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Technical Report
NREL/TP-5100-60958
Revised December 2015

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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 An internal 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 the 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 *Internal Standard:* The inclusion of a known amount of internal standard in samples and standards allows for the correction of the FAME quantification for extraction variability, 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 ± 3°C

6.3 Vacuum desiccator with dry desiccant (if no vacuum oven is available, samples can be stored overnight in a vacuum desiccator prior to analysis, given that a corresponding moisture measurement is available the same day for that material)

6.4 Digital dry block, capable of maintaining 85°C ± 3°C

6.5 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

6.6 Automated sampler compatible with the chosen GC system: Agilent 7693A Automatic Liquid Sampler or equivalent

6.7 Detector compatible with the chosen GC system and type of analysis: Agilent 7890A equipped with a flame ionization detector (FID) or equivalent

6.8 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)—may be prepared ahead of time and stored for up to 2 months

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—may be prepared ahead of time and stored for up to 2 months

7.1.7 Tridecanoic acid methyl ester (C13:0ME) standard (Sigma Aldrich #91558-5ML)

7.1.8 FAME Standard Calibration Mix C8:0–C24:0 (NuChek-Prep **GLC 461C**)

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 µL 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. Storage of samples should ideally be in an inert (nitrogen) atmosphere and at least at -20°C.

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. Either dry samples in vials overnight in a 40°C oven under vacuum or store in a desiccator under vacuum overnight. 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 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 internal 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 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. Store sealed for up to 6 months.

10.3 Transesterification of the samples

10.3.1 Preheat a digital dry block, or equivalent, to 85°C ± 3°C.

10.3.2 While the block is preheating, add the following to each of the sample vials:

- 25 µL of the pre-prepared C13:0ME internal standard (10 mg/mL) using a gas-tight syringe. This amount may be adjusted to more accurately reflect the estimated fatty acid content of the sample. Recap the C13:0ME vial after adding to all samples, it will be needed in section 10.5.
- 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, DO NOT use metal syringes

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 1 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 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 (no longer than 24 hours) in a freezer (-20°C). Always store vials upright.

10.4 Isolation and preparation of FAME for GC analysis

10.4.1 After cooling to room temperature, 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, vials must be re-capped and then stored overnight (no longer than 24 hours to avoid co-extraction of unwanted compounds) in a freezer (-20°C). Always store vials upright.

NOTE: If longer storage is required, transfer the hexane layer (after allowing for complete extraction of at least 1 hour) to a new 1.5-mL GC vial, cap the vial, and store in a freezer (-20°C) for up to a week.

10.4.3 Label a new set of 1.5-mL GC vials for the final hexane phase dilutions. The following is a general dilution outline. Add the following 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 Seal the 1.5-mL vials containing the dilutions with PTFE (or rubber) crimp caps.

10.5 Addition of internal standard to FAME standards

10.5.1 Label a set of 300-µL insert vials for each level of FAME standard (prepared as per Table 1). To each standard vial add:

- 5 µL of the ***SAME*** C13:0ME internal standard (10 mg/mL) used for the samples in step 10.3.2.

- 200 µL of standard prepared in step 10.6.1 (refer to Table 1).

10.5.2 Cap the 300-µL standard vials immediately with PTFE/silicone/PTFE crimp caps. Also recap the standard levels prepared in step 10.6.1.

10.5.3 Invert or vortex the 300-µL standard vials to thoroughly mix the contents.

10.5.4 Recap the FAME standard vials (levels 1-5 and CVS) before returning to the freezer. Also recap the C13:0ME vial used for the samples and the standards and store in the freezer until the data have been worked up and passed QA/QC.

10.6 Preparation of the FAME standards

10.6.1 If using the C8:0–C24:0 neat mix (**GLC 461C**), prepare a 10-mg/mL working solution using the following steps:

- Tare a clean, 10-mL, class A, volumetric flask.

- Score the neck of the vial using a GC column cutter or glass scorer. DO NOT snap the top off yet.

- Warm the 461C neat mix vial with your hands to be sure that all the mix is in the bottom of the vial and no compounds are crystallized in the top of the vial before breaking off the top.

- Using a glass Pasteur pipet, transfer the neat mix as quantitatively as possible to the tared 10-mL, class A, volumetric flask. Keep the Pasteur pipet and neat mix warm with your fingers to prevent the crystallization of saturated longer chain fatty acids while transferring.

- Record the weight to the nearest 0.1 mg.
- Bring to volume with HPLC grade hexane and mix well.
- Transfer approximately 1.2 mL of the mixture to labeled 1.5-mL GC vials using a glass Pasteur pipet, working quickly and carefully to avoid evaporation. Seal vials immediately with PTFE/silicone/PTFE crimp caps.

10.6.2 Prepare a series of calibration standard levels using the working solution prepared in 10.6.1. Use the recommendations in Table 1 to prepare levels 1–5 and a CVS (from an independent working solution):

- Label 1.5-mL GC vials with the standard level and date.
- Fully prepare each standard level before moving on to the next: add the necessary amount of HPLC grade hexane to the labeled 1.5-mL GC vial using a gas-tight syringe, place a PTFE/silicone/PTFE cap on the vial (you do not need to crimp it yet). Add the necessary amount of FAME working solution from step 10.6.1 using a gas-tight syringe and crimp the cap. Then move onto the next standard level.

Table 1. Standards Prepared Using the NuChek 461C Working Solution from Step 10.6.1

Standard Level	461C Standard Working Solution (µL)	Hexane (µL)
5	500	500
4	250	750
3	100	900
2	30	970
1	10	990
CVS	90	910

10.6.3 The working solution from step 10.6.1 and the prepared calibration standard levels may be stored in PTFE/silicone/PTFE capped (not punctured) 1.5-mL GC vials in a freezer (-20°C). Always store vials upright. Standards and working solution may be stored for up to 6 months.

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 (23 min total)
- FID: 280°C, 450 mL/min zero air, 40 mL/min H_2, 30 mL/min helium

10.7.2 Create a calibration table or update the existing calibration table with the amount of each compound in µg/mL based on the certificate of analysis (percent and purity) and the weight recorded in step 10.6.1 for the standard lot used.

10.7.3 Update the C13:0ME internal standard amount in µg/mL in the Chemstation software.

10.7.4 Quantification by Chemstation (Agilent) or respective GC software: use calibration response factors (corrected for internal standard recovery—in this case, C13:0ME—the software will do this for you once the correct value has been entered) 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; use 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/C16:4	C18:3n3
C18:4n3	C18:3n3
C20:4n3	C20:4n6

11. Calculations

11.1 Export the FAME concentrations (as µg/mL for each individual FAME [C8:0 – C24:0]) from GC software and normalize for the quantity of the internal standard C13:0ME (this may have already been done by the software). The outcome is the sum or total FAME content normalized for the recovery of the internal standard added at the start of the reaction (step 10.3.2):

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

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:

$$RMS = x_m = mean = \sqrt{\left(\frac{\sum_{1}^{n} x}{n}\right)^2}$$

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

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 *Control Charts:* 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 are outside (+) 1 SD or (-) 1 SD.

14.10.3 Two out of three consecutive points are outside (+) 2 SD or (-) 2 SD.

14.10.4 Eight consecutive points are all above or below the mean.

14.10.5 Eight consecutive points are 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
- Revision July 26, 2013, updated with calibration mixture preparation solutions and moisture correction
- Revision December 2, 2013, updated for public distribution
- Revision December 29, 2015, updated with C13 internal standard, NuChek GLC 461C FAME mix, and other minor revisions to add details

16. References

[1] Laurens, L.M.L.; Quinn, M.; Van Wychen, S.; Templeton, D.W.; Wolfrum, E.J. "Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification." *Anal. Bioanal. Chem.* (403:1), 2012; pp. 167–178. (Open Access: http://www.springerlink.com/content/g214r02011861712).

[2] Van Wychen, S.; Laurens, L.M.L. *Determination of Total Solids and Ash in Algal Biomass.* NREL/TP-5100-60956. Golden, CO: National Renewable Energy Laboratory. 2015. http://www.nrel.gov/docs/fy14osti/60956.pdf.

[3] Christie, W.W. *Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids,* 3rd ed.; Bridgwater, England: Oily Press, 2005.