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GC-MS-Based Analysis of Methanol: Chloroform-extracted Fatty Acids from Plant Tissues

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Abstract

Fatty acids (FAs) are carboxylic acids with long aliphatic chains that may be straight, branched and saturated or unsaturated. Most of the naturally occurring plant FAs contains an even number of carbon (C4-C24). FAs are used in food and pharmacological industries due to their nutritional importance. In addition, FAs are considered as a promising alternative for the production of biodiesel from terrestrial plant biomass. To establish commercial applications, more reliable analytical methods are needed for the identification, quantification, and composition determination of FAs. Here, we describe a relatively rapid and sensitive method for the extraction, identification, and quantification of FAs from a small quantity of plant tissue. The method includes steps of lipid extraction, conversion of lipid to fatty acid methyl esters (FAMEs) by transmethylation, identification and quantification of FAMEs using gas chromatography-mass spectrometry (GC-MS). In this protocol, an internal standard is added prior to GC-MS analysis. The amount of each FA is calculated from its peak area relative to the peak area of the internal standard.

Keyword: Biodiesel, Fatty acids, GC-MS, Lipids, Nutraceutical, Plant tissue

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Background

Synthesis of fatty acids is important for the storage of metabolic energy. The increasing population and energy cost have emphasized the need to produce sustainable renewable fuels. The source of second-generation biofuels is non-food oilseed crops or lignocellulosic biomass mainly comprising of wastes of crop plants like perennial grasses including switchgrass, husks, straw and forest residue (Hadar, 2013). In this context, plants can serve as an excellent system to study fatty acid for nutraceutical and biodiesel aspects. Further, in biodiesel production, clean burn properties of the fuel are influenced by structural features of FAs including chain length and the degree of unsaturation (Knothe, 2005). Lignocellulosic biomass is a greener alternative to produce these products directly from cost-effective resources. FA profiling using GC-MS permits the normalization, annotation and quantification of a relatively wide variety of fatty acids in a single plant extract. The efficiency of lipid extraction depends on the polarity of the solvent. Polar lipids (such as glycolipids or phospholipids) are more soluble in polar solvents (such as alcohols) and non-polar lipids (such as triacylglycerols) are more soluble in non-polar solvents (such as chloroform). Thus, the total lipid extraction depends on the nature of the organic solvent. Bligh and Dyer (1959) established a method for total lipid extraction using the mixture of chloroform and methanol as a solvent. The total lipids were converted to fatty acid methyl ester by transmethylation (Carreau and Dubacq, 1978). In one study, it was observed that the solvent system of chloroform/methanol is very effective for lipid extraction (Sheng *et al.*, 2011). Moreover, lipid recovery in terms of total lipid content, lipid class and FA composition of different microalgal extracts are also affected by different types of pretreatment and cell disruption techniques and solvents (Ryckebosch *et al.*, 2012). The chloroform/methanol mixture was also found to be useful for the extraction of lipids from microalgae (Ryckebosch *et al.*, 2012). Similarly, there are several other reports suggest that chloroform/methanol/phosphate buffer-based solvent system has a higher efficiency for the extraction of lipids from different microalgae and plants (Kumari *et al.*, 2013; Mishra *et al.*, 2015; Pandey *et al.*, 2015; Patel *et al.*, 2016). Chloroform/methanol mixture exhibits strong dissolving power for the entire range of polarity found in lipids. This mixture is also able to break up membranes and denature the proteins (Schreiner, 2006). The buffer helps to overcome the ionic adsorption effects of salt that may hinder lipid extraction in plant tissue. In most of the studies, methanolic NaOH and methanolic HCl were found to be appropriate derivatizing agents for the profiling of FAs in plants.

Based on this information, here we provide a detailed protocol for extraction of lipids, identification, and quantification of fatty acid methyl esters. We also provide a detailed formula to estimate the total saturated fatty acids (SFA), unsaturated fatty acids (MUFAs and PUFAs), unsaturation index (Poerschmann *et al.*, 2004), degree of unsaturation (Ramos *et al.*, 2009), atherogenic and thrombogenic indices (Simat *et al.*, 2015). Analyses of FAs are done by GC-MS. This protocol provides a relatively rapid and reproducible method. Moreover, this method can be used to profile fatty acids from different types of plant tissues. The produced information can be useful in several contexts of nutraceutical, pharmacological and industrial purposes.

Materials and Reagents

1. 5 ml, 2 ml and 1 ml glass pipettes (Borosil)
2. Filter paper 40 (GE Healthcare, Whatman, catalog number: 1440-110)
3. 50 ml graduated centrifuge tube, PP (Tarsons, catalog number: 546041)
4. 2 ml GC vials and caps (Agilent Technologies, catalog number: 5190-2240) with 250 µl glass inserts (Agilent Technologies, catalog number: 5181-1270)
5. Pyrex culture tube, screw cap with PTFE liner (Corning, catalog number: 9826-13)
6. Plant tissue (Leaf, Stem, Root, and Fruit)
7. Liquid nitrogen
8. Nitrogen gas (> 99% Purity)
9. HPLC-grade methanol (Merck, catalog number: 1060020500)
10. HPLC-grade chloroform (Merck, catalog number: 1024470500)
11. HPLC-grade water (Avantor Performance Materials, J.T. Baker, catalog number: 1.00577.2500)

12. HPLC-grade n-Hexane (Sigma-Aldrich, catalog number: 34859)
13. Potassium phosphate monobasic, ACS reagent, ≥ 99.0% (Sigma-Aldrich, catalog number: P0662)
14. Potassium phosphate dibasic, ACS reagent, ≥ 98% (Sigma-Aldrich, catalog number: P3786)
15. Sodium hydroxide (Sigma-Aldrich, catalog number: S8045)
16. F.A.M.E. Mix, C4-C24 (Sigma-Aldrich, catalog number: 18919-1AMP)
17. Nonadecanoic acid (Sigma-Aldrich, catalog number: N5252)
18. Solvent extraction solution A (see Recipe 1)
19. Solvent extraction solution B (see Recipe 2)
20. Methanolic NaOH (see Recipe 3)
21. Methanolic HCl (see Recipe 4)
22. Internal standard (see Recipe 5)

Equipment

1. 50 ml Flask (Borosil)
2. Funnel (Borosil)
3. Table-top centrifuge (Sigma Laborzentrifugen, model: 3-30KS)
4. Sample concentrator (Hangzhou Allsheng Instruments, catalog number: MD200-2)
5. VacSeal liquid nitrogen dewar (Jencons-PLS, catalog number: 238-112)
6. Table-top spinix-vortex (Tarsons, catalog number: 3002)
7. Rotospin-Rotary mixer (Tarsons, catalog number: 3092)
8. Shaking water bath (JULABO, catalog number: SW23)
9. -20 °C New Brunswick premium freezers (Eppendorf)
10. -80 °C New Brunswick premium freezers (Eppendorf)
11. GC-MS (Shimadzu, model: GCMS-QP2010) coupled with mass spectrometer and equipped with an auto-sampler (AOC-5000) and flame ionization detection (FID)
12. Balance (Sartorius, model: BSA2 24S-CW)
13. The RTx-5MS capillary column (60 meters, 0.25 mm ID, and 0.5 µm df) (Rastek, catalog number: 13455)

Software

1. GC-MS solution Version 2.70, Post-run analysis
2. Excel software (Microsoft office 2010)

Procedure

A. Extraction of lipid from plant tissue (Figure 1)

1. Collect the plant tissue (Leaf/Stem/Root/Fruit) and quickly freeze the sample in liquid N_2. To perform absolute quantification of fatty acids, determine the exact fresh weight (FW) of plant material quickly and accurately before freezing the sample. Store the sample at -80 °C in foil until further use.
   Critical Step: Sufficient plant material is necessary for the yield of satisfactory GC-MS signals. Usually, 100.00 to 500.00 mg tissue is enough for the analysis of fatty acid content. In this protocol, ~200 mg tissue is collected as one sample.
   Pause Point: Frozen samples can be stored at -80 °C.
2. Homogenize ~200.00 mg of plant tissue in liquid N_2 using mortar pestle and transfer the powder to a 50 ml graduated centrifuge tube.

3. Add 5 ml of pre-cooled (4 °C) solvent extraction solution A (see Recipe 1) and spinix-vortex vigorously for 3 min.
4. Centrifuge the sample for 10 min at 10,000 × *g* at 4 °C and collect the supernatant in a 50 ml flask A.
5. Add 2.5 ml of pre-cooled solvent extraction solution B (see Recipe 2) to the pellet and vortex vigorously.
6. Centrifuge sample for 10 min at 10,000 × *g* at 4 °C and transfer the supernatant in 50 ml flask A (Repeat the Steps A5-A6).
7. Filter the pooled supernatant by gravity filtration method through Whatman filter paper 40 using a funnel and wash by mixing with an equal volume of potassium phosphate buffer (50 mM; pH-7.5).
8. Centrifuge the mixture for 10 min at 10,000 × *g* at 4 °C and collect the lower organic phase into a 50 ml graduated centrifuge tube. Collect the lower phase with a glass pipette.
9. Dry total lipids under N_2 using a sample concentrator and determine total lipid contents gravimetrically.
   Pause Point: Total dried lipid can be stored at -20 °C prior to further analysis.

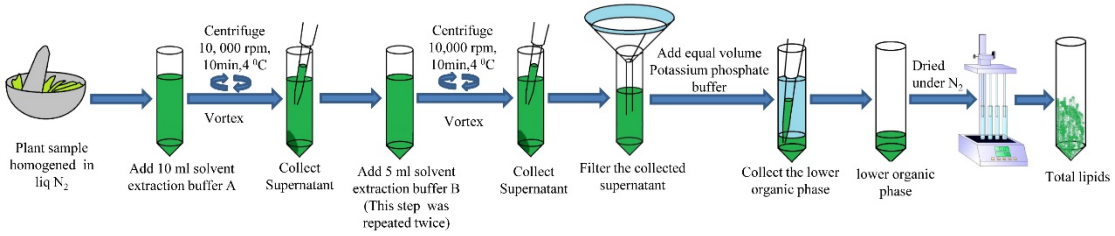

Figure 1. Workflow of extraction of total lipid from plant tissue.

B. Conversion of lipid to fatty acid methyl esters (FAMEs) by transmethylation (Figure 2)

1. Dissolve ~1-10 mg of total lipid in 1 ml of methanolic NaOH (see Recipe 3) and transfer in Pyrex culture tube.
2. Heat for 15 min at 55 °C in a water bath.
3. Add 2 ml of methanolic HCl (see Recipe 4) and vortex for 20 sec.
4. Again heat for 15 min at 55 °C in the water bath.
5. Add 10 µl of 1 mg ml^{-1} nonadecanoic acid (C19:0) into the lipid samples as an internal standard.
6. FAMEs extract through Rotospin-Rotary mixer (Tarson) by adding 1 ml of water (Milli Q) and subsequently add 2 ml of 100 % hexane.
7. Transfer upper phase (~2 ml) into a fresh Pyrex culture tube.
8. Extract FAMEs two more times each with 2 ml of 100% hexane and pool in the same Pyrex culture tube (Step B7).
9. Dry the pooled FAMEs under N_2 and finally dissolved in 100 µl of 100% hexane.
10. Transfer the FAMEs thus eluted into glass inserts, to 2 ml clear GC vials.
    Pause Point: Samples can be stored in capped vials at -20 °C for a month.

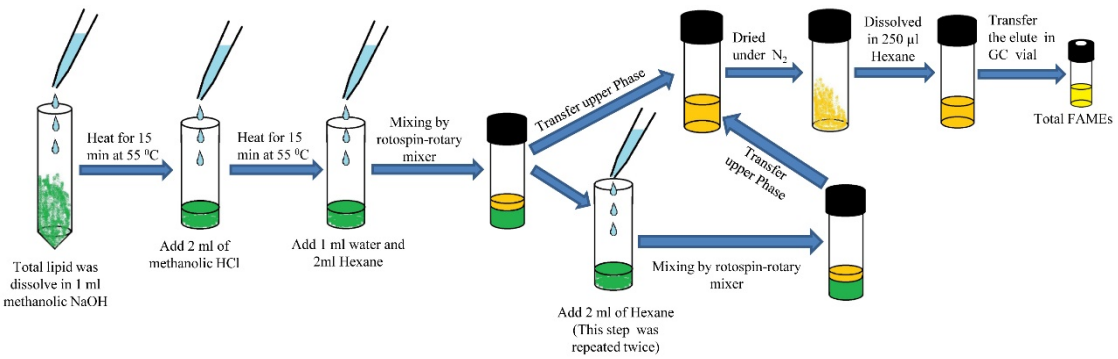

Figure 2. Workflow of conversion of lipid to fatty acid methyl esters (FAMEs) by transmethylation.

C. GC-MS analysis (Figure 3)

1. Carry out GC-MS analysis of FAMEs samples by gas chromatography coupled with a mass spectrometer (GC-MS QP-2010, Shimadzu, Japan) equipped with an auto-sampler (AOC-5000) using flame ionization detection (FID) and RTx-5MS capillary column (Table 1).
2. Use RTx-5MS capillary column (60 meters, 0.25 mm ID, and 0.5 µm df) from Rastek, USA with 1 ml min^{-1} flow rate of helium (99.9 % purity) as a carrier gas and a pre-column pressure of 112.9 kPa. Set the initial column temperature regime to 40 °C for 3 min, followed by a 5 °C min^{-1} increment up to 230 °C followed by 40 min at 230 °C. Following this, set the injection volume, time and temperature of 1 µl, 67 min and 230 °C respectively. Operate the mass spectrometer in electron compact mode with electron energy of 70 eV and keep the temperature of the ion sources and quadrupole at 200 °C.

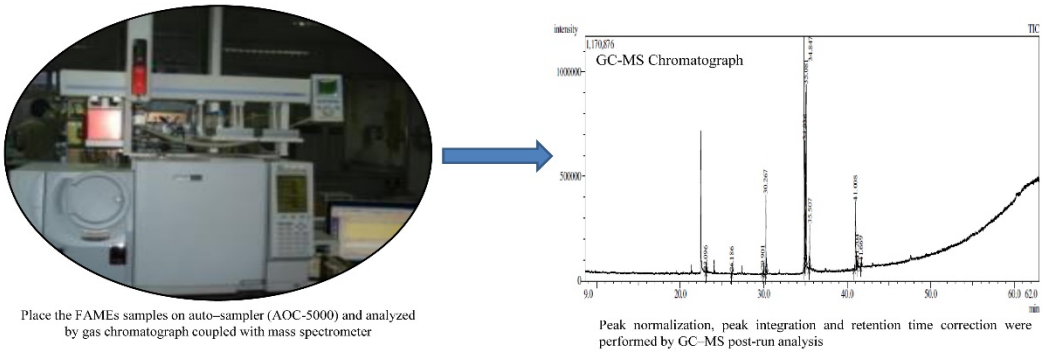

Figure 3. Workflow of GC-MS running and Post-run data analysis.

Table 1. GC-MS operating conditions for determination of fatty acid compositions.

Column	Type	RTx-5MS capillary column
	Length (m)	60
	ID (mm)	0.25
	Df (µm)	0.5
Carrier gas	Gas type	Helium (99.9% purity)
	Flow rate	1 ml min^{-1}
	Pressure	112.9 kPa
Inlet	Temperature	230 °C
	Injection volume	1 µl
	Injection mode	Split
	Total Flow	24.0 ml min^{-1}
	Column Flow	1.00 ml min^{-1}
	Purge Flow	3.0 ml min^{-1}
	Split Ratio	20.0
Oven Temp. Program	Isothermal heating	3 min at 40 °C
	Temperature gradient	5 °C min^{-1} increments up to 230 °C
	Final heating	3 min at 300 °C
Mass spectra	Scanning method	Total-ion-count (TIC)
	Scanning range	Start m/z: 45.00
		End m/z: 500.00
	Run time	Start Time: 6.00 min
		End Time: 63.00 min
	Acquisition mode	Q3 Scan
	Event time	0.300 sec
Metabolites annotated		Fatty acid profiling

Data analysis

1. Perform Peak finding, peak integration and retention time correction with the post-run analysis (GC-MS QP-2010) Shimadzu, Japan.
2. Compare the corresponding FAMEs and retention time indices with standards (FAME Mix C4-C24, Supelco, USA and 7-hexadecenoic acid methyl ester, Cayman Chemicals, USA) by GC-MS post-run analysis.
3. Next, normalize the integrated relative peaks against the FAME mix standard signal.
4. Fatty acids were quantified by area normalization of the relative peak area of each fatty acid using Microsoft Office Excel 2010. Figure 4 shows representative differential peak areas of total ion chromatograms of standard (FAME mix) and plant leaf.

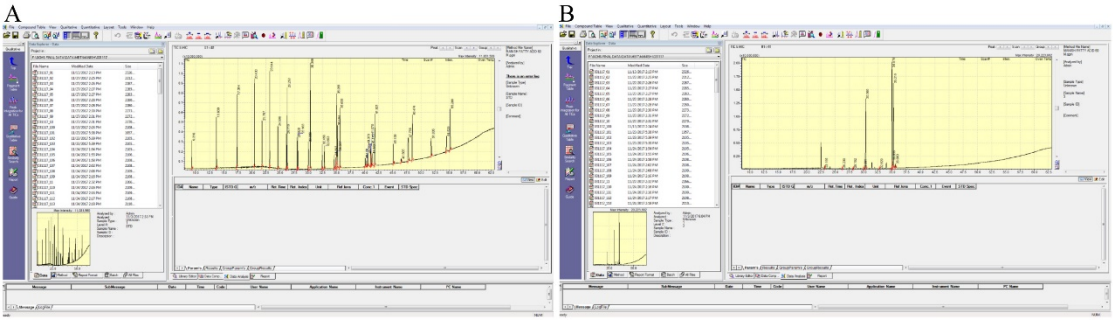

Figure 4. Representative total-ion-count (TIC) chromatograms.
A. Standard; B. Leaf.

5. Total saturated fatty acids (SFA) and unsaturated fatty acids (MUFA and PUFA) contents are estimated by summation of percent quantity of corresponding fatty acids (Patel *et al*., 2016). Unsaturation index (Poerschmann *et al*., 2004), degree of unsaturation (Ramos *et al*., 2009), atherogenic and thrombogenic indices (Simat *et al.*, 2015) were calculated using the following equations:

$$\text{Monounsaturated fatty acids (MUFAs)} = (Summestion\ of\ single\ dobule\ bond\ in\ long\ aliphatic\ chains)$$

$$\text{Polyunsaturated fatty acids (PUFAs)} = (Summestion\ of \geq \text{two}\ dobule\ bond\ in\ long\ aliphatic\ chains)$$

$$Degree\ of\ unsaturation\ (DU) = (MUFA\ w\%) + 2 \times (PUFA\ w\%)$$

$$Unsaturation\ index\ (UI) = \Sigma(FA\ w\% \times number\ of\ double\ bonds)$$

$$Atherogenic\ index\ (AI) = \frac{C12{:}0+4\times C14{:}0+C16{:}0}{\Sigma n3PUFA+\Sigma n6PUFA+\Sigma MUFA}$$

$$Thrombogenic\ index\ (TI) = \frac{C14{:}0+C16{:}0+C18{:}0}{(0.5\times MUFA)+(0.5\times n6PUFA)+(3\times n3PUFA)+(n3/n6)}$$

Recipes

1. Solvent extraction solution A
Chloroform:methanol, 1:2 (v/v)

2. Solvent extraction solution B
Chloroform:methanol, 1:1 (v/v)

3. Methanolic NaOH
1% NaOH in MeOH
The solution can be stored at 4 °C up to one month

4. **Methanolic HCl**
   5% Conc. HCl in MeOH (Conc. HCl-36%; Molarity: 11.65)
   The solution can be stored at 4 °C up to one month

5. **Internal standard**
   1 mg·ml^{-1} nonadecanoic acid (C19:0)

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Competing interests

The authors declare no conflicts of interest or competing interests.

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