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1. Introduction

- 1.1 This Laboratory Analytical Procedure (LAP) covers the determination of total lipids expressed as fatty acid methyl esters (FAME). Results are reported as the percent FAME content based on the dry weight of the sample. The procedure is based on a whole biomass transesterification procedure of lipids to FAME, which eliminates the need for extraction and therefore is able to access all fatty acids in the biomass and represent an accurate reflection of the biofuels potential.
- 1.2 Lipids are present in many forms and play various roles within an algal cell, from cell membrane phospholipids to energy stored as triacylglycerols. The ability to identify and accurately quantify the fatty acid content of these lipids as well as free fatty acids is essential to evaluating fuel potential and establishing a comprehensive compositional analysis of algae.
- 1.3 Acid-catalyzed transesterification is one way to measure total fatty acid content. The procedure first solubilizes the lipids and then frees the fatty acids by transferring a methyl group from methanol onto the -acyl chains of the lipids. During this reaction, the ester bond between the fatty acids and the glycerol backbone (e.g., triacylglycerol) will be replaced by an ester bond between the fatty acid and a methyl group, producing methyl esters of the fatty acids (FAME) and free glycerol.
- 1.4 The FAME are then extracted from the polar methanol phase with hexane, leaving the polar compounds, e.g., glycerol or phosphatidic acid, behind.
- 1.5 A surrogate/recovery standard consisting of an odd chain fatty acid (e.g., C13:0ME) that does not naturally occur in algae is transesterified with the sample and used to quantify the total FAME content on a gas chromatograph.
- 1.6 A full description of the method, precision and bias and optimization with respect to catalyst can be found in reference [1].

2. Scope

- 2.1 This procedure is developed and optimized specifically for microalgal biomass, residual algal biomass after extraction, and algal extractives.

3. Terminology

- 3.1 *Oven Dry Weight (ODW)* – The weight of the biomass corrected for the percent moisture determined by drying the biomass overnight in a 60°C atmospheric pressure or 40°C vacuum convection oven according to *LAP Determination of Total Solids and Ash in Algal Biomass* [2].
- 3.2 *Lipids* – Lipids are defined as “fatty acids and their derivatives” [3]. For our purposes, the lipid determination as total FAME is an accurate reflection and can be used for the quantification of total lipid fraction.

- 3.3 *Transesterification* – The process of exchanging the organic group of an ester with the organic group of an alcohol.
- 3.4 *Extractives/Extracted Lipids* – The portion of algal biomass that is soluble in any type of solvent used to extract the sample. Extractives may include sugars, proteins, lipids, and glycoproteins, as well as an array of other compounds.
- 3.5 *Extractives-free Biomass/Residual Biomass* – Algal biomass that has been exhaustively extracted with any solvent (e.g., chloroform:methanol) that solubilizes and removes some portion of the algal lipids.
- 3.6 *Fatty Acid Methyl Esters (FAME)* – The result of the transesterification of lipids, where a methyl group from methanol forms an ester bond with a fatty acid.
- 3.7 *Surrogate/Recovery Standard* – The inclusion of a known amount of surrogate/recovery standard allows for the correction of the FAME quantification for FAME extraction variability and evaporation of the extraction solvent during the FAME procedure.
- 3.8 *Internal Standard* – The inclusion of a known amount of internal standard in samples and standards allows for the correction of the FAME quantification for analytical instrument variability and solvent evaporation during the FAME analysis.

4. Significance and Use

- 4.1 This procedure is used in conjunction with other procedures to determine the amounts of biofuel-relevant fatty acids present in algal biomass.
- 4.2 This procedure may be used in conjunction with other compositional analysis procedures to determine the summative mass closure of algal biomass.

5. Interferences

- 5.1 Samples that are moldy or wet or that have been exposed to an oxygen-rich environment may be compromised, resulting in erroneous lipid values.
- 5.2 Samples with greater than 20% moisture may undergo hydrolysis during the reaction, resulting in erroneous lipid values.

6. Apparatus

- 6.1 Analytical balance, accurate to 1 mg or 0.1 mg (e.g., Mettler Toledo XP205)
- 6.2 Vacuum oven set to 40°C ± 1°C or drying oven set to 60°C ± 1°C
- 6.3 Digital dry block, capable of maintaining 85°C
- 6.4 Gas chromatograph (GC) equipped with a variable split-flow injector or equivalent device: Agilent 7890A GC system equipped with a split/splitless inlet or equivalent

- 6.5 Automated sampler compatible with the chosen GC system: Agilent 7693A Automatic Liquid Sampler or equivalent
- 6.6 Detector compatible with the chosen GC system and type of analysis: Agilent 7890A equipped with a flame ionization detector (FID) or equivalent
- 6.7 Capillary column with polyethylene glycol stationary phase: Agilent J&W GC Column DB-Wax length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm or equivalent

7. Reagents and Materials Needed

7.1 Reagents

- 7.1.1 Chloroform, high-performance liquid chromatograph (HPLC) grade (Acros Organics 404635000)
- 7.1.2 Methanol, HPLC grade (Fisher Chemical A4525K-1)
- 7.1.3 Hexane, HPLC grade (Sigma Aldrich 34859-1L)
- 7.1.4 Hydrochloric acid (HCl), concentrated (36.5%-38%) (~12M) (J.T. Baker 9535-03)
- 7.1.5 Chloroform:methanol solution (2:1, v/v)
- 7.1.6 HCl:methanol solution [0.6M (2.1% v/v) HCl in methanol], prepared by adding 5 mL of concentrated HCl (12M) to 95 mL of methanol
- 7.1.7 Tridecanoic acid methyl ester (C13:0ME) standard (Sigma Aldrich #91558-5ML)
- 7.1.8 Pentadecane (Sigma Aldrich #76509-5ML)
- 7.1.9 F.A.M.E. Mix, C4:0-C24:0, (37 compound calibration mix, Sigma Aldrich #18919-1AMP), alternative acceptable calibration mixes are C8-24 mix (Sigma Aldrich # 18918-1AMP) and GLC-80 (Sigma Aldrich #1898)

7.2 Materials

- 7.2.1 Volumetric flask (class A), 10 mL
- 7.2.2 Gas-tight syringes, covering ranges from 5–1000 μL
- 7.2.3 Adjustable pipet, covering ranges from 100–300 μL
- 7.2.4 Vials, clear, crimp tops, 1.5 mL fill volume (Agilent #5182-0543 or equivalent)

- 7.2.5 Vials with inserts, clear, crimp tops, 300 μ L fill volume (Agilent #9301-1388 or 5188-6572(amber))
- 7.2.6 Crimp caps, polytetrafluoroethylene (PTFE)/silicone/PTFE septa (Agilent #5181-1211)

8. ES&H Considerations and Hazards

- 8.1 Methanol is a slight health hazard and is moderately flammable.
- 8.2 Hydrochloric acid is toxic and corrosive.
- 8.3 Hexane is a significant health hazard and is extremely flammable.
- 8.4 Chloroform is a trihalomethane and is considered a possible human carcinogen.
- 8.5 Follow all applicable chemical handling procedures.

9. Sampling, Test Specimens and Test Units

- 9.1 Care must be taken to ensure a representative and ground/homogenized (particle size <1 mm) sample is taken for analysis.
- 9.2 Limit sample contact with oxygen, heat, and moisture to lessen the possibility of lipid degradation.

10. Procedure

- 10.1 Preparation of the samples for transesterification
 - 10.1.1 Label 1.5-mL GC vials using a permanent marker for each of the samples to be analyzed. Each sample should be analyzed in triplicate at a minimum (unless the amount of sample prohibits this). In addition, a quality control (QC) biomass or oil standard should be included in triplicate with each batch of samples. Ensure sufficient quantities of the standard are available for QC charting over multiple analyses.
 - 10.1.2 Record the weights of all labeled vials to the nearest 0.1 mg. Written labels should be dry before the weight is recorded.
 - 10.1.3 *Whole or residual biomass:* Weigh between 5 to 10 mg of sample into the labeled and pre-weighed GC vials. Record the weight to the nearest 0.1 mg. Dry samples in vials overnight in a 40°C oven under vacuum. After drying, take a final weight to calculate the moisture-free sample amount.
 - 10.1.4 *Neat lipids or extracts:* Weigh or aliquot a known volume (estimated lipid weight of 5 to 10 mg) into a labeled and pre-weighed GC vial. If the sample drips on the outside of the vial during transfer, label and weigh a new vial.

Dry lipids/extracts in vials overnight or in a 40°C oven under vacuum until the samples are completely dry. After the sample is dry, take a final weight to calculate the moisture-free sample amount.

- 10.1.5 If no vacuum oven is available, dry a representative aliquot of biomass for moisture determination on the same day and correct the biomass weights prior to calculating the FAME concentration.

NOTE: In steps 10.1.3 and 10.1.4, allow samples to cool in a desiccator under vacuum before recording a final weight.

10.2 Preparation of the surrogate/recovery standard

- 10.2.1 To make up a 10 mg/mL solution, weigh out approximately 100 mg of the methyl tridecanoate (C13:0ME) into a 10-mL, class A, volumetric flask. Record the weight of the C13:0ME to the nearest 0.1 mg.
- 10.2.2 Bring to volume with HPLC grade hexane and mix well.
- 10.2.3 Transfer the hexane-C13:0ME mixture into 1.5-mL GC vials and seal immediately with PTFE/silicone/PTFE crimp caps. Take extra care while transferring to limit evaporation as it is very important to maintain the recorded surrogate concentration.
- 10.2.4 Record the date of preparation, concentration, and any other pertinent information on the vials and place upright in a laboratory freezer (-20°C) for storage.

10.3 Transesterification of the samples

- 10.3.1 Preheat a digital dry block, or equivalent, to 85°C.
- 10.3.2 While the block is preheating, add the following to each of the sample vials:
- 20 µL of the pre-prepared C13:0ME surrogate (10 mg/mL) using a gas-tight syringe. This amount may need to be adjusted to more accurately reflect the estimated fatty acid content of the sample
 - 200 µL of chloroform:methanol (2:1, v/v) using a gas-tight syringe
 - 300 µL of 0.6M HCl:methanol using a pipet with a plastic or glass pipet tip
- 10.3.3 Seal all vials with the PTFE/silicone/PTFE crimp caps and vortex well to mix the contents.
- 10.3.4 Place the sealed vials into the preheated block at 85°C for one hour. Once the reagents have been added to the samples, the samples must be heated. Do not allow samples to sit around as this may affect the reaction.

- 10.3.5 After 1 hour on the digital dry block, remove the vials and allow to cool for at least 15 minutes, but no longer than an hour, at room temperature. If the rest of the procedure cannot be completed on the same day, cooled samples may be stored overnight in a freezer (-20°C). Always store vials upright.

10.4 Isolation and preparation of FAME for GC analysis

- 10.4.1 After cooling, add 1.0 mL HPLC grade hexane to each of the vials using a gas-tight syringe. This can be done without removing the vial caps by using a small hollow-core needle to penetrate the cap while adding the hexane with the syringe through a second hole in the cap. Be sure the opening in the hollow-core needle faces away from the direction of the added hexane to avoid hexane being expelled back up through the needle.
- 10.4.2 Vortex well to mix the vial contents and let them stand undisturbed at room temperature for at least 1 hour, but no more than 4 hours, to allow the phases to separate. If the rest of the procedure cannot be completed on the same day, samples must be re-capped and then stored overnight in a freezer (-20°C). Always store vials upright.
- 10.4.3 Samples may need to be diluted to fall within the calibration curve. The following is a general dilution outline. Add the following to labeled 1.5-mL GC vials, using gas-tight syringes:
- *Whole Biomass* – estimated low lipid content (e.g., early harvest): 400 µL HPLC grade hexane and 100 µL of the upper phase of the sample.
 - *Whole Biomass* – estimated high lipid content (e.g., late harvest): 450 µL HPLC grade hexane and 50 µL of the upper phase of the sample.
 - *Residual Biomass (Extractives-Free)* – Use the upper phase of the sample undiluted. At least 300 µL is needed in the new vial.
 - *Extracts/Isolated Lipids* – Add the following using gas tight syringes: 450 µL HPLC grade hexane and 50 µL of the upper phase of sample.
 - The upper phase of the sample can be withdrawn without removing the caps. Use caution not to disturb the lower phase when drawing the upper phase of the sample. Rinse the syringe thoroughly with hexane between samples. Work in groups of 10 samples or less to minimize evaporation of hexane and cap immediately after adding the sample.
- 10.4.4 Arrange and label new 300-µL GC vials with inserts for each sample.
- 10.4.5 Seal the 1.5-mL vials containing the dilutions with PTFE/rubber crimp caps.

10.5 Preparation and addition of the internal standard

- 10.5.1 Weigh out approximately 10 mg of pentadecane standard into a labeled 1.5-mL vial. Record the weight to the nearest 0.1 mg.
- 10.5.2 Add 1 mL of HPLC grade hexane to the vial and seal with a PTFE/silicone/PTFE crimp cap.
- 10.5.3 Dilute this standard 1:10 before adding to samples and standards.
- 10.5.4 To each labeled 300- μ L GC vial prepared in step 10.4.4 add:
- 5 μ L of the 1:10 diluted pentadecane. Only add pentadecane to 10 vials at a time.
 - 200 μ L of diluted sample from the vials prepared in step 10.4.5 or from top hexane phase for undiluted samples.
- 10.5.5 Cap the vials immediately with PTFE/rubber crimp caps.
- 10.5.6 Label a set of 300- μ L insert vials for the standards (prepared as per section 10.6). To each standard vial add:
- 5 μ L of the ***SAME*** 1:10 diluted pentadecane used for the samples in step 10.5.4.
 - 200 μ L of standard prepared in step 10.6.1 (refer to Table 1).
- 10.5.7 Cap the standard vials immediately with PTFE/silicone/PTFE crimp caps.
- 10.5.8 Once all the samples and standards have been prepared with the internal standard, vortex all vials to mix the contents.

10.6 Preparation of the FAME standards

- 10.6.1 Prepare a series of calibration standards containing the compounds that are to be quantified, i.e., C4:0-C24:0 (37 compound calibration mix, Sigma Aldrich #18919-1AMP). Refer to Table 1 for suggested calibration standards. The range will depend on the compound amount and purity in the original neat standard mix, but it will typically range from 1–6 μ g/mL to 100–200 μ g/mL depending on the individual FAME concentration.
- 10.6.2 If using the C4:0-C24:0 neat mix (18919-1AMP), prepare a 10-mg/mL working solution using the following steps:
- Using HPLC grade hexane, transfer the neat mix as quantitatively as possible to a clean 10-mL, class A, volumetric flask.
 - Bring to volume with HPLC grade hexane and mix well.

- Transfer the mixture to labeled 1.5-mL GC vials, working quickly and carefully to avoid evaporation. Seal vials immediately with PTFE/silicone/PTFE crimp caps.

Table 1: Standards Prepared using the C4:0-C24:0 Working Solution from Step 10.6.2.

Standard Level	C4-C24 Working Solution	HPLC Grade Hexane
	<i>ul</i>	<i>ul</i>
5	500	500
4	250	750
3	100	900
2	30	970
1	10	990
CVS	90	910

NOTE: Once prepared as calibration concentrations, standards can be stored up to 6 months.

NOTE: Actual standard concentrations will depend on the batch of calibration mix and can be calculated based on the certificate of analysis (see calculation spreadsheet Appendix A).

10.6.3 In addition to the standards, a calibration verification standard (CVS) containing all the compounds in the standards should also be prepared at a level that is not included in the calibration curve (see Table 1), preferably from an independent batch of FAME standards.

10.6.4 Store all working solutions and standards in 1.5-mL vials sealed with PTFE/silicone/PTFE crimp caps in a freezer (-20°C). Always store vials upright.

10.7 Setting up the GC for FAME analysis

10.7.1 Suggested GC analysis follows a temperature and flow program as detailed below for DB-WAX 30 m x 0.25 mm inner diameter x 0.25 µm film thickness:

- 1-µL injection at 10:1 split ratio, inlet temperature of 250°C
- Constant flow: 1 mL/min helium
- Oven temperature: 100°C for 1 min, 25°C/min up to 200°C and hold for 1 min, 5°C/min up to 250°C and hold for 7 min
- FID: 280°C, 450 mL/min zero air, 40 mL/min H₂, 30 mL/min helium

- 10.7.2 Quantification by Chemstation (Agilent) or respective GC software: use calibration response factors (corrected for internal standard recovery – in this case, pentadecane) for each individual fatty acid and only work up data when calibration quality of correlation is 0.999 or better. Response factors can be significantly different between different fatty acids; using only respective response factors for quantification and for novel fatty acids that are not present in the calibration mix, determine respective response factors by transferring existing response factors from structurally similar compounds. Suggestions are given in Table 2:

Table 2: Response Factors to be used for Compounds Not Present in Calibration Standard.

Compound	Compound in Calibration Curve Response Factor to be used
All C16:1	C16:1n7
C16:2	C18:2n6
C16:3	C18:3n3
C18:1n7	C18:1n9
C18:4n3	C18:3n3
C20:4n3	C20:4n6

11. Calculations

- 11.1 Export the FAME concentrations (as µg/mL for each individual FAME [C4:0 – C24:0]) from GC software and normalize for the quantity of the recovery standard C13:ME. The outcome is the sum or total FAME content normalized for the recovery of the surrogate standard added at the start of the reaction (step 10.3.2):

$$Total\ FAME_{C13\ normalized} = \sum_{C4-C24} \frac{Amount_{Measured\ FAME\ C_i}}{Amount_{Measured\ FAME_{C13}}} \times Amount_{Added\ FAME_{C13}}$$

- 11.2 Calculate the total FAME as a percent of the dry weight of the sample. For the FAME analysis, the dry weight refers to the weight after drying the sample overnight at 40°C in a vacuum oven:

$$\% Total\ FAME = \frac{Total\ FAME_{C13\ normalized}}{ODW_{sample}} \times 100$$

- 11.3 To report or calculate the root mean square deviation (RMS) or the standard deviation (STDEV) of the samples, use the following calculation:

$$RMS = x_m = mean = \sqrt{\frac{\sum_{i=1}^n x_i^2}{n}}$$

$$RMSdeviation = \sigma = stdev = \sqrt{\frac{\sum_{i=1}^n (x_i - x_m)^2}{n}}$$

where:

x_m = the root mean square of all x values in the set

n = number of samples in set

x_i = measured value from the set

- 11.4 To report or calculate the relative standard deviation (RSD) of the triplicates, use the following calculation:

$$RSD = \left(\frac{stdev}{X_{mean}} \right) \times 100$$

where:

stdev = RMS deviation from the calculation in step 11.3

X_{mean} = the mean % total FAME of the triplicates

12. Report Format

- 12.1 For replicate analyses of the same sample, report the average, standard deviation, and RSD.
- 12.2 Report the triplicate average percent FAME in the sample on a dry weight basis.

13. Precision and Bias

- 13.1 Precision and bias need to be determined by a round robin experiment using standard method verification biomass. The target RSD should be less than 10%.
- 13.2 Improper sample preparation and/or storage may bias the results low due to the degradation of oxygen- or heat-sensitive lipids.

14. Quality Control

- 14.1 *Reported Results* – Report results with two decimal places. Report the average, standard deviation, and RSD.

- 14.2 *Replicates* – Run all samples in triplicate when sufficient sample is available, keeping the minimum weight of the balance in account.
- 14.3 *RSD Criterion* – Each set of triplicates must reproduce % total FAME at <10% RSD.
- 14.4 *Sample Size* – Approximately 7 to 10 mg of sample or lipid should be weighed out for transesterification.
- 14.5 *Sample Storage* – Before transesterification, all samples containing lipids should be freeze-dried or dried under nitrogen and stored in an air-tight container in a freezer (-20°C or -80°C depending on preservation needs).
- 14.6 *Blank* – HPLC grade hexane – the same batch of hexane used during sample analyses.
- 14.7 *Standard Preparation* – Standards and calibration verification standard should be prepared with HPLC grade hexane.
- 14.8 *Standard Storage* – GC standards should be stored upright in a freezer (-20°C) until needed.
- 14.9 Quality assurance/QC material should be control charted to verify reproducibility.
- 14.10 The analysis is out of control and must be repeated if the QC sample data meet any of the following conditions:
- 14.10.1 Any result falls outside of three standard deviations (SD) of the mean.
- 14.10.2 Four out of five consecutive points outside (+) 1 SD or (-) 1 SD.
- 14.10.3 Two out of three consecutive points outside (+) 2 SD or (-) 2 SD.
- 14.10.4 Eight consecutive points all above or below the mean.
- 14.10.5 Eight consecutive points all increasing or decreasing.
- 14.10.6 Non-random patterns are observed.

15. Appendices

- 15.1 Calibration standards concentration [TBD]
- 15.2 GC method and settings for Agilent 6890N GC-FID [TBD]
- 15.3 List of revisions/updates
- Distribution of May 16, 2013 DRAFT version.
 - July 26, 2013, updated with calibration mixture preparation solutions and moisture correction.
 - December 2, 2013.

16. References

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