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Laboratory Analytical Procedure (LAP)

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1. Introduction

1.1 This Laboratory Analytical Procedure (LAP) covers the determination of total sterols in freeze-dried microalgal biomass. Results are reported as the percent sterol content based on the oven dry weight of the sample. The procedure is based on the National Renewable Energy Laboratory's fatty acid methyl esters LAP for total lipid quantification [1], making it easy to perform two analyses at once if desired, and saving significant time when compared to other sterol methods. Because this method is based on an acid-catalyzed transesterification and hydrolysis, free sterols are produced from their complex composition, and analyzed by gas chromatography. The following procedure does not produce phytol as another terpenoid-derived product, which is typically formed when a base-catalyzed conversion reaction is used.

1.2 Phytosterols in algae are found in various configurations, including free sterols, sterol esters with higher fatty acids, sterol glycosides, and acylsterol glycosides. These compounds are important in cell membrane functions and properties. The method described here focuses specifically on the analysis of free sterols.

1.3 This procedure first solubilizes algal cell contents and then hydrolyzes bound sterols using dilute acid hydrolysis. The sterols are then extracted from the polar phase with hexane, utilizing the addition of a salt solution to improve free sterol extraction kinetics.

1.4 An internal standard consisting of a sterol-like compound not likely to be found in microalgae (e.g., 5α-cholestane) is utilized to correct for sterol extraction efficiency.

2. Scope

2.1 This procedure is developed and optimized specifically for microalgal biomass.

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 *Phytosterols:* Steroid alcohols produced by photosynthetic organisms that are: biosynthetically derived from the terpenoid pathway, can range in concentration in algae between 0.2% and 5% of the dry biomass, and exist as free sterols, sterol esters with higher fatty acids, sterol glycosides, or acylsterol glycosides.

3.3 *N,O-Bis(trimethylsilyl)trifluoroacetamide: Trimethylchlorosilane (BSTFA:TMCS):* A silylation reagent used to derivatize non-volatile compounds to trimethylsilyl (TMS) derivatives for easier separation and better peak shape in gas chromatography.

3.4 *Trimethylsilyl (TMS):* Functional group consisting of three methyl groups and a silicon atom.

3.5 *Internal Standard:* A known amount used in samples and standards, allowing for the correction of the sterol quantification for extraction efficiency, analytical instrument variability, and solvent evaporation/miscibility during the analysis.

4. Significance and Use

4.1 This procedure is used to determine the amounts of phytosterols present in microalgal biomass.

4.2 This procedure may be used in conjunction with other compositional analysis procedures to determine the summative mass closure of microalgal 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 sterol values.

5.2 Samples with greater than 20% moisture may undergo unwanted hydrolysis during the reaction, resulting in erroneous 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 ± 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 ± 3°C

6.5 Centrifuge (e.g., Beckman Allegra X-12), capable of spinning gas chromatograph (GC) vials (vials may be placed inside of other containers, e.g., Falcon tube for centrifugation)

6.6 GC equipped with a variable split-flow injector or equivalent device: Agilent 7890A GC system equipped with a split/splitless inlet or equivalent

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

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

6.9 Optional: mass spectrometer (MS) compatible with the chosen GC system (for sterol identification)

6.10 Capillary column with polysiloxane stationary phase: Agilent J&W GC Column DB-5ms, 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 (Millipore Sigma 34859-1L)

7.1.4 Hydrochloric acid (HCl), concentrated (36.5%–38%) (~12M) (J.T. Baker 9535-03)

7.1.5 Sodium chloride (NaCl), ACS grade (Fisher Chemical S271-500)

7.1.6 Chloroform:methanol solution (2:1, v/v)—may be prepared ahead of time and stored for up to 2 months

7.1.7 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

7.1.8 NaCl solution (2%, w/v)—may be prepared ahead of time and stored for up to 6 months

7.1.9 N,O-Bis(trimethylsilyl)trifluoroacetamide: Trimethylchlorosilane (BSTFA, 1% TMCS)(Millipore Sigma 15238-10X1ML)

7.1.10 Pyridine (Millipore Sigma 270970-4X25ML)

7.1.11 5α-cholestane internal standard (Millipore Sigma C8003)

7.1.12 Cholesterol (Millipore Sigma C8667)

7.1.13 Ergosterol (Millipore Sigma 45480)

7.1.14 Stigmasterol (Millipore Sigma S2424)

7.2 Materials

7.2.1 Volumetric flask (class A), 10 mL

7.2.2 Gas-tight syringes, covering ranges from 1–1,000 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)

7.2.5 Vials, amber, crimp tops, 1.5 mL fill volume (Agilent 5181-3376)

7.2.6 Vials with inserts, clear, crimp tops, 300 µL fill volume (Agilent 9301-1388)

7.2.7 Crimp caps, polytetrafluoroethylene (PTFE)/silicone/PTFE septa, 11 mm (Agilent 5181-1211)

8. Environment, Safety, and Health 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 BSTFA (1% TMCS) is corrosive and extremely flammable.

8.6 Pyridine is toxic and extremely flammable.

8.7 Follow all applicable chemical handling and waste disposal 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 sterol 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 hydrolysis

10.1.1 Label clear, 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 standard should be included in triplicate with each batch of samples. Ensure sufficient quantities of the standard are available for QC charting over multiple batches of sterols analysis.

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 Weigh 9–11 mg of freeze-dried sample into the labeled and pre-weighed GC vials. Record the weight to the nearest 0.1 mg.

10.1.4 It is also recommended to weigh out an ergosterol reference sample as ergosterol is a mix of isomers and each of the isomers undergoes an isomerization reaction during the acid hydrolysis, forming multiple peaks in the chromatography that are shifted from the un-hydrolyzed standard. Weigh out approximately 1 mg of the ergosterol used to prepare the standards. Recording a weight is not necessary for this reference as it is only to identify peak locations after hydrolysis. The reference should be treated as a sample for the remainder of the analysis.

10.1.5 Samples should be dried in vials in a 40°C vacuum oven or stored in a desiccator under vacuum overnight.

NOTE: Allow samples to cool and dry and then take a final weight to calculate the ODW of the sample.

10.1.6 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 sterol concentration [2].

10.2 Preparation of the internal standard

10.2.1 Prepare a 1 mg/mL solution of internal standard as follows:

- Tare a clean, 10-mL, class A, volumetric flask.
- Weigh out about 10 mg of the 5α-cholestane into the tared flask.
- Record the weight to the nearest 0.1 mg.
- Bring to volume with HPLC grade chloroform and mix well.

10.2.2 Transfer approximately 1 mL of the mixture to labeled 1.5-mL amber GC vials using a glass Pasteur pipet, working quickly and carefully to avoid evaporation. Seal vials immediately with PTFE/silicone/PTFE crimp caps.

10.2.3 Record the date of preparation, concentration, and any other pertinent information on the vials and place upright in a freezer (-20°C) for storage. Store sealed for up to 6 months.

10.3 Hydrolysis of the samples

10.3.1 Preheat a digital dry block, or equivalent, to 85 ± 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 5α-cholestane internal standard (1 mg/mL) using a gas-tight syringe. Recap the 5α-cholestane vial after adding to all samples; it will be needed in step 10.7.2.
- 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.

NOTE: Vials do not need to be capped after addition of the internal standard. Evaporation of the other reagents should be kept to a minimum, either by working quickly but carefully or by setting caps on the vials in between steps.

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, heat the samples. 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 1 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 Extraction of the sterols

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 or pipet tip compatible with hexane.

10.4.2 Add 200 µL 2% NaCl solution to each vial using a plastic pipet.

NOTE: It is easiest to perform steps 10.4.1 and 10.4.2 without the caps on the vials.

10.4.3 If uncapped, recap the vials with PTFE/silicone/PTFE crimp caps.

10.4.4 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.

10.4.5 To get complete phase separation, vials must sometimes be centrifuged due to emulsification of some of the residual biomass. To separate phases, centrifuge the GC vials by placing in compatible centrifugation tubes at 930 rcf for 5–10 minutes.

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

10.4.6 Label a new 300-µL GC insert vial for each sample.

10.4.7 Remove 300 µL of the hexane upper phase and place in the new insert vial. Do not cap the insert vials. The hexane 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.

10.4.8 Allow the samples from step 10.4.7 to evaporate under vacuum, at room temperature or at 40°C. Increase the vacuum slowly to prevent the solvent from evaporating too quickly. To avoid sterol degradation, do not allow samples to sit out in the light and ambient atmosphere for extended periods of time.

10.4.9 Once the samples are dry, they can either be capped and stored in a freezer (-20°C) for up to 1 week, or immediately derivatized and analyzed.

10.5 Preparation of the sterol working standard

10.5.1 Prepare a 1 mg/mL sterol working standard as follows:

- Tare a clean, 10-mL, class A, volumetric flask.
- Weigh out approximately 10 mg each of the cholesterol, ergosterol, and stigmasterol directly into the flask.
- Record the exact weight of each sterol to the nearest 0.1 mg.
- Bring to volume with HPLC grade chloroform and mix well.

10.5.2 Transfer approximately 1 mL of the standard mixture to labeled 1.5-mL amber GC vials using a glass Pasteur pipet, working quickly and carefully to avoid evaporation. Seal vials immediately with PTFE/silicone/PTFE crimp caps.

10.5.3 Record the date of preparation, concentration, and any other pertinent information on the vials and place upright in a freezer (-20°C) for storage. Store sealed for up to 6 months.

10.5.4 Repeat steps 10.5.1 through 10.5.3 to prepare a calibration verification standard (CVS).

10.6 Preparation of the sterol standard levels

10.6.1 Prepare a series of sterol standard levels and a CVS as described in Table 1.

Table 1. Sterol Standard Levels Prepared Using the Sterol Working Standard from Section 10.5

Standard Level	Sterol Working Standard (µL)	Chloroform (µL)
6	300	700
5	150	850
4	90	910
3	60	940
2	30	970
1	10	990
CVS*	70	930

* For the CVS, use the CVS working standard solution.

10.6.2 Prepare each level in a 1.5-mL amber GC vial using gas-tight syringes. Seal vials immediately with PTFE/silicone/PTFE crimp caps.

10.6.3 Record the date of preparation, concentration, and any other pertinent information on the vials and place upright in a freezer (-20°C) for storage. Store sealed for up to 6 months. Replace levels every month if being pierced frequently. Always recap after using to preserve the concentration.

10.7 Derivatization of the samples and standards

10.7.1 Prepare the BSTFA(1% TMCS):pyridine derivatization reagent (1:1, v/v) as follows:

- Label a 1.5-mL amber GC vial.
- Add 500 µL BSTFA (1% TMCS) to the vial using a pipet tip compatible with the chemical or a gas-tight syringe (rinse thoroughly following use with methanol, followed by chloroform).
- Add 500 µL pyridine to the vial using a pipet tip compatible with the chemical or a gas-tight syringe (rinse thoroughly following use with methanol, followed by chloroform).
- Seal the vial with a PTFE/silicone/PTFE crimp cap and vortex to mix.

NOTE: It is possible to prepare more than 1 mL of derivatization reagent by preserving the 1:1 (v/v) ratio of the two chemicals when prepping. Dispose of any excess reagent immediately after the derivatization. Ascertain that the pyridine is a fresh container, as any moisture present in pyridine can interfere with the silylation chemistry and thus present with inaccurate results.

10.7.2 Prepare the standard level vials for derivatization as follows:

- Label a 300-µL GC insert vial for each standard level and the CVS.
- Add 2.5 µL of the 5α-cholestane internal standard used in step 10.3.2 to each of the 300-µL insert vials using a gas-tight syringe. Allow to evaporate. Recap the internal standard vial immediately, it may be needed later if a re-run is necessary (store it in the freezer in the event it needs to be reused).
- Add 75 µL of the standard level to the appropriately labeled 300-µL insert vial using a gas-tight syringe and set a PTFE/silicone/PTFE crimp cap on the vial to prevent evaporation.
- Add 25 µL of the derivatization reagent prepared in step 10.7.1 to each of the vials using a gas-tight syringe.
- Seal the vials immediately with the PTFE/silicone/PTFE crimp caps and vortex well to mix.

  NOTE: Recap the pierced derivatization reagent to use for the samples in the next step.

10.7.3 Prepare the 300-µL sample vials from step 10.4.9 for derivatization as follows:

- Add 75 µL of HPLC grade chloroform to each vial using a gas-tight syringe and set a PTFE/silicone/PTFE crimp cap on the vial to prevent evaporation.
- Add 25 µL of the derivatization reagent prepared in step 10.7.1 to each of the vials using a gas-tight syringe.
- Seal the vials immediately with the PTFE/silicone/PTFE crimp caps and vortex well to mix.

10.7.4 Derivatized standards and samples should be analyzed on the GC within 3 days of being prepared.

10.8 Setting up the GC for sterols analysis

10.8.1 Suggested GC analysis follows a temperature and flow program as detailed below for DB-5ms 30 m x 0.25 mm inner diameter x 0.25 µm film thickness:

- 1-µL injection, inlet temperature of 280°C
- Constant flow: 0.9 mL/min helium
- Oven temperature: 100°C for 5 min, 15°C/min up to 325°C and hold for 15 min (35 min total)
- FID: 300°C, 400 mL/min zero air, 40 mL/min H_2, 30 mL/min helium

10.8.2 Run several blank chloroform samples before running the calibration set, CVS, and samples. Run a chloroform blank every 10 samples and finish the run with the CVS followed by several injections of a methanol blank (to scavenge any remaining derivatization reagent).

10.8.3 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 10.5.1 for the standards used.

10.8.4 Update the 5α-cholestane internal standard amount in µg/mL in the Chemstation software.

10.8.5 To quantify by Chemstation (Agilent) or respective GC software, use calibration response factors (corrected for internal standard recovery—in this case, 5α-cholestane—the software will do this once the correct internal standard value has been entered) for each individual sterol and only work up data when correlation is 0.999 or better.

10.8.6 Frequently, the sterols present in microalgae are not available commercially and may not match what is in the standard mixture. Sterol identification should be confirmed on a GC-MS. Response factors from structurally similar sterols may be used to quantify identified sterols using the FID signals obtained for the peaks. The MS should not be used for quantification as small molecular differences of the respective free sterols can produce large differences in signal response, so for compounds without an exact calibration standard, quantification may be inaccurate on the MS.

10.8.7 For the ergosterol standard, more than one peak will be observed. For calibration and quantification purposes, all observed peaks are summed. The hydrolyzed samples will have shifted ergosterol peaks, due to isomerization reactions; use the ergosterol reference sample weighed out in step 10.1.4 to identify the isomerized peaks for quantification.

11. Calculations

11.1 Export the sterol concentrations (as µg/mL for each individual sterol) from GC software and normalize for the quantity of the internal standard (this may have already been done by the software).

11.2 Calculate the total sterols as a percent of the ODW of the sample. The ODW refers to the weight after drying the sample overnight at 40°C in a vacuum oven (or approved equivalent):

$$\%\ Total\ Sterols = \frac{Total\ Sterols\ 5\alpha\ cholestane\ normalized}{ODW_{sample}}\ x\ 100$$

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 sterols 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 sterols in the sample on an ODW 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 sterols or sterol conjugates.

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 sterols at <10% RSD.

14.4 *Sample Size:* Approximately 9 to 11 mg of sample should be weighed out for hydrolysis.

14.5 *Sample Storage:* Before the analysis, all samples containing sterols 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 chloroform and methanol should be from the same bottle used for analysis.

14.7 *Standard Preparation:* Standards and calibration verification standard should be prepared with HPLC grade chloroform.

14.8 *Standard Storage:* GC standards should be stored upright in a freezer (-20°C) until needed (see notes throughout this LAP for acceptable storage periods).

14.9 *Control Charts:* Quality assurance/QC material should be charted to verify reproducibility. The analysis is out of control and must be repeated if the QC sample data meet any of the following conditions:

- Any result falls outside of three standard deviations (SD) of the mean
- Four out of five consecutive points outside (+) 1 SD or (-) 1 SD
- Two out of three consecutive points outside (+) 2 SD or (-) 2 SD
- Eight consecutive points all above or below the mean

- Eight consecutive points all increasing or decreasing
- Non-random patterns observed

15. Appendices

15.1 List of revisions/updates

- Posted to the National Renewable Energy Laboratory website December 2018.

16. References

[1] Van Wychen, S.; Ramirez, K.; Laurens, L.M.L. *Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by* in situ *Transesterification.* NREL/TP-5100-60958. Golden, CO: National Renewable Energy Laboratory, 2015. http://www.nrel.gov/docs/fy14osti/60958.pdf.

[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.