0.03	0.95 ± 0.03	1.00 ± 0.03	0.99 ± 0.03
$Recovery_{IS}$[f] ± SD (%)	96 ± 4	97 ± 4	93 ± 3	96 ± 4	97 ± 4	93 ± 3
IS normalized MF[g] ± SD	1.02 ± 0.06	0.96 ± 0.04	1.03 ± 0.04	1.03 ± 0.06	1.00 ± 0.04	0.98 ± 0.06
IS normalized recovery[h] ± SD (%)	92 ± 7	95 ± 7	95 ± 3	86 ± 5	90 ± 5	88 ± 5

[a]PA_{Ana} = mean peak area of analyte.
[b]MF_{Ana} = (PA_{Ana} in extracted algal matrix)/(PA_{Ana} in solvent).
[c]$Recovery_{Ana}$ = [(PA_{Ana} in algal matrix)/(PA_{Ana} in extracted algal matrix)] × 100%.
[d]PA_{IS} = mean peak area of internal standard.
[e]MF_{IS} = (PA_{IS} in extracted algal matrix)/(PA_{IS} in solvent).
[f]$Recovery_{IS}$ = [(PA_{IS} in algal matrix)/(PA_{IS} in extracted algal matrix)] × 100%.
[g]IS normalized MF = (MF_{Ana})/(MF_{IS}).
[h]IS normalized recovery = [($Recovery_{Ana}$)/($Recovery_{IS}$)] × 100%.
Note: The concentration of the IS (tridecanoic-2,2-d_2 acid) is 50.0 µM. All the samples were taken in three replicates.

time in algal samples, but also optimized the reaction conditions in the real sample matrix, which were not done in the previous studies. In detail, to maximize the product yields and improve the reproducibility of the reaction between fatty acids or glycerolipids and BCl_3-MeOH, factors (such as the mole ratio of MeOH to analyte, reaction temperature, and reaction time) that affect esterification of fatty acids and transesterification of glycerolipids were investigated using analyte concentrations at the upper limits of calibration curves (500 µM for fatty acids and 200 µM for glycerolipid).

As shown in Fig. 3, a steady-state production of FAME compounds could be reached at mole ratios of MeOH to analyte ≥ 10 for fatty acids and ≥ 25 for glycerolipid (Fig. 3a) with reaction temperatures ≥ 100°C for both fatty acids and glycerolipid (Fig. 3b) and reaction times ≥ 15 min for fatty acids and ≥ 50 min for glycerolipid (Fig. 3c). Therefore, for the subsequent studies, the optimum reaction conditions chosen were as follows: a mole ratio of MeOH to analyte at 162 times over the ones needed to reach the steady-state production of FAMEs, a reaction temperature of 100°C, and a reaction time of 15 min for esterification of fatty acids or 50 min for transesterification of glycerolipids. These optimized conditions showed reductions in both reaction temperature (from 120 to 100°C) and reaction time (from 90 to 50 min) in comparison to the previous reports [31–35].

Under the optimum reaction conditions, the conversion rates of fatty acids (i.e., palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid at three different concentrations of 1.50, 200 and 400 µM), and glycerolipid (i.e., glyceryl tri(hexadecanoate-2,2-d_2) acid at three different concentrations of 0.600, 80.0 and 160 µM) to their corresponding FAME compounds were examined, and the conversion yields were calculated using individual calibration curve prepared by individual FAME standard solutions. As summarized in Table S1 (Supporting Information), the conversion yields ranged from 97 to 100% for fatty acids and 92 to 94% for glycerolipid.

In our work, we observed that the esterification and transesterification reaction involving BCl_3-MeOH was sensitive to trace levels of water. Water tends to hinder the reaction producing lower yields of FAMEs. Hence, 2,2-dimethoxy propane was used as a water scavenger and its amount was optimized for the reaction conditions in the real sample matrix. From our experiments, we observed that the levels of 2,2-dimethoxy propane above 15 µL were producing spurious peaks between 3 and 5 min on the chromatogram. Therefore, 15 µL was chosen as an optimum amount for the reaction.

3.2 | Method validation

3.2.1 | Recovery and matrix effect

The recovery and MF data were summarized in Table 2. As shown in the table, the absolute and the IS normalized recoveries were 88–92 and 92–95% for pentadecanoic-2,2-d_2-acid,

TABLE 3 Intra- and inter-run precision and accuracy of the fatty acids and glycerolipid

Analyte name	Fatty acid chain	[Nominal] (µM)	Intra-run (n = 5) [Measured] (µM)	SD (µM)	CV (%)	RE (%)	Inter-run (n = 5) [Measured] (µM)	SD (µM)	CV (%)	RE (%)
Palmitic acid	C16:0	0.500 (LLOQ)	0.52	0.05	9	4	NM[a]	NM	NM	NM
		1.50	1.57	0.09	6	4	1.42	0.1	7	−6
		200	210	9	4	5	190	9	5	−5
		400	382	19	5	−5	379	18	5	−6
Palmitoleic acid	C16:1	0.500 (LLOQ)	0.49	0.03	6	−2	NM	NM	NM	NM
		1.50	1.43	0.08	6	−5	1.58	0.1	7	5
		200	211	10	5	5	190	12	6	−5
		400	414	22	5	3	420	22	5	5
Stearic acid	C18:0	0.500 (LLOQ)	0.51	0.05	9	3	NM	NM	NM	NM
		1.50	1.53	0.05	3	2	1.43	0.1	8	−5
		200	193	8	4	−4	209	9	4	4
		400	383	18	5	−4	384	16	4	−4
Oleic acid	C18:1	0.500 (LLOQ)	0.48	0.03	5	−4	NM	NM	NM	NM
		1.50	1.44	0.08	6	−4	1.58	0.1	7	5
		200	211	8	4	5	189	8	4	−6
		400	387	23	6	−3	378	24	6	−6
Linoleic acid	C18:2	0.500 (LLOQ)	0.46	0.03	6	−7	NM	NM	NM	NM
		1.50	1.45	0.04	3	−3	1.49	0.1	9	−1
		200	210	9	4	5	195	8	4	−3
		400	423	27	6	5	418	22	5	4
Linolenic acid	C18:3	0.500 (LLOQ)	0.47	0.03	7	−7	NM	NM	NM	NM
		1.50	1.47	0.06	4	−2	1.42	0.09	6	−6
		200	192	9	5	−4	189	8	4	−6
		400	381	21	6	−5	415	26	6	4
Glyceryl tri(hexa decanoate-2,2-d$_2$)	C16:0-d$_2$	0.200 (LLOQ)	0.18	0.01	9	−9	NM	NM	NM	NM
		0.600	0.58	0.04	7	−3	0.57	0.04	7	−5
		80.0	84	6	7	5	83	7	7	4
		160	168	13	8	5	168	19	9	5

[a]NM = not measured.

Note: The concentration of the IS (tridecanoic-2,2-d$_2$ acid) is 50.0 µM. All the samples were taken in five replicates.

TABLE 4 Stability studies of fatty acid and glyerolipid in stock solutions and algal matrix

Test conditions	Temp.(°C)	Pentadecanoic-2,2-d$_2$ acid			Glyceryl tri(hexadecanoate-2,2-d$_2$)		
		[Analyte] (µM)	Recovery ± SD (%)		[Analyte] (µM)	Recovery ± SD (%)	
			6 h	24 h		6 h	24 h
Bench-top	23	200 (stock)	95 ± 2	93 ± 3	80.0	94 ± 3	92 ± 4
		400 (stock)	96 ± 3	93 ± 2	160	94 ± 4	90 ± 3
		1.50 (LQC)	98 ± 3	87 ± 4	0.600	94 ± 4	86 ± 3
		400 (HQC)	99 ± 3	86 ± 3	160	95 ± 4	84 ± 3
Autosampler	23	1.50 (LQC)	91 ± 4	89 ± 2	0.600	93 ± 3	89 ± 3
		400 (HQC)	93 ± 4	89 ± 3	160	92 ± 3	89 ± 2
3 Freeze-thaw cycles	−20 to 23	1.50 (LQC)		90 ± 4	0.600		88 ± 4
		400 (HQC)		90 ± 4	160		90 ± 4

Note: The concentration of the IS (tridecanoic-2,2-d$_2$ acid) is 50.0 µM. All the samples were taken in five replicates.

and 82–86 and 86–90% for glyceryl tri(hexadecanoate-2,2-d$_2$); and the absolute and the IS normalized MFs were 0.96–1.02 and 0.96–1.03 for pentadecanoic-2,2-d$_2$-acid, and 0.97–1.00 and 0.98–1.03 for glyceryl tri(hexadecanoate-2,2-d$_2$), respectively.

In comparison to the previous analytical methods for algal sample analyses [18,24], which did not carry out method validation in algal matrix and simply applied solvent calibrators for quantitation of FAME compounds, our studies addressed the concern of matrix effect and revealed that matrix effect on analytical signals were insignificant and negligible, and the recoveries of the FAME compounds from algal matrix were high enough for accurate quantitation with the use of internal standard. Therefore, solvent calibrators instead of matrix calibrators were used in the subsequent quantitative studies.

3.2.2 | Calibration curves and lower limits of quantitation

The linear calibration curves of methyl esters of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were established from 0.500 to 500 µM, and the calibration equations were provided in Table S2 (Supporting Information). The LLOQs of these FAME compounds derived from free fatty acids and glycerolipid were 0.500 and 0.200 µM, respectively, which had accuracy and precision of ≤ ±7% and ≤ 9% for free fatty acids, and ≤ ±9% and ≤ 9% for glycerolipid, by five replicate measurements in solvent matrix (Table 3).

## 3.2.3 | Accuracy and precision

As shown in Table 3, the intra-assay accuracy and precision were ≤ ±5% and ≤ 6% for the fatty acids and ≤ ±5% and ≤ 8% for the glycerolipid, and the inter-assay accuracy and precision were ≤ ±6% and ≤ 9% for the fatty acids and ≤ ±5% and ≤ 9% for the glycerolipid, indicating the method developed was both accurate and precise.

3.2.4 | Stability

The stability studies of the standard stock solutions of pentadecanoic-2,2-d$_2$ acid (10.0 mm) and glyceryl tri(hexadecanoate-2,2-d$_2$) (10.0 mm), and low- and high-QCs of pentadecanoic-2,2-d$_2$ acid (1.50 and 400 µM) and glyceryl tri(hexadecanoate-2,2-d$_2$) (0.600 and 160 µM) in algal matrix were conducted. As summarized in Table 4, at room temperature (23°C), the stock solutions and the algal matrix QCs were stable for up to 24 h on bench top (pre-preparative) and in autosampler (post-preparative) at room temperature (23°C) with recoveries ranged 86–99% for pentadecanoic-2,2-d$_2$ acid, and 84–95% for glyceryl tri(hexadecanoate-2,2-d$_2$). For three freeze–thaw cycles, the recoveries were 90% for pentadecanoic-2,2-d$_2$ acid, and 88–90% for glyceryl tri(hexadecanoate-2,2-d$_2$). The data indicated that there were no significant losses of analytes during sample analyses.

3.3 | Analysis of fatty acids in algae *Scenedesmus dimorphus*

The validated GC–MS method was applied to the determination of saturated and unsaturated fatty acids with carbon numbers of 16 and 18 (i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3)) in the fractions of free fatty acids and glycerolipids from total lipid extract of algae *Scenedesmus dimorphus* dry mass. By the procedures described in Section 2.11, the free fatty acids and glycerolipids were converted into FAMEs and detected by the GC–MS method developed (Fig. 4). The quantitation of individual fatty acid was accomplished by individual FAME calibration curves whose data were obtained concurrently with those of algal samples. As shown in Table 5, the sum of six fatty acids from the free fatty acid fraction of the total lipid extract was 4.44% of the algal dry mass,
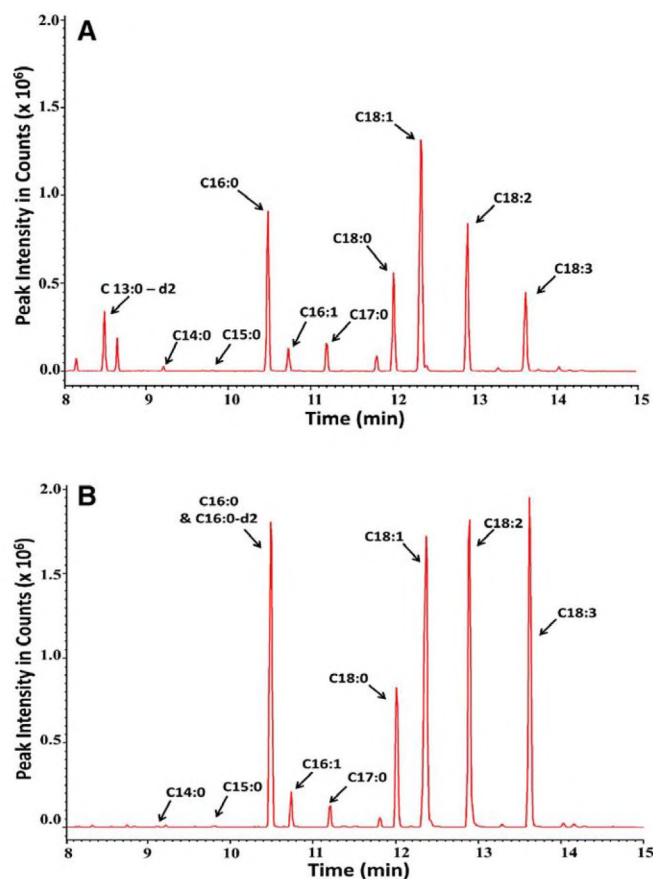
FIGURE 4 Total ion chromatograms of FAMEs from algae *Scenedesmus dimorphus* dry mass. (A) The fraction of free fatty acids from total algal lipid extract (tridecanoic-2,2-d$_2$ acid was used as the IS at the concentration of 50.0 μM); and (B) the fraction of glycerolipids from total algal lipid extract [glyceryl tri(hexadecanoate-2,2-d$_2$) was used as the IS at the concentration of 20.0 μM]. Experimental conditions were the same as those described in the Section 2.11

whereas the sum of six fatty acids from the glycerolipid fraction was 6.83%. These data are in concordance with the previous findings [18,24]. The compositions of fatty acids found in the fraction of free fatty acids from the total algal lipid extract match those reported in the Superpathway of Fatty Acid Biosynthesis II of the green algae family Scenedesmaceae [36], whereas the fatty acids found in the fraction of glycerolipids from the total algal lipid extract match those reported in the Biosynthesis Pathway of Diacylglycerol and Triacylglycerol [37].

4 | CONCLUSION

This paper detailed the development and validation of a quantitative analytical method for the determination of fatty acids in both free fatty acid and glycerolipid fractions of total lipid extract from algae *Scenedesmus dimorphus* dry mass. In this work, the total lipids were first extracted by

a modified Folch method, then free fatty acids and glycerolipids were fractionated by aminopropyl SPE cartridges. This method employed a time-efficient one-step esterification and transesterification reaction to convert free fatty acids and glycerolipids to FAME compounds, which were optimized in terms of reactant ratio, reaction temperature and time. The separation and quantitation of FAME compounds were accomplished by GC–MS. The method developed has been validated in terms of matrix effect, recovery, LLOQ, linear calibration range, accuracy and precision, as well as stabilities. It has been successfully applied to the analysis of fatty acids in algae *Scenedesmus dimorphus*, and can be used for quality assessment of biodiesel produced from algal biomass.

TABLE 5 The content of fatty acids as FAMEs from the fractions of free fatty acids and glycerolipids of algal lipid extract

Fraction of algal lipid extract	Fatty acid chain	Content of fatty acid in algal dry mass (%)
Free fatty acids[a]	C16:0	0.685
	C16:1	0.327
	C18:0	0.174
	C18:1	0.804
	C18:2	1.19
	C18:3	1.26
Sum of free fatty acids		4.44
Glycerolipids[b]	C16:0	1.04
	C16:1	0.531
	C18:0	0.240
	C18:1	1.58
	C18:2	1.63
	C18:3	1.81
Sum of fatty acids from glycerolipids		6.83

[a]Tridecanoic-2,2-d$_2$ acid was used as the IS at a concentration of 50.0 μM.
[b]Glyceryl tri(hexadecanoate-2,2-d$_2$) was used as the IS at a concentration of 20.0 μM.
Note: All the samples were taken in three replicates.

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