Identification and Quantification of Photosynthetic Pigments in Algae

Laboratory Analytical Procedure (LAP)

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1. Introduction

1.1 This Laboratory Analytical Procedure (LAP) describes the extraction, identification, and quantification of pigments and was optimized for extraction and analysis of freeze-dried (lyophilized) microalgae biomass. A High-Performance Liquid Chromatography (HPLC) system coupled with a Diode Array Detector (DAD) is utilized to identify and quantify common pigments in microalgae.

1.2 Reported methods for pigment extraction typically focus on the detection and quantification of pigments; however, the extraction efficiency is either overlooked or normalized to 100% efficiency for the extraction with the greatest yield. This procedure addresses the issue of extraction efficiency by establishing a measurement of extraction yield based on determination of phytol from chlorophyll in the initial biomass and the residual biomass after extraction.

1.3 The chromatographic separation described in this LAP is based on work by García-Plazaola and Becerril [1] but was modified for optimal performance with the system and column described herein.

2. Scope

This procedure is optimized for the extraction of pigments from freeze-dried (lyophilized) microalgae biomass and may be modified for fresh/wet biomass from other sources, including macroalgae, with careful consideration and results validation.

3. Terminology

3.1 Pigment: Organic compounds produced by some living organisms (for example, microalgae) that absorb light in the visible region of the electromagnetic spectrum in such a way that they are perceived as having color, generally grouped into Chlorophylls, Carotenoids, and Xanthophylls.

3.2 Chlorophyll: Porphyrin-containing organic molecules present in microalgae, and other photosynthetic organisms, that are green in color and are the primary facilitators of photosynthesis.

3.3 Carotenoid: A class of conjugated organic compounds that are terpenoid-derived and have yellow, orange, or red color. Carotenoids are composed of only carbon and hydrogen atoms.

3.4 Xanthophyll: A class of conjugated organic compounds that are terpenoid-derived and have yellow, orange, or red color. Xanthophyll structures resemble those of carotenoids but have oxygen functionalities, mainly in the form of alcohols and carbonyls.
3.5 **HPLC**: High-performance liquid chromatography, an analytical technique that utilizes a chromatographic column packed with adsorbent material which provides separation between chemicals with different structures.

3.6 **DAD**: Diode Array Detector, a detector that is used in conjunction with HPLC to determine the UV and Visible wavelength spectrum for analytes separated via HPLC.

3.7 **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].

4. **Significance and Use**

4.1 This procedure was developed for freeze-dried microalgal biomass and has not been optimized for foods or other types of biomass.

4.2 The procedure described in this work is used to determine the total concentration of carotenoids, chlorophylls, and xanthophylls present in microalgae, as well as the concentration of individually identified pigments.

NOTE: Pigment identification was based on standards when available. When no standard was available, UV/Vis spectra were used to identify pigments. However, the comparison of retention and spectral characteristics should always be compared with a standard if available.

5. **Apparatus**

5.1 Analytical balance, 0.1 mg (e.g., Mettler Toledo XP205 DeltaRange)

5.2 Bead beating apparatus

5.3 Vortex mixer

5.4 Ice bath

5.5 HPLC system equipped with a degasser, binary pump, and Diode Array Detector with the following column: Phenomenex Kinetix 5 µm C18 column with 100 Å pore size and dimensions of 150 mm x 4.6 mm or equivalent

5.6 Ultra-low temperature lab freezer (approximately -80 °C)

6. **Reagents and Materials Needed**

6.1 Purified water, equivalent to 18.2 MΩ water

6.2 Chloroform (HPLC-grade)
6.3 Methanol (HPLC-grade)
6.4 Acetonitrile (HPLC-grade)
6.5 Ethyl acetate (HPLC-grade)
6.6 1.5 mL centrifuge tubes, such as Eppendorf tubes
6.7 2 mL bead mill tubes
6.8 Stainless steel beads, 2.4 mm
6.9 Volumetric flask (class A), 1L and 10 mL
6.10 Graduated cylinder, 50 mL and 500 mL
6.11 Gas-tight syringes, covering ranges from 100-500 µL

7. ES&H Considerations and Hazards

The organic solvents used in this method are hazardous and should be handled in a fume hood. Consult the material safety data sheets for each reagent prior to handling chemicals.

8. Sampling, Test Specimens, and Test Units

8.1 Care must be taken to preserve the oxidation state of pigments as close to the biologically-relevant environment. Oxidation or degradation of pigments due to air, light, heat, and biological activity is common. Proper storage (nitrogen atmosphere and -80 °C) may limit the likelihood of oxidation.

8.2 Samples should be freeze-dried immediately after harvesting and stored at -80 °C until ready for extraction.

NOTE: Previous work has shown that filtered biomass pigments are well preserved for 60 days at -90 °C, and for more than 300 days at -198 °C. [3]

8.3 Microalgal samples should ideally be stored in amber vials if available, and samples may be wrapped in aluminum foil to prevent light penetration during storage or use.

8.4 This LAP is designed for freeze-dried material. Samples are typically generated by centrifugation, followed by freeze-drying (from -80 °C) avoiding material thawing. The extraction procedure listed here may be modified for other types of samples, but care must be taken to ensure proper and complete extraction of pigments, which falls outside of the scope of this LAP.

8.5 Representative samples must be collected for analysis from an algal biomass. Samples should be prepared by freeze-drying, moisture should be less than 10%, and samples should be ground/homogenized to a particle size <1mm.
9. Procedure

9.1 Each analytical batch should be accompanied with a quality control or reference sample, ideally for the same matrix, e.g., algal biomass. For example, reference samples could be representative aliquots of the same species or well-characterized biomass, e.g., *Nannochloropsis* and *Scenedesmus* samples that were harvested at the Arizona Center for Algae Technology and Innovation at Arizona State University, for which vacuum-sealed samples are available at NREL and have been certified by a team of analytical chemists. These samples should be freeze-dried, vacuum-sealed, and stored at least at -20 °C (ideally colder) to ensure proper preservation. [4]

9.2 Cell disruption and pigment extraction prior to analysis

9.2.1 Weigh approximately 100 mg of freeze-dried biomass into a 2 mL bead milling tube.

9.2.2 Add 10-20 beads to the tube and mill them under cold conditions for 1 minute on high level or approximately 4800 rpm.

9.2.3 Weigh approximately 15 mg of the bead-milled biomass into labeled and tared 1.5 mL centrifuge tubes. Record the weight of the sample to the nearest 0.1 mg in a lab notebook.

NOTE: Technical replicates are not performed for bead milling, i.e., only one aliquot is subjected to bead milling for each sample. However, each sample subjected to bead milling is then portioned into 2-3 vials, and the extraction of pigments is performed in duplicate or triplicate for each sample.

9.2.4 After all bead-milled samples have been weighed into centrifuge tubes, add 100 μL of purified water and vortex for 15 seconds.

NOTE: The goal of step 9.2.4 is to rehydrate the cells to provide a more effective extraction of pigments. If samples do not look wetted, i.e., dry spots remain in the biomass, more vortexing may be required.

9.2.5 Store tubes on ice for 30 minutes to limit biological activity during rehydration.

9.2.6 After 30 minutes on ice, add 0.5 mL of methanol and vortex for 15 seconds.

9.2.7 Add 0.5 mL of chloroform and vortex for another 15 seconds.

9.2.8 Centrifuge samples at 5000 RCF for 5 minutes and transfer the supernatant to pre-weighed and labeled vials by pipetting.

9.2.9 Add 0.5 mL methanol and then 0.5 mL chloroform to the extracted biomass residue and vortex for 15 seconds. Then centrifuge as described above and
transfer the supernatant to the same pre-weighed and labeled vials as in 9.2.8. This step should be repeated until the supernatant is clear.

NOTE: Our experiments have shown that 3-6 rounds of extraction with methanol:chloroform is typically sufficient to remove most of the pigments. The resulting biomass should be white or grey in color.

9.2.10 The extracted samples are then dried under a gentle stream of clean nitrogen gas and re-dissolved in 1 mL of methanol:acetone (8:2 v/v).

9.2.11 The methanol:acetone dissolved samples should be filtered through a 0.45 µm PTFE syringe filter and transferred to HPLC vials. Amber vials are preferred.

9.2.12 If samples are not analyzed immediately, they may be stored in solution at -80 °C for up to one month. This recommendation is based on previous stability work. [3]

9.3 Measurement of extraction efficiency

9.3.1 To determine the approximate extraction efficiency for pigments, phytol in the biomass prior to extraction and in the residual biomass after extraction can be measured using a standard base saponification method. Phytol in this case is used as a proxy for the other pigments.

9.3.2 For reference, the extraction method described in section 9.2 resulted in greater than 95% extraction efficiency based on phytol results for the species tested. Phytol results, as well as photos of the biomass after the first and fourth extractions, are provided in Appendix A (Table 3 and Figure 12).

9.3.3 A modified method of work by Ahmed et al. was utilized to determine the phytol content for each sample. [5] The procedure used may be found in Appendix B.

9.4 Preparation of standards

9.4.1 Standards should be selected based on the expected/desired pigments. It is always best practice to use standards for proper identification and quantification.

9.4.2 Weigh 10 mg of standard into a labeled and tared vial then add 1 mL of solvent (see NOTE below) to dissolve.

NOTE: Solubility of standards will vary based on chemical functional group polarity. Most xanthophylls and chlorophylls will be soluble in methanol or acetone, but carotenoids may only be soluble in acetone. Acetone is suggested as it provides the broadest coverage in solubility for pigment standards.
9.4.3 Quantitatively transfer the standard solution to a 10 mL, class A, glass volumetric flask, rinsing the original vial with extra solvent to ensure quantitative transfer.

9.4.4 Fill the volumetric flask to the 10 mL line with solvent.

9.4.5 Dilute standards to the desired concentration for calibration by use of volumetric flasks or gastight syringes.

9.5 HPLC analysis of extracted pigments

9.5.1 Mobile phase A should be prepared by accurately adding 50 mL of ultrapure water, measured in a graduated cylinder, to a 1000 mL, class A, glass volumetric flask. Bring to volume with HPLC grade acetonitrile and mix well before transferring to a chemically compatible HPLC bottle.

9.5.2 Mobile phase B should be prepared by accurately adding 320 mL of ethyl acetate, measured in a graduated cylinder, to a 1000 mL, class A, glass volumetric flask. Bring to volume with HPLC grade methanol and mix well before transferring to a chemically compatible HPLC bottle.

9.5.3 After column installation in the HPLC system, flush the column with at least 10 column volumes of mobile phase A.

NOTE: If using a newly purchased column or an unused column, please follow the manufacturer's instructions to prepare the column for use before completing step 9.5.3.

9.5.4 For new columns, disconnect the mobile phase flow from the DAD detector and make ~ 10 blank injections to ensure no column debris becomes dislodged and enters the detector cell.

9.5.5 Reconnect the solvent line to the DAD detector and collect several solvent blank injections with the gradient program and detector conditions found in Appendix A.

NOTE: Always record backpressure at the beginning of HPLC use. This record will be helpful if the need to troubleshoot should arise.

9.5.6 Once blank injections have been evaluated and are observed to be free of interference signals, insert samples into a dark autosampler and program your analysis method (HPLC method and chromatographic conditions are located in Table 1, Table 2, and Figure 1 of Appendix A).

NOTE: To prevent photo-oxidation of the samples, the autosampler should be shielded from ambient light as best as possible. For autosamplers with windows, foil or adhesive films may be used to cover windows and block out light.
9.5.7 See Appendix A (Figure 2) for representative sample chromatogram and UV-vis spectra for select pigments (Figures 3-7).

10. Calculations

A. Report pigments on an ODW basis by correcting the original biomass weight with the measured moisture content, or corrected for ash and expressed on an ash-free dry weight basis (AFDW). [2]

10.1 The concentration of each pigment should be calculated using the established calibration curves from the appropriate standards. The concentration of pigments present in the initial biomass may be calculated using the equations below:

\[
Pigment(\% \text{ of Biomass}) = \frac{Pigment_{(mg)}}{ODW_{(Sample, \ mg)}}
\]

\[
Mass\ of\ Pigment\ (mg) = Pigment\ Concentration_{(mg/mL)} \times Solution\ Volume_{mL}
\]

NOTE: Solution volume is from step 9.2.10.

10.2 If standards are not available for a given pigment, the calibration curve for a pigment with similar molar absorptivity may be used for quantification reported as concentration equivalents. For example, a calibration curve for Lutein may be used to quantify Zeaxanthin since their molar absorptivities are similar. The amount of Zeaxanthin measured would then be reported in Lutein equivalents.

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

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

\[
RMS\ deviation = \sigma = stddev = \sqrt{\frac{\sum_{i=1}^{n} (x_i - x_m)^2}{n}}
\]

where:
\[x_m = \text{the root mean square of all x values in the set}\]
\[n = \text{number of samples in set}\]
\[x_i = \text{measured value from the set}\]
10.4 To report or calculate the relative percent difference (RPD) between duplicates of the same sample, use the following calculation:

\[
RPD = \left( \frac{X_1 - X_2}{X_{mean}} \right) \times 100
\]

where:
- \( X_1 \) and \( X_2 \) = measured values
- \( X_{mean} \) = the mean of \( X_1 \) and \( X_2 \)

11. **Report Format**

11.1 Report the total pigment concentration as a percent of the ODW. Individual pigments may also be reported as a percent of the ODW.

11.2 For replicate analyses of the same sample, report the average and standard deviation (for triplicates) or %RPD (for duplicates).

11.3 Report and track the recovery of the QC or reference sample included in each analytical batch.

12. **Precision and Bias**

Precision and bias need to be determined by a round robin experiment using standard method verification biomass.

13. **Quality Control**

13.1 *Reported Results:* Report results with two decimal places. Report the average and standard deviation or %RPD.

13.2 *Replicates:* Run all samples in duplicate or triplicate, unless prohibited by the amount of sample available (extraction only).

13.3 *RPD Criterion:* Determined by data quality objectives and laboratory-specific *Quality Assurance Plan*.

13.4 *CVS:* CVSs should be independently prepared and analyzed as per the procedure. Required agreement for calibration verification standard quantification relative to the theoretical concentration should be within 5% RPD.

13.5 *Sample Size:* 15 ± 2.5 mg for each replicate.

13.6 *Sample Storage:* Freeze-dried samples may be stored in the freezer at -80°C for up to one month without degradation.
13.7 *Standard Storage:* HPLC standards should be stored as described by the manufacturer prior to being opened. Once opened, standards should be stored in solution at -80 °C.

13.8 *Definition of a Batch:* Any number of samples that are analyzed and recorded together.

13.9 *Control Charts:* Quality assurance/quality control material should be control charted to verify reproducibility.

14. **Appendices**

14.1 Appendix A: Supplemental data to aid in the description of the procedure described herein.

14.2 Appendix B: Procedure for detection and quantification of phytol from microalgal biomass.

15. **References**


Appendix A

Table 1. Gradient program for HPLC analysis of pigments. Solvent A consists of Acetonitrile:Water (95:5, v/v) and Solvent B consists of Methanol:Ethyl Acetate (68:32, v/v).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>2.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1. Graphical representation of the gradient profile used for pigment separation.

Table 2. Chromatographic conditions

<table>
<thead>
<tr>
<th>Chromatographic Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume (µL)</td>
</tr>
<tr>
<td>Autosampler Temperature (°C)</td>
</tr>
<tr>
<td>Flow Rate (mL/min)</td>
</tr>
<tr>
<td>Column Temperature (°C)</td>
</tr>
<tr>
<td>DAD Wavelength Range (nm)</td>
</tr>
</tbody>
</table>
Figure 2. Example chromatogram with select labeled pigments.

Figure 3. UV/vis spectrum of Chlorophyll a from LC-DAD.
Figure 4. UV/vis spectrum of Chlorophyll b from LC-DAD.

Figure 5. UV/vis spectrum of Violaxanthin from LC-DAD.
Figure 6. UV/vis spectrum of Lutein from LC-DAD.

Figure 7. UV/vis spectrum of Zeaxanthin from LC-DAD.
Figure 8. Calibration curve for Chlorophyll a.

Figure 9. Calibration curve for Chlorophyll b.
Figure 10. Calibration curve for Zeaxanthin.

Figure 11. Calibration curve for β-carotene.
Table 3. Phytol recoveries for algae biomass and extracted algae biomass residue.

<table>
<thead>
<tr>
<th>Algae Species</th>
<th>% Phytol in Biomass</th>
<th>% Phytol in Extraction Residue</th>
<th>Extraction Residue (Mass %)</th>
<th>Extraction Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nannochloropsis sp.</td>
<td>0.48</td>
<td>0.02</td>
<td>65.66%</td>
<td>97.07%</td>
</tr>
<tr>
<td>Scenedesmus sp.</td>
<td>0.48</td>
<td>0.03</td>
<td>79.56%</td>
<td>95.41%</td>
</tr>
</tbody>
</table>

Figure 12. Photos of biomass after the first extraction and the fourth extraction for a *Monoraphidium sp.* sample.
Appendix B

This appendix describes a method for phytosterol analysis in algae using a base saponification method as described by Ahmed, 2015. In the context of the photosynthetic pigment laboratory procedure above, the base saponification method is used for the isolation and quantification of phytol, derived from chlorophyll.

1. **Apparatus**

   1.1 Analytical balance, accurate to 1 mg or 0.1 mg (e.g., Mettler Toledo XP205)

   1.2 Vacuum oven set to 40 ± 3°C

   1.3 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)

   1.4 Digital dry block, capable of maintaining 75 ± 3°C

   1.5 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)

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

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

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

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

   1.10 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

2. **Reagents and Materials Needed**

   2.1 Reagents

      2.1.1 Potassium hydroxide (KOH)

      2.1.2 Methanol, HPLC grade (Fisher Chemical A4525K-1)

      2.1.3 Hexane, HPLC grade (Millipore Sigma 34859-1L)
2.1.4 Sodium chloride (NaCl), ACS grade (Fisher Chemical S271-500)

2.1.5 Chloroform, high-performance liquid chromatograph (HPLC) grade (Acros Organics 404635000)

2.1.6 Methanolic KOH solution (10%, w/v) \((10g \text{ KOH} + 100ml \text{ methanol})\) — prepare fresh weekly

2.1.7 NaCl solution (0.9%, w/v) — may be prepared ahead of time and stored for up to 6 months

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

2.1.9 Pyridine (Millipore Sigma 270970-4X25ML)

2.1.10 \(5\alpha\)-cholestane internal standard (Millipore Sigma C8003)

2.1.11 Phytol (Millipore Sigma 139912)

2.2 Materials

2.2.1 Volumetric flask (class A), 10 mL

2.2.2 Gas-tight syringes, covering ranges from 1–1,000 µL

2.2.3 Adjustable pipet, covering ranges from 100–300 µL

2.2.4 Vials, clear, crimp tops, 1.5 mL fill volume (Agilent 5182-0543)

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

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

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

3. Procedure

3.1 Preparation of the samples for hydrolysis

3.1.1 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.
3.1.2 Record the weights of all labeled vials to the nearest 0.1 mg. Written labels should be dry before the weight is recorded.

3.1.3 Weigh 10 +/- 2.5 mg of freeze-dried sample into the labeled and pre-weighed GC vials. Record the weight to the nearest 0.1 mg.

3.1.4 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.

3.1.5 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 sterol concentration.

3.2 Preparation of the internal standard

3.2.1 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.

3.2.2 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.

3.2.3 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.

3.3 Saponification of the samples

3.3.1 Preheat a digital dry block, or equivalent, to 75 ± 3°C.

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

- 34 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 3.7.2.

- 500 µL of methanolic KOH (10%) using a pipet with a glass or plastic tip.
3.3.3 Seal all vials with the PTFE/silicone/PTFE crimp caps and vortex well to mix the contents.

3.3.4 Place the sealed vials into the preheated block at 75°C for 120 minutes (2 hours). Once the reagent has been added, heat the samples. Do not allow samples to sit around as this may affect the reaction. Vials should be vortexed every 15 minutes during the reaction.

3.3.5 After 2 hours on the digital dry block, remove the vials and cool for at least 15 minutes, but no longer than 1 hour, at room temperature.

3.4 Extraction of the sterols

3.4.1 After cooling to room temperature, add 200 µL 0.9% NaCl solution to each vial using a plastic pipet.

3.4.2 Add 850 µL HPLC grade hexane to each of the vials using a gas-tight syringe or pipet tip compatible with hexane.

**NOTE:** It is easiest to perform steps 3.4.1 and 3.4.2 without the caps on the vials.

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

3.4.4 Vortex well to mix the vial contents and let sit for about 10 minutes.

3.4.5 To get complete phase separation, vials may need to be centrifuged. To separate phases, centrifuge the GC vials by placing in compatible centrifugation tubes at 930 rcf for 5–10 minutes.

3.4.6 Label a new 1.5-mL amber GC vial for each sample.

3.4.7 Remove 700 µL of the hexane phase and place in the new vial. Allow to evaporate under nitrogen or under vacuum at a maximum temperature of 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.

3.4.8 Repeat the extraction and centrifugation step two more times (for a total of 3 hexane extractions of 850 µL each – DO NOT ADD MORE SALT). Each time remove 700 µL of the hexane phase and dry down in the 1.5-mL extract vial (same vial for each extraction).

3.4.9 Once fully dry, bring extract up in 300 µL of chloroform, using a gas tight syringe, and cap the vial to prevent evaporation. Save the samples for derivatization with the standards (Step 3.7.3).

3.5 Preparation of the sterol working standard
3.5.1 Prepare a 1 mg/mL sterol working standard as follows:

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

3.5.2 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.

3.5.3 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.

3.5.4 Repeat steps 3.5.1 through 3.5.3 to prepare a calibration verification standard (CVS).

3.6 Preparation of the sterol standard levels

3.6.1 Prepare a series of sterol standard levels and a CVS as described in Table 4.

Table 4. Sterol Standard Levels Prepared Using the Sterol Working Standard from Section 3.5

<table>
<thead>
<tr>
<th>Standard Level</th>
<th>Sterol Working Standard (µL)</th>
<th>Chloroform (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>300</td>
<td>700</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>850</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>910</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>940</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>970</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>990</td>
</tr>
<tr>
<td>CVS*</td>
<td>70</td>
<td>930</td>
</tr>
</tbody>
</table>

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

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

3.6.3 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.
3.7 Derivatization of the samples and standards

3.7.1 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.

**NOTE:** It is also possible to run samples and standards without derivatization. The derivatization step is included here as it aids in cleaning up the chromatography for samples with complex biochemical matrices.

3.7.2 Prepare the standard level vials for derivatization as follows:

- Label a 300-µL GC insert vial for each standard level and the CVS.
- Add 4 µL of the 5α-cholestan internal standard used in step 3.3.2 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 3.7.1 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.

3.7.3 Prepare the sample extracts from step 3.4.9 for derivatization as follows:

- Label a 300-µL GC insert vial for each sample.
- Transfer 75 µL of the sample in 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 3.7.1 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.

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

3.8 Setting up the GC for sterols analysis

3.8.1 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 H₂, 30 mL/min helium

3.8.2 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).

3.8.3 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 3.5.1 for the standards used.

3.8.4 Update the 5α-cholestane internal standard amount in µg/mL in the Chemstation software.
3.8.5 To quantify by Chemstation (Agilent) or respective GC software, use calibration response factors (corrected for internal standard recovery—in this case, 5α-cholestan— 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.

3.8.6 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.