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Validation of Thermally Assisted Hydrolysis and Methylation−Gas Chromatography Using a Vertical Microfurnace Pyrolyzer for the Compositional Analysis of Fatty Acid Components in Microalgae

Yasuyuki Ishida,*[,†] Takayuki Honda,[†] Shigemi Mabuchi,[‡] and Osamu Sueno[‡]

[†]Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai 487-8501, Japan

[‡]Scientific Department, Nikken Sohonsha Company, 1-32 Asahira, Fukujyu-cho, Hashima 501-6255, Japan

ABSTRACT: Thermally assisted hydrolysis and methylation−gas chromatography (THM-GC) in the presence of trimethylsulfonium hydroxide, using a vertical microfurnace pyrolyzer, was validated for the compositional analysis of fatty acid components in microalgae. The chromatograms of a microalga, *Pavlova lutheri*, obtained under optimized THM conditions clearly showed a series of fatty acid methyl esters including thermally labile polyunsaturated fatty acid components. On the basis of these peak areas, their chemical compositions were rapidly determined without using any tedious sample pretreatment with a precision of <8% relative standard deviation. Moreover, the compositions thus obtained were in good agreement with those obtained by the conventional technique involving solvent extraction. Finally, the THM-GC technique was applied for the compositional analysis of fatty acid components in a newly found microalga, *Coccomyxa gloeobotrydiformis*. The obtained data showed a high abundance (24 mol %) of α-linolenic acid components, suggesting its potential usefulness as feed sources and/or functional foods.

KEYWORDS: *thermally assisted hydrolysis and methylation−GC, validation, trimethylsulfonium hydroxide, microalgae, Coccomyxa gloeobotrydiformis, polyunsaturated fatty acid*

INTRODUCTION

Some microalga species are known to synthesize various classes of lipids containing polyunsaturated fatty acid (PUFA) residues, which play important roles in vital functions. For example, one microalga species, *Pavlova lutheri*, has been reported to possess the ability to synthesize and accumulate a large amount of PUFAs with longer carbon chains, such as eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6).[1−6] These two PUFAs are grouped into n-3 fatty acid components, where the first double bond is located at the third carbon atom from the methyl end of the carbon chain. Many lines of evidence have supported the proposal that n-3 fatty acids such as EPA and DHA are effective for the prevention of cardiovascular diseases.[7,8] Therefore, the microalgae containing a large amount of n-3 PUFA components are attracting a great deal of attention not only as feed sources used in aquaculture but also as functional foods to improve human health. To evaluate nutritional values of microalgae, a practical method for the analysis of fatty acid components including PUFAs accumulated in algal cells needs to be developed.

Generally, fatty acid components in microalgae have been determined by gas chromatography (GC) with preliminary sample treatments such as solvent extraction, saponification, and/or derivatization.[9−11] A high-performance liquid chromatography (HPLC) system with a fluorescence detector was also used for compositional analysis of fatty acid components extracted from microalgae.[12] However, these techniques require tedious and time-consuming sample pretreatments prior to the final analytical measurements.

On the other hand, thermally assisted hydrolysis and methylation−GC (THM-GC) in the presence of an organic alkali has been widely utilized as a rapid and convenient technique to characterize fatty acid components of lipids contained in a small amount of various biological samples.[13,14] The THM reaction under optimized conditions allowed quantitative detection of fatty acid moieties in lipids as their corresponding methyl esters. On the basis of the peak areas of the fatty acid methyl esters observed in the resulting chromatograms, the chemical composition of fatty acid components in the lipid samples can be determined without doing any cumbersome sample pretreatments.

Furthermore, the authors reported that the THM-GC method in the presence of trimethylsulfonium hydroxide [$(CH_3)_3SOH$; TMSH] as an organic alkali, using a vertical microfurnace pyrolyzer, enabled highly sensitive detection of thermally labile PUFA components such as EPA and DHA.[15] After that, THM-GC in the presence of TMSH became a powerful method for practical and precise lipid analyses for biological samples containing a large amount of PUFA residues, such as marine lipids,[16] microalgae,[17,18] aquatic microorganisms,[19] and raw human plasma and whole blood.[20] Among them, Blokker et al. and Akoto et al. analyzed the chemical composition of fatty acid components including PUFAs in microalgae by means of THM-GC using a direct

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thermal desorption interface.[17,18] Moreover, the authors successfully applied the THM-GC technique using a vertical microfurnace pyrolyzer to the lipid characterization of various zoo- and phytoplankton samples with dry masses on the order of a few tens of micrograms.[21,22] However, there has been no report concerning the validation of the THM-GC system with a microfurnace pyrolyzer for the characterization of fatty acid components in microalgae.

In this study, the validation of the technique using a microfurnace pyrolyzer for the analysis of fatty acid composition in microalgae was carried out by using *P. lutheri* as a sample. First, the methylation efficiency of fatty acid components in microalgae was examined by focusing on the reaction temperature. Then, the values of the fatty acid composition obtained under thus-optimized THM conditions were compared with those obtained by the conventional method involving solvent extraction. Finally, the THM-GC technique was applied to the compositional analysis of fatty acid components in a newly found microalga, *Coccomyxa gloeobotrydiformis*.

MATERIALS AND METHODS

Culture Conditions. The monoalgal strain of *P. lutheri* was used for the optimization and validation of the technique in this work. The enriched seawater (SE) medium prepared at pH 7.5 was used for the culture of *P. lutheri*. The temperature of the medium was maintained at 25 °C, and the air enriched with 1% (v/v) CO_2 was bubbled at the bottom of the reactor. In addition, the monoalgal strain Nikken of *C. gloeobotrydiformis* newly found on the surface of a stone collected at Point Barrow, AK, USA, was also used for the compositional analysis of fatty acid components. This strain was cultured with the Bold's basal medium (BBM) under conditions similar to those for *P. lutheri*. The microalgae harvested by centrifugation at the growth stage of 14 days were subjected to the THM-GC measurement after washing and freeze-dried.

An ingredient analysis of these microalgae revealed that *C. gloeobotrydiformis* contained a much larger amount of carbohydrates and proteins than *P. lutheri*; the mass ratio among carbohydrates, proteins, and lipids was 2.5:4.9:1.0 for *C. gloeobotrydiformis* but 1.5:2.1:1.0 for *P. lutheri*.

Reagents. A methanol solution of trimethylsulfonium hydroxide (TMSH) (0.25 M) purchased from Fluka (Buchs, Switzerland) was used as an organic alkali reagent. Methanol containing 10% HCl supplied from Fluka was also used for transmethylation of the extracted lpids. A standard solution of 31 fatty acid methyl esters with different chain lengths and degrees of unsaturation was purchased from Nu-Chek Prep (Elysian, MN, USA). This standard solution was used for the assignment of the peaks observed in the chromatograms of microalgae on the basis of the retention times.

Conditions for THM-GC. The procedure for THM-GC is basically the same as that described in our previous papers.[21,22] A vertical microfurnace pyrolyzer [Frontier Lab (Koriyama, Japan) PY-2020iS] was directly attached to a gas chromatograph [Agilent (Palo Alto, CA, USA) 4890] equipped with a flame ionization detector (FID). After a microalga sample weighing about 300 μg was added into a small platinum cup, 3 μL of the organic alkali solution was added to the same sample cup. Here, this sample size was selected to permeate the organic alkali solution through microalgal powders in the sample cup, although the maximum size of the sample that could be introduced into the cup was about 1 mg. Next, the sample cup was first mounted on the waiting position of the pyrolyzer near room temperature and then dropped into the heated center of the pyrolyzer maintained at 350 °C under the flow of helium carrier gas.

A metal capillary column (Frontier Lab, Ultra ALLOY-CW, 30 m × 0.25 mm i.d.) coated with poly(ethylene glycol) (0.25 μm film thickness), immobilized by chemical cross-linking, was used for GC separation. The 50 mL/min carrier gas flow rate at the pyrolyzer was reduced to 1 mL/min at the capillary column by means of a splitter. The column temperature was initially set at 50 °C and then programmed to 220 °C at a rate of 10 °C/min. Identification of the peaks on the chromatograms was also carried out using a GC-MS system [Shimadzu (Kyoto, Japan) QP5050] with an electron ionization (70 eV) source to which the pyrolyzer was directly attached.

Procedure for Solvent Extraction and Transmethylation followed by GC Analysis. The results for *P. lutheri* obtained by THM-GC were compared with those obtained by the conventional solvent extraction and transmethylation−GC method. Lipid extraction from the sample was performed according to the Bligh−Dyer method.[23] Then the extracted lipids were subjected to transmethylation with methanol containing 10% HCl at 75 °C for 30 min, and the resulting fatty acid methyl esters were analyzed by an ordinary GC system under the same chromatographic conditions as for the THM-GC measurements.

RESULTS AND DISCUSSION

Optimization and Validation of THM-GC. At first, the effect of the reaction temperature on the THM procedure of fatty acid components was evaluated by using *P. lutheri* as a microalga sample. Figure 1 shows a typical chromatogram of the *P. lutheri* sample obtained by THM-GC-FID in the

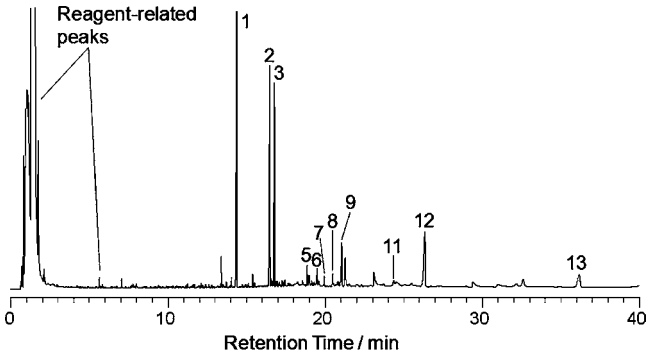

Figure 1. Typical chromatogram of *P. lutheri* sample obtained by THM-GC at 350 °C in the presence of TMSH. The peaks are assigned in Table 1.

Table 1. Identification of the Characteristic Peaks on the Chromatograms Shown in Figures 1 and 2

peak	compound	structure of the original fatty acid, C*i*:*j*[a]	ECN[b]
1	methyl myristate	C14:0	13.75
2	methyl palmitate	C16:0	15.75
3	methyl palmitoleate	C16:1	15.65
4	methyl stearate	C18:0	17.75
5	methyl oleate	C18:1	17.65
6	methyl linoleate	C18:2	17.55
7	methyl γ-linolenate	γC18:3	17.45
8	methyl α-linolenate	αC18:3	17.45
9	methyl stearidonate	C18:4	17.35
10	methyl arachidate	C20:0	19.75
11	methyl arachidonate	C20:4	19.35
12	methyl eicosapentaenoate.	C20:5	19.25
13	methyl docosahexaenoate	C22:6	21.15

[a]The number C*i*:*j*, for example, indicates the carbon number *i* and *j* double bond(s). [b]Empirically estimated values based on the structures.[24].

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Table 2. Influence of Reaction Temperature on Relative Peak Intensities of Fatty Acid Methyl Esters Observed in Chromatograms

	relative peak intensities for fatty acid components[a]/%											
T/°C	C14:0	C16:0	C16:1	C18:1	C18:2	γC18:3	αC18:3	C18:4	C20:4	C20:5	C22:6	total
300	24.8	22.1	19.3	1.95	2.31	1.24	1.45	6.22	0.607	14.9	5.07	100
	(10.1	5.9	6.3	13.5	14.8	12.2	11.3	10.9	13.5	18.2	28.9)[b]	
350	24.0	22.4	19.5	1.84	1.98	1.17	1.53	6.50	0.659	15.5	5.00	100
	(2.7	2.3	1.3	3.7	4.2	3.0	2.8	3.0	5.9	4.1	8.2)[b]	
400	29.3	23.0	20.0	1.89	1.89	0.993	1.65	5.51	0.587	11.4	3.70	100
	(1.3	1.0	1.2	7.0	5.6	0.8	3.0	0.8	3.0	4.0	4.8)[b]	

[a]The number Ci:j, for example, indicates the carbon number i and j double bond(s). [b]Values in parentheses are relative standard deviations (n = 3).

Table 3. Distributions of Fatty Acid Components (Mole Percent) for *Pavlova lutheri* Determined by THM-GC and Conventional Transmethylation followed by GC[a]

	fatty acid composition[b]/mol %													
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	γC18:3	αC18:3	C18:4	C20:0	C20:4	C20:5	C22:6	total
						Pavlova lutheri								
THM-GC	28.1	22.8	19.9	nd	1.67	1.81	1.07	1.40	6.01	nd	0.547	12.9	3.79	100
	(2.7	2.3	1.3		3.7	4.2	3.0	2.8	3.0		5.9	4.1	8.2)[c]	
conventional GC[d]	26.2	27.5	18.2	nd	2.01	1.65	1.03	1.02	4.50	nd	0.752	13.0	4.08	100
	(0.8	0.4	0.6		2.6	4.6	2.2	2.0	0.9		1.4	1.7	2.7)[c]	
						Coccomyxa gloeobotrydiformis								
THM-GC	7.76	24.6	7.75	4.90	8.81	13.0	1.09	24.2	nd	3.71	2.67	1.53	nd	100
	(3.3	1.0	6.3	4.1	1.9	2.7	2.2	3.9		12.6	9.4	10.9	)[c]	

[a]Calculated from the peak areas corrected by ECN. [b]The number Ci:j, for example, indicates the carbon number i and j double bond(s). [c]Values in parentheses are relative standard deviations (n = 3). [d]Obtained by sample pretreatments, such as solvent extraction and offline transmethylation, followed by GC measurement.

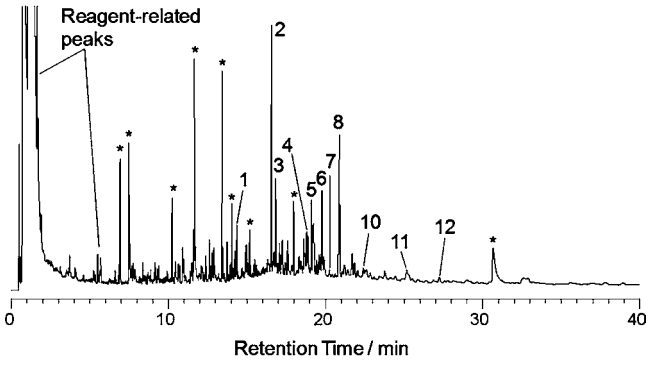

Figure 2. Typical chromatogram of *C. gloeobotrydiformis* sample obtained by THM-GC at 350 °C in the presence of TMSH. The peaks are assigned in Table 1. ∗, these peaks might be derived from polysaccharides and proteins contained in *C. gloeobotrydiformis* sample.

presence of TMSH at 350 °C. On this chromatogram, a series of sharp peaks of fatty acid methyl esters derived from the lipid components were clearly observed after the elution of the reagent-related peaks such as methanol and dimethyl sulfide. Table 1 summarizes the assingment of these characteristic peaks together with their abbreviations and effective carbon numbers (ECNs), which correspond to the relative molar sensitivity of FID.[24] As shown in this table, this alga sample contains not only saturated and monounsaturated fatty acids but also a series of PUFA components such as EPA (C20:5, peak 12) and DHA (C22:6, peak 13), as was reported earlier.[1−6]

Next, Table 2 summarizes the influence of the THM temperature on relative peak areas (percent) of fatty acid methyl esters, normalized by the total peak areas, observed in the chromatograms. Here, the data for the relative standard deviations (RSD) based on three repeated runs are also shown. In this table, RSD obtained under 300 °C showed significantly higher values because of insufficient thermal energy to promote the THM reaction quantitatively. Moreover, the THM-GC measurements at 400 °C resulted in the relatively lower areas for PUFA components such as the methyl esters of EPA and DHA presumably due to their unwanted degradation during the THM reaction at an elevated temperature. Therefore, the following measurements were carried out at 350 °C as the reaction temperature.

According to the method proposed in our previous papers,[21] the chemical composition of fatty acid components for the *P. lutheri* sample was calculated on the basis of the peak areas for the fatty acid methyl esters observed in the chromatograms using the equation

$$\text{fatty acid composition (mol \%)} = \frac{A_{i:j}/\text{ECN}_{i:j}}{\sum (A_{i:j}/\text{ECN}_{i:j})} \times 100 \quad (1)$$

where $A_{i:j}$ and $\text{ECN}_{i:j}$ are the observed peak area and the ECN value for the methyl ester of $C_{i:j}$ fatty acid containing i carbon(s) and j double bond(s), respectively. Table 3 summarizes the distributions of the main fatty acid components (mole percent) for *P. lutheri* estimated by the THM-GC method along with those obtained by the conventional GC

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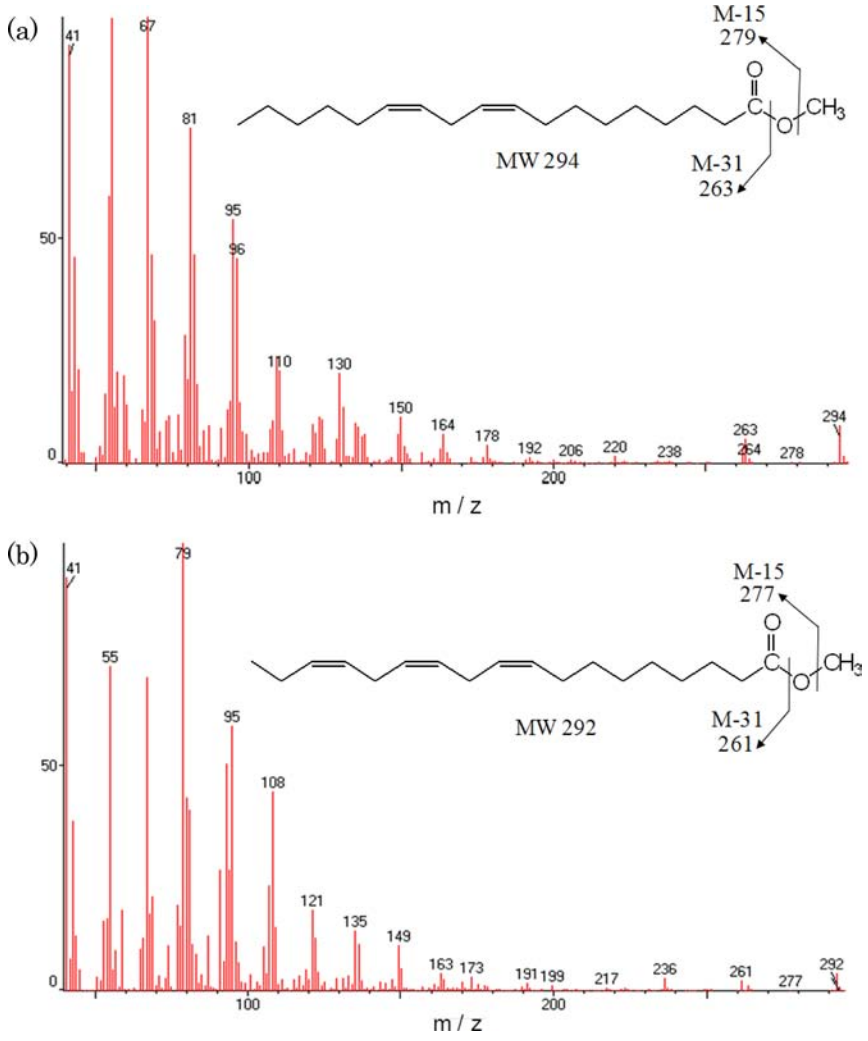

Figure 3. EI mass spectra of (a) peak 6 and (b) peak 8 observed in the chromatogram of Figure 2.

technique. As shown in this table, the fatty acid compositions obtained by THM-GC were in fairly good agreement with those obtained by the conventional GC method for every fatty acid component. Although the RSD values for the fatty acid compositions observed by THM-GC were slightly higher than those by the conventional method, a precision of <8% RSD was sufficient to perform the screening of microalgae based on the fatty acid composition. Moreover, the minimum mass of the analyte for the fatty acid analysis was estimated at about 10 μg, on the basis of the ratio between the peak height of the most minor fatty acid (C20:4, peak 11) and 10 times background noise. These results suggested that the THM-GC technique enabled the analysis of the fatty acid composition for microalgae precisely and accurately using a very minute sample size on the order of 100 μg.

Compositional Analysis of Fatty Acid Components in *C. gloeobotrydiformis*. Finally, the THM-GC technique in the presence of TMSH was applied to the determination of fatty acid compositions of the *C. gloeobotrydiformis* sample. Figure 2 shows a typical chromatogram of the *C. gloeobotrydiformis* sample obtained through THM-GC-FID at 350 °C. The assignment of the peaks on this chromatogram is summarized in Table 1 along with their abbreviations and ECNs. Similarly to the case for the *P. lutheri* sample, the methyl esters of C14 (peak 1) and C16 fatty acids (peaks 2 and 3) were clearly observed as main peaks after the elution of the reagent's related peaks. Futhermore, the two peaks (peaks 6 and 8) were unambiguously detected as the characteristic PUFA components to this alga sample, although PUFAs with longer carbon chains such as EPA and DHA were almost missing. Here, the mass spectra of the two peaks 6 and 8 are shown in Figure 3, panels a and b, respectively. In the spectrum of the peak 6 (panel a), the molecular ion at m/z 294 and the fragment ions at m/z 278 and 263 formed through elimination of methyl and methoxyl groups from the molecule, respectively, were observed. On the basis of this mass spectrum and the information on the retention time of the corresponding authentic fatty acid standard, this peak was assigned to linoleic acid (C18:2) methyl ester (MW = 294). Similarly, peak 8 was identified as α-linolenic acid (α-C18:3) methyl ester (MW = 292) on the basis of the retention time of the standard and the mass spectrum, where the molecular ion and the fragment ion formed through elimination of a methyl group were observed at m/z 292 and 277, respectively.

In addition, the peaks marked with asterisks in the chromatogram of *C. gloeobotrydiformis* might be derived from carbohydrates and proteins owing to their high abundance. Some of these peaks were assigned to hydroxy acetaldehyde [retention time (RT) = 7 min] and 2-furanmethanol (RT = 10 min) by GC-MS, which were reported to be pyrolysis products

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from carbohydrates.[25] Regardless of the appearance of these products, a series of fatty acid methyl esters were clearly observed as sufficiently separated peaks to integrate their areas without any interference.

The fatty acid composition of the *C. gloeobotrydiformis* sample calculated from the peak areas of fatty acid methyl esters is summarized in Table 3 together with their RSD values for three repeated measurements. As shown in this table, this alga contains a large amount of α-linolenic acid (α-C18:3) residue (ca. 24 mol %) grouped into the n-3 fatty acid components such as EPA and DHA. This observation suggests that *C. gloeobotrydiformis* might be used as a feed source in aquaculture and/or a functional food to improve human health. Furthermore, the reproducibility for the composition of main fatty acid components was around 5% based on the RSD values, indicating that THM-GC can be used as a precise and practical method for the compositional analysis of fatty acid components contained in microalgae.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81 568 51 6324. Fax: +81 568 51 6324. E-mail: yishida@isc.chubu.ac.jp.

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Notes

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