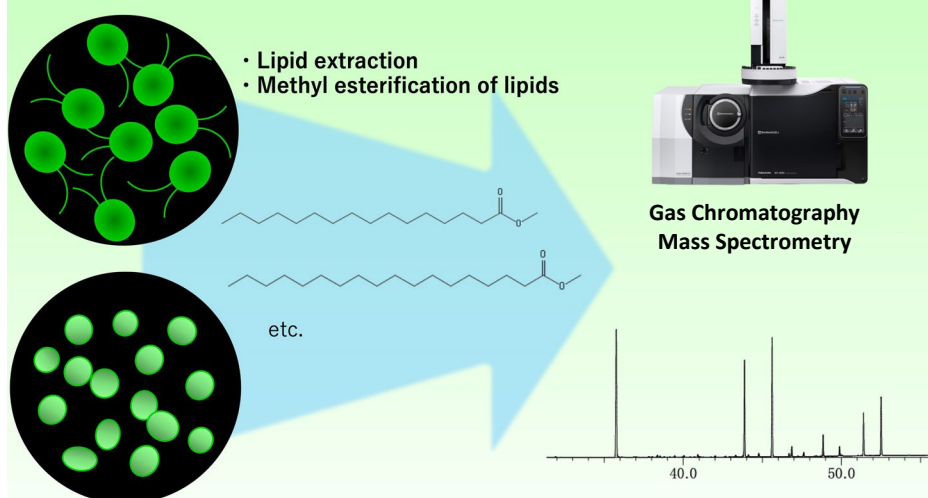


GC-MS-Based Evaluation of Fatty Acid Composition in Microalgae

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■ Abstract

Given the urgent need to mitigate climate change and reduce dependence on fossil fuels, there has been a substantial increase in research and development efforts focused on the production of Sustainable Aviation Fuel (SAF) from microalgae. As the fatty acids contained in microalgae are used as raw materials for SAF, the standardization of analytical methods for these fatty acids is imperative for accurately evaluating the potential of microalgae as a SAF source. This paper introduces a case study in which we validated an analytical method for fatty acids in microalgae biomass using the GCMS-TQTM8040 NX, a gas chromatography-mass spectrometer capable of high-resolution and high-sensitivity detection.

1. Introduction

Microalgae, a diverse group of unicellular photosynthetic organisms, are capable of synthesizing and storing various valuable substances, including lipids, proteins, carbohydrates, and carotenoids. Due to their rapid growth rate and high lipid content, microalgae have emerged as a promising feedstock for the production of Sustainable Aviation Fuel (SAF). However, the lack of standard analytical methods and evaluation criteria has hindered the ability to conduct fair comparisons and evaluations among different studies, limiting the advancement of this research field. For instance, productivity metrics, such as biomass concentration (g-biomass L⁻¹ d⁻¹ or g-biomass m⁻² d⁻¹),

cell density (cells L⁻¹ d⁻¹), and optical density (ΔOD d⁻¹), are notated differently among research groups, hindering direct comparisons¹⁻⁸⁾. Similarly, methods for lipid composition analysis, including fatty acid profiling, differ among research groups, with factors such as the choice of extraction solvents and their ratios influencing results (Table 1)⁹⁻¹⁴⁾.

Such issues are also observed for simple environmental parameters like light intensity, water temperature, pH, aeration rate, and various gas concentrations. Consequently, these findings are often difficult to compare and verify adequately, leading to a fragmented and isolated research landscape. This situation arises from the lack of standardized methodologies and evaluation criteria for experiments and analyses, representing the challenge that requires urgent attention.

As we have outlined in the preceding paragraphs, the absence of standardized protocols for microalgae cultivation and analysis has hindered progress in the field. To overcome this issues, it is imperative to establish standardized procedures for cultivation, harvesting, lipid extraction, and analysis. Particularly, there is urgent need to standardize analytical methods for fatty acid profiling in various algal species to accurately assess the potential of microalgae as SAF source, since fatty acids from microalgae serve as raw materials for SAF. In this study, we utilized gas chromatography-mass spectrometer, GCMS-TQ8040 NX, to compare and validate various extraction solvents, their ratios, and methods employed for the analysis of fatty acids derived from microalgae.

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• **Transesterification to Fatty Acid Methyl Esters with the Extraction Process**

We determined the fatty acid composition of microalgae by converting the lipids into fatty acid methyl esters (FAME) and subsequent analysis using GC-MS (Fig. 1). Specifically, 10 mg of lyophilized microalgae biomass was disrupted in 1 mL of various extraction solvents, each containing tripentadecanoin as an internal standard, using bead beater with 0.1 mm zirconia beads (Fig. 1A). Bead beating was performed at 2,500 rpm for 1 minute followed by a 2-minute interval for a total of 8 cycles. Subsequently, 3 mL of extraction solvent and 2.5 mL of Tris buffer (50 mM Tris, 1 M NaCl, pH 7) were added to the cell lysate and stirred for 5 minutes. After centrifugation, the organic layer was collected. If color remained in the residue, another 1 mL of extraction solvent was added, followed by stirring for 5 minutes and centrifugation to recover the organic layer. The collected samples were dried by blowing nitrogen gas over them.

The prepared samples were dissolved in 3 mL of 5% $H_2SO_4/MeOH$ and reacted at 70° C for 3 hours (Fig. 1B). After adding 3 mL of ultrapure water and 3 mL of hexane, followed by stirring, the mixtures were centrifuged, and the upper organic layer was collected. Subsequently, anhydrous sodium sulfate was added to remove residual water. The resulting samples were analyzed for FAME composition using the GCMS-TQ8040 NX.

• ***in situ* Transesterification to Fatty Acid Methyl Esters without the Extraction Process**

Ten milligrams of lyophilized microalgae biomass were reacted with 1 mL of toluene, containing tripentadecanoin as an internal standard, and 2 mL of 0.5 M $CH_3ONa/MeOH$ at 90° C for 30 minutes (Fig. 2). After cooling the reaction mixture to room temperature, 2 mL of 14% $BF_3/MeOH$ was added. The mixture was then reacted at 90° C for another 30 minutes. After cooling to room temperature, 3 mL of hexane and 3 mL of saturated NaCl solution were added and reacted at 90° C for 10 minutes. The upper organic layer was collected after centrifugation. Anhydrous sodium sulfate was added to remove residual water from the samples. The resulting samples were analyzed for FAME composition using the GCMS-TQ8040 NX.

• **GC-MS Analysis Methods**

GCMS-TQ8040 NX (Fig. 3A) was employed for FAME analyses. The detailed analytical condition is shown in Table 2. Retention times of target compounds were predicted using the Smart Metabolites Database™ (Fig. 3B) based on the retention indices of n-alkanes, and the analysis method was automatically created. Quantification of FAMES was performed using Supelco 37 Component FAME Mix (Sigma-Aldrich). All values are presented as mean $\pm$ SD.

Table 1. Example of Extraction Solvent for Lipid Analysis from Previous Literature

Literature	Extraction Solvent	Algal species
Guido et al., (2013), <i>Journal of Visualized Experiments</i> ; e50628:1–9.	$CHCl_3/MeOH$ (4:5)	<i>Scenedesmus obliquus</i> , <i>Phaeodactylum tricornutum</i>
Halim et al., (2011), <i>Bioresource Technology</i> ;102(1):178–85.	Hexane, hexane/IPA (3:2)	<i>Chlorococcum</i> sp.
Lee et al., (1998), <i>Biotechnology Techniques</i> ;7:553–6.	$CHCl_3/MeOH$ (2:1) , hexane/IPA (3:2), dichloroethane/ $MeOH$ (1:1), dichloroethane/ $EtOH$ (1:1), acetone/dichloromethane (1:1)	<i>Botryococcus braunii</i>
Nagle and Lemke, (1990), <i>Applied Biochemistry and Biotechnology</i> ;24:355–61.	1-butanol, hexane/IPA (2:3), $MeOH/CHCl_3$ (1:1)	<i>Chaetoceros muelleri</i> , <i>Monoraphidium minutum</i>

highlighted in red: conditions with high extraction efficiency in the literature

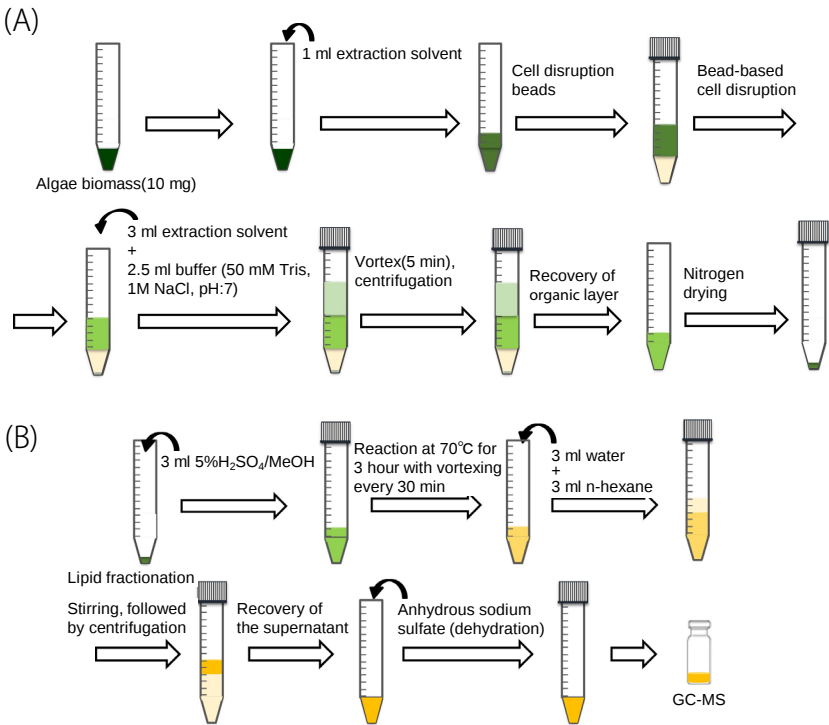


Fig. 1. Analytical Methods for Fatty Acids in Microalgae Biomass
(A) Lipid extraction from microalgal biomass
(B) Transesterification to fatty acid methyl esters



Fig. 2. *in situ* methyl esterification of fatty acids in microalgae



Fig. 3 GCMS-TQ™8040 NX (A) and Smart Metabolites Database™ (B)

Table 2 GC-MS analysis parameters (Smart Metabolites Database: FAME analysis method)

[GC]

Column	SP-2560(0.25 mm I.D. × 100 m, 0.20 μm, Supelco)
Injection Temperature	250°C
Injection Volume	2 μL
Injection Mode	Split
Carrier Gas	He
Control Mode	Linear velocity
Oven Temperature	40°C(2 min) – (4°C/min) – 240°C(2 min)

[MS]

IF Temperature	250°C
Ion source Temperature	200°C
Ionization Mode	EI
MS Mode	Scan

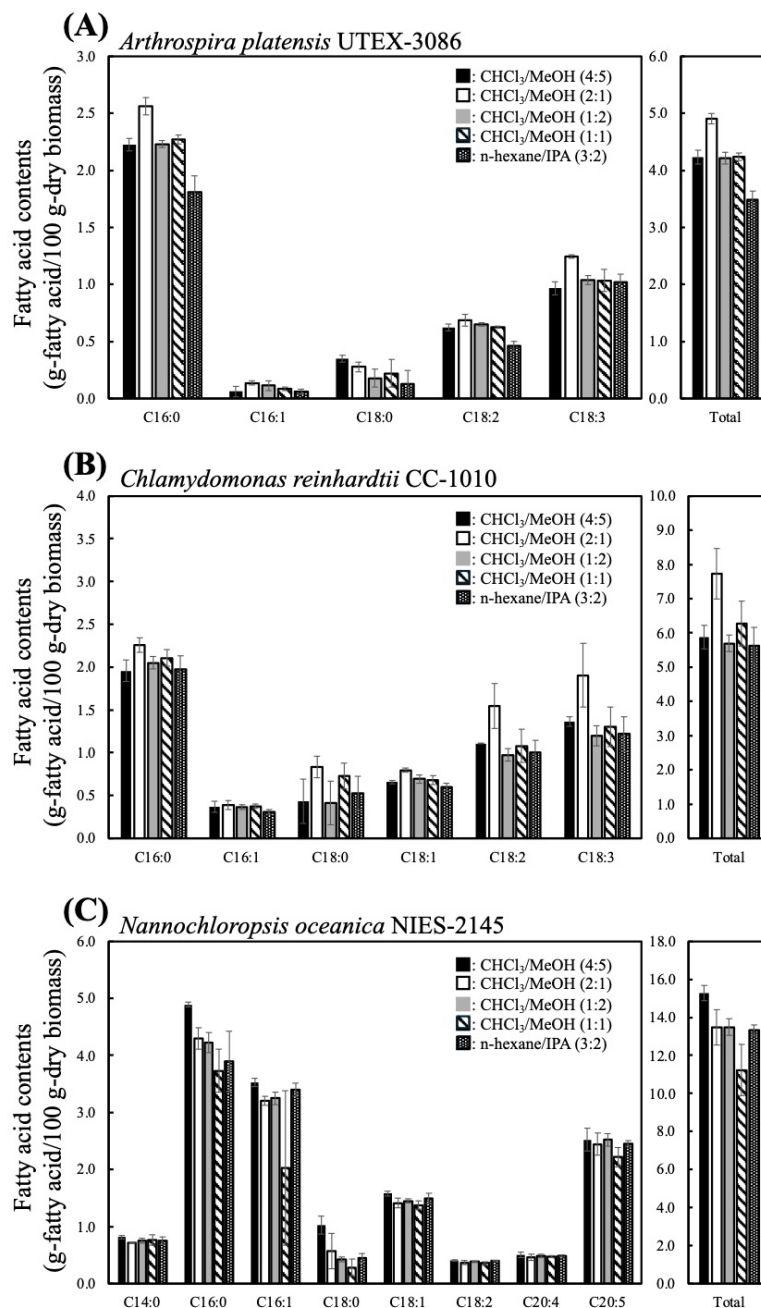


Fig. 4 Comparison of various extraction conditions in fatty acid composition analyses for *Spirulina* (A), *Chlamydomonas* (B), and *Nannochloropsis* (C)

3. Results and Discussion

Microalgae are positioned to become a primary feedstock to produce SAF, because of their rapid growth rates and high lipid content. However, the analysis of microalgal fatty acids has been hindered by the lack of standardized protocol, as diverse solvents and solvent ratios have been employed, leading to inconsistent results across research groups⁹⁻¹⁴. To address this issue and ensure reliable and comparable data for subsequent studies, systematic evaluation of extraction conditions on fatty acid composition analysis is necessary. In this study, we firstly examined the impacts of different types and ratios of extraction solvents on the fatty acid composition analysis for various microalgal species: *Spirulina* (*Arthrospira platensis* UTEX-3086), *Chlamydomonas* (*Chlamydomonas reinhardtii* CC-1010), and *Nannochloropsis* (*Nannochloropsis oceanica* NIES-2145).

GCMS-TQ8040 NX analysis showed marked differences in the relative abundances of fatty acid methyl esters across solvents, although the detected types of fatty acids were consistent (Fig. 4). Namely, *Spirulina* exhibited an increased content of C16:0 and C18:3 upon extraction with CHCl₃/MeOH (2:1), resulting in higher total fatty acid content compared to other solvents

(Fig. 4A). *Chlamydomonas* similarly showed increased levels of fatty acids, including C18:2 and C18:3, under the same extraction conditions (Fig. 4B). In contrast, *Nannochloropsis* exhibited the highest overall fatty acid content when extracted with CHCl₃/MeOH (4:5), with elevated levels of C16:0, C18:0 (Fig. 4C). These results indicate that the amounts of fatty acids detected in microalgae are influenced by the type and ratio of solvents used for extraction process, suggesting that the choice of the extraction process can potentially underestimate the total fatty acid content of microalgae. Moreover, considering that the optimal conditions vary among microalgal species, it is necessary to assess different solvents for each species.

Recent studies on the analysis of fatty acids in microalgae have seen a growing interest in *in situ* methylation, direct methylation method that bypasses the extraction process, enabling more rapid and efficient analysis of fatty acid profiles within the microalgal biomass¹⁵. Furthermore, while the Algae Biomass Organization (ABO) has recommended *in situ* methylation as a promising technique for fatty acid analysis of microalgae biomass¹⁶, there is a paucity of comprehensive

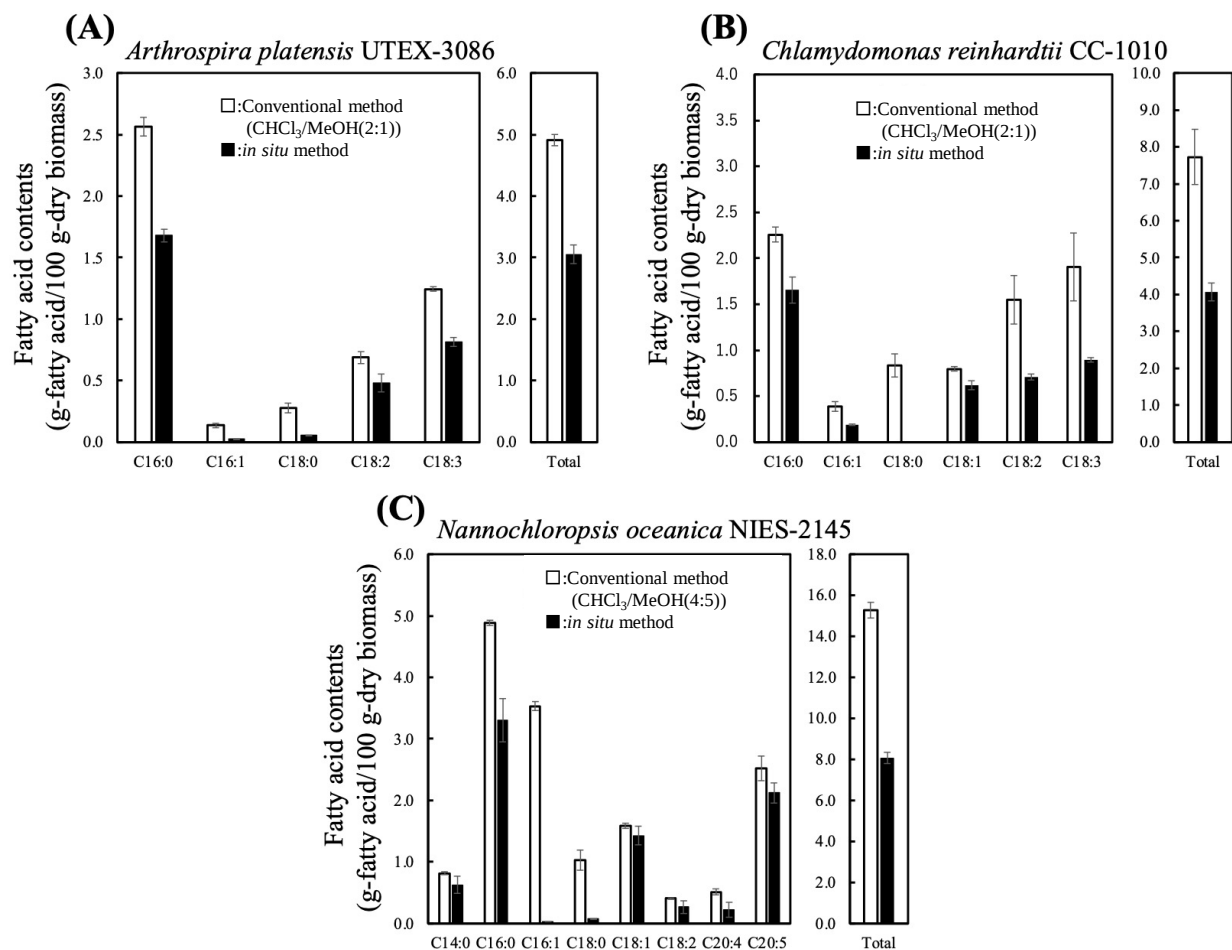


Fig. 5 Comparison with and without extraction process in fatty acid composition analyses (Conventional method and *in situ* method, respectively) for *Spirulina* (A), *Chlamydomonas* (B), and *Nannochloropsis* (C)

comparative studies directly contrasting this method with conventional extraction-based approaches. Therefore, we conducted a comparative validation of both methods. As a result, while no differences were observed in the methyl fatty acids detected between the conventional and *in situ* methods, significant discrepancies were found in their quantities (Fig. 5). Namely, the *in situ* method tended to show lower overall fatty acid quantities compared to the conventional method across all microalgal species. These results suggest that the *in situ* method, despite its rapidity and convenience, requires further validation and improvement to accurately quantify fatty acids in microalgal biomass.

4. Conclusion

This application note presented a comparative validation of fatty acid analysis methods for microalgae using the gas chromatography-mass spectrometer GCMS-TQ8040 NX. Our results from conventional fatty acid analysis, which involved an extraction process, showed that the quantity of fatty acids detected in microalgae was influenced by the type and ratio of extraction solvents. Additionally, since optimal conditions differ among microalgal species, consideration of extraction solvents is necessary in analyses involving extraction processes. In contrast, the *in situ* analysis without an extraction process was found to result in lower fatty acid detection compared to the extraction-based method, indicating that further improvements are needed.

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