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Laboratory Analytical Procedure (LAP)

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National Renewable Energy Laboratory
15013 Denver West Parkway
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1. Introduction

1.1 This Laboratory Analytical Procedure (LAP) uses a two-step sulfuric acid hydrolysis to hydrolyze polymeric forms of carbohydrates in algal biomass into monomeric subunits. The monomers are then quantified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or a suitable spectrophotometric method.

1.2 Algal biomass in the context of these LAPs originates from either microalgae or macroalgae (seaweed) feedstocks. Carbohydrates as defined here include monomers and polymers of sugars, which in their broadest interpretation include neutral monosaccharides, uronic acids, amino-sugars (e.g. glucosamine), and reduced sugar alcohols (e.g. mannitol).

1.3 An optimized hydrolysis procedure should yield complete hydrolysis of all polymeric structural and storage carbohydrates into monomeric sugars. Due to the diversity of complex polysaccharides in and between algae species (micro and macro), complete hydrolysis of all polysaccharides may require the use of different inorganic acids and hydrolysis conditions. Here, we present a two-step sulfuric acid hydrolysis, followed by the quantification of released monosaccharides. This method works well for most glucose dominant polysaccharides (e.g., cellulose, starch, glycogen, laminarin, glucomannan). For use with other polysaccharides, it is recommended to run a study with a control (pure polysaccharide) to determine monomeric recoveries.

1.4 Portions of this procedure are substantially similar to ASTM E1758-01 “Standard Method for the Determination of Carbohydrates by HPLC” and the LAP developed for terrestrial feedstocks and reference [1].

2. Scope

2.1 This procedure was originally optimized for terrestrial biomass but has been modified in this LAP to apply to freeze-dried algal 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 at 40°C–60°C in an atmospheric pressure or vacuum convection oven according to the LAP *Determination of Total Solids and Ash in Algal Biomass* [2].

3.2 *Total Carbohydrates:* The sum of monomeric sugars produced after analytical acid hydrolysis with an inorganic acid (such as sulfuric acid); this fraction will also include monosaccharides derived from storage polysaccharides, such as starch. This value should be subtracted from the reporting of total structural carbohydrates.

3.3 *Storage Carbohydrates, specifically starch*: The fraction of monomeric glucose measured after enzymatic hydrolysis of algal biomass with an enzyme cocktail of α-amylase and

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amyloglucosidase. This fraction of storage carbohydrates is referred to as starch, but reflects any portion of α-1,4 linked glucose polymer in algal biomass.

3.4 *HPAEC-PAD:* High-performance anion exchange chromatography with pulsed amperometric detection, which separates monosaccharides based on their pK_a values. Details of a comparison between chromatography columns and conditions can be found in reference [3].

4. Significance and Use

4.1 This procedure was originally developed for use on terrestrial feedstocks, and its efficacy was tested on a variety of micro- and macroalgal species and related polysaccharides. Although it is not optimized for each individual species or polysaccharide, it has been shown to produce consistent results and effectively hydrolyze a range of polysaccharides common to micro- and macroalgae.

4.2 The procedure described here is used to determine the total monomeric carbohydrate content of freeze-dried algal biomass samples after a two-step sulfuric acid hydrolysis.

4.3 In this procedure, we describe separation and quantification of algae-specific monosaccharides using an HPAEC-PAD system. If using other chromatography columns or systems, care should be taken with respect to the monosaccharide resolution of complex mixtures of sugars. Refer to reference [3] for a comparison of different chromatography systems and columns.

4.4 Alternatively, spectrophotometric quantification of carbohydrates as monosaccharides can be achieved by complexing the free aldehyde group with MBTH (3-methyl-2-benzothiazolinone hydrazone), which has been shown to give an almost equal response of different aldehyde sugars in solution, and matched high-performance liquid chromatography (HPLC) quantification of complex mixtures of monosaccharides even in complex matrices [4].

4.5 This procedure is typically used in conjunction with other compositional analysis procedures to determine the summative mass closure for algal biomass.

5. Interferences

5.1 Samples with ash content >10% may need caution when analyzed with this procedure, as some components of ash could cause side reactions during hydrolysis. If the sample has a high ash content, as is the case with marine algae species, it is recommended to check the pH of the hydrolysate after autoclaving. A pH of ~1 or less is desirable.

5.2 Samples with moisture content >10% may not be suitable for this procedure as the excess moisture will interfere with appropriate acid concentrations.

5.3 Samples that are moldy or wet or that have been exposed to an oxygen-rich environment may be compromised, resulting in erroneous carbohydrate values.

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5.4 This procedure is not suitable for samples containing added acid, base, or catalyst.

5.5 Carbohydrates in the hydrolysates should not be measured using a phenol-sulfuric acid spectrophotometric quantification procedure. This quantification is known to be susceptible to significant interferences, as well as exhibiting substantially different responses for different monosaccharides.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg (e.g., Mettler Toledo XP205 DeltaRange).

6.2 Vortex mixer.

6.3 Water bath, set to 30°C ± 3°C.

6.4 Autoclave, suitable for autoclaving liquids, set to 121°C ± 3°C.

6.5 Digital dry block, capable of maintaining 80°C ± 3°C (for spectrophotometric carbohydrate determination)—compatible with glass tubes.

6.6 Spectrophotometer (for spectrophotometric carbohydrate determination), set to measure at 620 nm.

6.7 HPAEC system equipped with a gradient pump, pulsed amperometric detector (PAD), a CarboPac PA1 column (Thermo Scientific PN: 035391) with CarboPac PA1 guard column (Thermo Scientific PN: 043096), and AS-AP autosampler or equivalent.

7. Reagents and Materials Needed

7.1 Reagents and standards:

7.1.1 Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C) (Ricca Chemical Company R8191600-1A).

7.1.2 Sodium hydroxide, 50% w/w (Fisher Scientific SS254-500).

7.1.3 Sodium acetate, anhydrous (Sigma-Aldrich 791741).

7.1.4 Calcium carbonate, ACS reagent grade (Sigma Aldrich 239216).

7.1.5 Ultrapure water, 18.2 MΩ-cm.

7.1.6 HPAEC-PAD method:

7.1.6.1 Monomeric sugar standards: D-mannitol (Sigma M4125), L(-) fucose (Sigma F2252), L(-) rhamnose monohydrate (Sigma R3875), D(+) galactosamine hydrochloride (Sigma G0500), D(-) arabinose (Sigma A3131), D(+) glucosamine hydrochloride

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(Sigma G4875), D(+) galactose (Sigma G0750), D(+) glucose (Sigma G7528), D(+) mannose (Sigma M2069), D(+) xylose (Sigma X1500), and D(-) ribose (Sigma R7500).

7.1.6.2 Uronic acid standards: D(+) galacturonic acid monohydrate (Sigma 48280-5G-F) and D-glucuronic acid (Sigma G5269).

7.1.7 MBTH spectrophotometric method:

7.1.7.1 High-purity sugar standards: D(+) glucose (Sigma Aldrich G7528).

7.1.7.2 MBTH (Sigma Aldrich 129739).

7.1.7.3 NaOH, ACS reagent grade (Sigma Aldrich 221465).

7.1.7.4 Dithiothreitol (DTT) (Sigma Aldrich G0632).

7.1.7.5 Ferric ammonium sulfate dodecahydrate (Fluka 09730).

7.1.7.6 Sulfamic acid (Sigma Aldrich 242772).

7.1.7.7 Hydrochloric acid (HCl), concentrated (36.5%–38%) (J.T. Baker 9535-03).

7.2 Materials:

7.2.1 Pressure tubes and caps, suitable for use with acid in an autoclave.

7.2.2 Adjustable pipet and tips, covering 20 µL to 10 mL.

7.2.3 Disposable syringes (VWR BD301073) and compatible 0.2-µm nylon filters (VWR 28144-592), if planning to filter as opposed to centrifuging to remove solids.

7.2.4 15-mL centrifuge tubes.

7.2.5 Cuvettes—glass, quartz, or plastic (spectrophotometric method only).

7.2.6 13 × 100-mm glass test tubes or equivalent (spectrophotometric method only).

7.2.7 Glass marbles or caps to cover or plug the 13 × 100-mm glass test tubes (spectrophotometric method only).

7.2.8 1.5-mL vials with split septum caps (Thermo Scientific 055427) (HPAEC method only).

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8. Environmental Safety and Health (ES&H) Considerations and Hazards

8.1 Sulfuric acid is corrosive and should be handled with care.

8.2 Sodium hydroxide is corrosive and should be handled with care.

8.3 MBTH procedure: Hydrochloric acid, sodium hydroxide, and sulfamic acid are moderate health hazards. MBTH and ammonium ferric sulfate dodecahydrate are moderate health hazards. Dithiothreitol is a moderate health hazard with mild flammability and reactivity. Follow all applicable chemical handling procedures.

8.4 Use caution when handling hot pressure tubes after removal from the autoclave. The pressurized tubes are a possible explosion hazard if not cooled properly.

9. Sampling, Test Specimens, and Test Units

9.1 The LAP *Determination of Total Solids and Ash in Algal Biomass* [2] should be performed in concurrence with this LAP to allow for accurate reporting on a dry weight or ash-free dry weight basis.

9.2 Care must be taken to ensure a representative sample is taken for analysis from an algal biomass prepared and dried by freeze drying, spray drying, etc., ensuring moisture is <10% and is ground/homogenized to a particle size <1 mm.

10. Procedure

10.1 Prepare samples for hydrolysis:

10.1.1 Weigh out all samples in parallel with the LAP *Determination of Total Solids and Ash in Algal Biomass* [2]. The results from this LAP will be used later to correct the biomass weight. The hydrolysis and moisture correction samples should be weighed out on the same day, if possible, to avoid differences in moisture content.

10.1.2 Weigh 25 mg ± 2.5 mg of freeze-dried algal biomass into a labeled and tared glass pressure tube. Less sample may be weighed out to accommodate for limited volume samples. Record the weight of the sample to the nearest 0.1 mg. If not hydrolyzed promptly, cap the sample to preserve the moisture content and to limit the sample exposure to contaminants.

NOTE: It is recommended to include a quality control sample weighed out in triplicate with each set to monitor the success of the method.

10.1.3 After all the samples have been weighed out, add 250 µL of 72% (w/w) sulfuric acid to each tube while slowly vortexing the sample. This prevents clumping of the sample. After wetting the sample, vortex thoroughly and

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carefully to ensure all solids remain at the bottom of the tube and immersed in the acid.

NOTE: Do not use sulfuric acid that has started to yellow or brown, as its efficacy might be compromised.

10.1.4 Place tubes in a 30°C ± 3°C water bath with a water level set to just above the sample/acid level in the tubes. Incubate samples for 1 hour, vortexing each tube vigorously every 10 minutes. Vortex samples carefully to ensure all solids remain at the bottom of the tube and immersed in acid.

10.1.5 After 1 hour, remove tubes from the water bath and add 7 mL of 18.2 MΩ-cm water to each tube. This will bring the sulfuric acid concentration to 4% (w/w).

10.1.6 Cap the tubes and thoroughly vortex the contents.

10.1.7 Place the tubes in a rack suitable for autoclaving. Be sure to place the rack in a tray for secondary containment. Secondary containment should contain any tubes and their contents if they were to break during the autoclave cycle.

10.1.8 Autoclave the sealed samples for one hour at 121°C using the liquids (slow) setting.

10.1.9 After completion of the autoclave cycle, when the pressure has dropped to 0, carefully open the autoclave door (stand to the side to avoid steam burns). Do NOT remove tubes yet; allow to cool for approximately 15 minutes before removing them from the autoclave.

10.1.10 Once the samples are removed from the autoclave, allow the samples to cool for 30 minutes to 1 hour or until tubes reach room temperature.

10.2 Prepare samples for HPAEC-PAD analysis:

10.2.1 Once cool, either filter an aliquot of the sample (vortex thoroughly before taking an aliquot for filtering) through a 0.2-µm nylon filter (use the disposable syringes and compatible filters) OR centrifuge to remove ALL solids. If hydrolysates cannot be analyzed the same day, they should be stored in the freezer and analyzed within 2 weeks for uronic acids and within 1 month for other carbohydrates.

10.2.2 The filtered hydrolysate can be diluted directly into 1.5-mL vials for analysis.

10.3 Prepare samples for spectrophotometric analysis:

10.3.1 Once cool, either filter an aliquot of sample (vortex thoroughly before taking an aliquot for filtering) through a 0.2-µm nylon filter (use the disposable

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syringes and compatible filters) OR centrifuge to remove solids. If hydrolysates cannot be analyzed the same day, they should be stored in the freezer and analyzed within 2 weeks for uronic acids and within 1 month for other carbohydrates.

10.4 HPAEC-PAD analysis for monomeric sugars (not including uronic acids):

10.4.1 Prepare ~2-mg/mL stock standard using the sugars listed in Section 7.1.6.1 (use a volumetric flask for volume accuracy and bring to volume using 18.2-MΩ-cm water). Record all weights to the nearest 0.1 mg. Store the ~2-mg/mL stock standard in 1-mL aliquots in sealed vials in a freezer for up to 6 months.

NOTE: When calculating the standard concentrations for each sugar, be sure to correct for purity and compound formulas (e.g., rhamnose may be formulated as a monohydrate).

10.4.2 To prepare calibration standards, first prepare a 0.1-mg/mL working standard from the ~2-mg/mL stock standard using 18.2-MΩ-cm water.

10.4.3 Prepare a series of calibration standards from the 0.1-mg/mL working standard. Refer to **Table 1** for suggested calibration points. Due to the quadratic fit of the calibration curve on this system, it is recommended to use at least a nine-point calibration curve, calibrating with Levels 1–5 and 5–9.

Table 1. Suggested Calibration Curve for HPAEC-PAD Analysis Using the 0.1-mg/mL Working Standard

Standard Level	0.1 mg/mL Working Standard (µL)	18.2 MΩ-cm Water (µL)
1	2.5	997.5
2	5	995
3	10	990
4	20	980
5	40	960
6	100	900
7	200	800
8	400	600
9	600	400

NOTE: Standards 1–3 in **Table 1** can be prepared by serial dilution, starting with a 2x dilution of Standard 4 (500 µL of Standard 4, 500 µL of 18.2-MΩ-cm water).

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10.4.4 Prepare all eluents following the guidelines outlined in the Thermo Scientific TN-71: *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection*. Use 18.2-MΩ-cm water filtered through the recommended filters (TN-71) and sonicated to prepare eluents. For the method described here, Line A is filtered and sonicated 18.2-MΩ-cm water and Line B is 200-mM NaOH.

10.4.5 Using an HPAEC-PAD equipped with a CarboPac PA1 guard and column (4 × 250 mm), run the calibration standards and samples. Run calibration checks (e.g., separately prepared calibration verification standards, or use Standards 3 and 7) every 10 samples. It is recommended to start the run with at least 3 blanks to equilibrate the system and to monitor the detector response.

- Sample injection volume: 10 µL.
- Flow rate: 1 mL/min.
- Column temperature: 35°C.
- Detector temperature: 35°C.
- Eluent regime: 10 min at 100% Line B, 30 min at ~14-mM NaOH (optimize for individual column and system), 20 min at ~14-mM NaOH (this should match the optimized concentration from the previous 30 min).
- PAD setting: Waveform A (from Thermo Scientific manual).

10.4.6 Quantify the monomeric sugars using Chromeleon (Thermo Scientific). Correlation for each calibrated compound should be 0.999 or better.

10.5 HPAEC-PAD analysis for uronic acids:

10.5.1 Prepare ~2-mg/mL stock standard using the uronic acids listed in Section 7.1.6.2 (use a volumetric flask for volume accuracy and bring to volume using 18.2-MΩ-cm water). Record all weights to the nearest 0.1 mg. Store the ~2-mg/mL stock standard in a sealed vial or tube in a freezer for up to 2 weeks.

NOTE: When calculating the standard concentrations for each uronic acid, be sure to correct for purity and compound formulas (e.g., galacturonic acid may be formulated as a monohydrate).

10.5.2 To prepare calibration standards, first prepare a 0.1-mg/mL working standard from the ~2-mg/mL stock standard using 18.2-MΩ-cm water. Prepare fresh the same day the calibration standards are to be prepared.

10.5.3 Prepare a series of calibration standards from the 0.1-mg/mL working standard. Refer to **Table 2** for suggested calibration points.

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Table 2. Suggested Calibration Curve for HPAEC-PAD Uronic Acid Analysis Using the 0.1-mg/mL Working Standard

Standard Level	0.1-mg/mL Working Standard (μL)	18.2 MΩ-cm Water (μL)
1	20	980
2	40	960
3	100	900
4	200	800
5	400	600
6	600	400

10.5.4 Prepare all eluents following the guidelines outlined in the Thermo Scientific TN-71: *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection*. Use 18.2-MΩ-cm water filtered through the recommended filters (TN-71) and sonicated to prepare eluents. For the method described here, Line A is filtered and sonicated 18.2-MΩ-cm water, Line B is 200-mM NaOH, and Line C is 1-M sodium acetate/100-mM NaOH.

NOTE: DO NOT leave 1-M sodium acetate/100-mM NaOH on the system for more than a week. Fill that bottle with a small amount of 200-mM NaOH and flush Line C fully between runs. This prevents the potential growth of bacteria in the sodium acetate.

10.5.5 Using an HPAEC-PAD equipped with a CarboPac PA1 guard and column (4 × 250 mm), run the calibration standards and samples. Run a calibration check (e.g., separately prepared calibration verification standard, or use Standard 4) every 10 samples. It is recommended to start the run with at least 3 blanks to equilibrate the system and to monitor the detector response.

- Sample injection volume: 10 μL.
- Flow rate: 1 mL/min.
- Column temperature: 35°C.
- Detector temperature: 35°C.
- Eluent regime: 10 min at 100% Line B, 20 min at 17% Line C (optimize for individual column and system), 10 min at 17% Line C (this should match the optimized concentration from the previous 20 min).
- PAD setting: Waveform A (from Thermo Scientific manual).

10.5.6 Quantify the uronic acids using Chromeleon (Thermo Scientific). Correlation for each calibrated compound should be 0.999 or better. For guluronic and mannuronic acids (found in polysaccharides like alginic acid), use the galacturonic acid calibration curve. If possible, establish correction

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factors for the differences in response between guluronic and galacturonic and mannuronic and galacturonic by running at least one set of calibration curves prepared with guluronic and mannuronic acids (available from Carbosynth).

10.6 Spectrophotometric analysis for monomeric sugars and uronics using MBTH [4]:

10.6.1 Preparation of reagents/solutions—use 18.2-MΩ-cm water for all preparations requiring water:

- 0.5-M NaOH solution—store at room temperature in a plastic (HDPE) container and remake once a precipitate forms (looks like silvery flakes), typically about 2–3 weeks.
- 3-mg/mL MBTH—store in the dark at 4°C for up to 1 week.
- 1-mg/mL DTT—store in the dark at 4°C for up to 1 week.
- 0.25-M HCl—prepare in a 200-mL volumetric flask and store at room temperature in the dark for up to 1 month:
    - Add ~150 mL of 18.2-MΩ-cm water to the volumetric flask.
    - Pipet 4.16 mL of concentrated HCl (~36.5%–38%) into the flask.
    - Bring to volume with 18.2-MΩ-cm water.
    - Cap and mix thoroughly.
- Ferric solution: 0.5% ferric ammonium sulfate dodecahydrate, 0.5% sulfamic acid (w/v), and 0.25-M HCl. Dissolve 200 mg of ferric ammonium sulfate dodecahydrate and 200 mg of sulfamic acid into 40 mL of 0.25-M HCl. This solution may be stored at room temperature in the dark for up to 1 month.

10.6.2 Preparation of working solution, made up fresh right before adding to samples:

- MBTH working solution: 1:1 (v/v) of 3-mg/mL MBTH and 1-mg/mL DTT.

10.6.3 Preparation of standards:

- Prepare glucose standard and Calibration Verification Standard (CVS) stock solutions (these must be made up separately):

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- Weigh approximately 25 mg of glucose into a clean, tared, Class A, 100-mL volumetric flask.
- Record the weight to the nearest 0.1 mg.
- Bring to volume with 18.2-MΩ-cm water.
- Cap and mix thoroughly.

- The standard and CVS working solution concentrations should be approximately 0.25-mg/mL glucose. Due to balance limits, start with a higher stock glucose concentration and dilute to 0.25 mg/mL. ALWAYS prepare the standards and CVS from separate stocks so that you can check the accuracy of your calibration with the CVS.

- Glucose stock solutions should be stored in a freezer (−20°C) for up to 2 months or 5 days in the refrigerator (4°C).

- Create a six-point calibration with the 0.25-mg/mL glucose stock solution using the dilutions outlined in **Table 3**. Prepare dilutions directly into the glass reaction vials (13 × 100 mm or equivalent).

Table 3. Suggested Standard Concentrations for the Spectrophotometric Method

Concentration of Stock/Working (mg/mL)	Dilution Stock + Water (µL)	Final Concentration (mg/mL)
0.25	0 + 500	0
0.25	20 + 480	0.010
0.25	30 + 470	0.015
0.25	50 + 450	0.025
0.25	75 + 425	0.0375
0.25	100 + 400	0.050

- Prepare the CVS at the center of the calibration range: 75 µL CVS glucose stock solution (0.25 mg/mL) and 425 µL 18.2-MΩ-cm water. Prepare the CVS dilution directly into the glass reaction vials.

10.6.4 Reaction:

- Preheat a digital dry block that fits 13 × 100-mm glass test tubes (or equivalent reaction vials) to 80°C ± 3°C.

  Prepare sample dilutions directly in the glass reaction vials: Start with a 1:10 (50-µL hydrolysate) or greater dilution of the filtered/centrifuged acidic hydrolysate. Dilute the sample to a total volume of 0.5 mL. If the sample is known to be high in carbohydrates, start with a 1:20 dilution. The

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goal is to have the sample absorbance fall in the middle of the calibration curve.

NOTE: DO NOT use neutralized hydrolysate, as the soluble salts left over from the neutralization will affect the reaction.

NOTE: DO NOT use less than a 1:10 dilution on acidic hydrolysates, as the excess acid can have an effect on the reaction.

- ALWAYS run a set of standards and a CVS with each batch of samples.
- Add 500 µL 0.5-M NaOH to each glass tube.
- Add 500 µL MBTH working solution (Section 10.5.2) to each glass tube, vortex carefully to mix, and cover the tubes with a glass marble or cap. Immediately place the tubes with solutions in the preheated dry block at 80°C for 15 min ± 1 min. Do not exceed the incubation time. Work in smaller sample sets (<20). Do not allow the samples and standards to sit around after addition of the NaOH or MBTH working solution—they must be placed on the hot block within a few minutes of adding these solutions.
- After the 15-minute incubation time, turn off the block and immediately add 1 mL of the ferric solution while the glass tubes are still on the block (Section 10.5.1).
- Once the ferric solution has been added, remove the glass tubes from the hot block and carefully vortex to mix.
- Allow the samples and standards to react with the ferric solution for 10–15 minutes while the samples cool to room temperature. Tubes may remain open during this time. Allow them to cool in a hood to control fumes.
- Once the samples are at room temperature, add 2.5 mL 18.2-MΩ-cm water; mix by pipetting or vortexing.
- Once mixed, place an aliquot of the samples and standards into the appropriate cuvettes and obtain an absorbance on the spectrophotometer at 620 nm. Take an absorbance within an hour after the final dilution step. Zero the spectrophotometer on 18.2-MΩ-cm water. DO NOT zero the spectrophotometer on the 0 standard from the calibration standard set.
- Use the glucose calibration curve and linear regression to quantify the total carbohydrate concentration in mg/mL. Be sure to include the 0 standard as a point in the calibration. Remember to correct the sample carbohydrate concentration for the amount of hydrolysate that was used in the dilution (for a 1:10, use 50 µL) and then get a total carbohydrate content by multiplying that concentration by the total volume from the hydrolysis—

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7.25 mL. When building a linear calibration curve, DO NOT force the curve through the origin.

11. Calculations

11.1 Calculate the ODW of the sample using the average total solids content as determined by the LAP *Determination of Total Solids and Ash in Algal Biomass* [2]:

$$ODW = \frac{Weight_{sample} \times \%\ Total\ Solids}{100}$$

11.2 Calculate monomeric sugar concentrations (mg/mL) for each sample using the linear regression coefficients for the spectrophotometric glucose determination or HPAEC-PAD calibration. For the HPAEC-PAD method, it is recommended to quantify using two different calibration curves (Standards 1–5 and 5–9) to better cover the quadratic response of the detector.

11.3 Calculate monomeric sugar content (mg) for each sample using the following equation:

$$Total\ \ Monosaccharides_{(mg)} = Monosaccharide_{(mg/mL)} \times 7.25_{(mL)}$$

11.4 Sum the monomeric sugars to get total sugars in mg (for the HPAEC-PAD method).

11.5 Calculate the amount of monomeric (total) sugar in the sample on a percent ODW basis (if starch was not measured, total carbohydrates are calculated as follows without subtracting the starch content):

$$\%\ MonomericSugar = \left(\frac{Total\ \ Monosaccharides_{(mg)}}{ODW_{Sample}} \right) - \%\ Starch \times 100$$

11.6 To report or calculate the relative percent difference (RPD) between two samples, 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.

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11.7 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.

12. Report Format

12.1 Report the total carbohydrate results as a percent of the ODW. Individual sugars may also be reported as a percent of the ODW.

12.2 For replicate analyses of the same sample, report the average, standard deviation, and %RPD.

12.3 Report the CVS recovery.

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 RPD should be less than 10%.

14. Quality Control

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

14.2 *Replicates:* Run all samples in triplicate, unless prohibited by the amount of sample available (hydrolysis only).

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

14.4 *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. Acceptable criteria should be based on laboratory-specific quality assurance plan.

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14.5 *Sample Size:* 25 mg ± 2.5 mg for each replicate. Less sample may be weighed out to accommodate for limited volume samples.

14.6 *Sample Storage:* Hydrolysis liquors may be separated from the acid insoluble solids and stored (acidic or neutralized) in a freezer (−20°C) for 2 weeks for uronics and up to a month for other carbohydrates.

14.7 *Standard Storage:* HPAEC-PAD standards should be stored in a freezer (−20°C) and removed when needed. Thaw and vortex standards prior to use. Filter if necessary.

14.8 *Standard Preparation:* Standards should be prepared as described in the procedure.

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

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

15. Appendices

15.1 List of revisions/updates:

- Distribution of May 16, 2013, DRAFT version.
- Revision July 8, 2013, addition of additional experimental details and dilution descriptions.
- Revision July 17, 2013, correction to dilutions in Table 2.
- Revision July 26, 2013, addition of CVS recovery target precision.
- Revision December 29, 2015, update of spectrophotometric and HPLC methods and sample storage.
- Revision September 13, 2023, update to include HPAEC-PAD method and uronic acid analysis to accommodate macroalgae-specific compositional analysis.

16. References

[1] ASTM International. 2003. "ASTM E1758-01: Standard Method for the Determination of Carbohydrates by HPLC." *2003 Annual Book of ASTM Standards*, Volume 11.05. Philadelphia, PA: ASTM International.

[2] S. Van Wychen and L.M.L. Laurens. 2023. *Determination of Total Solids and Ash in Algal Biomass.* Golden, CO: National Renewable Energy Laboratory. NREL/TP-2700-87520. http://www.nrel.gov/docs/fy24osti/87520.pdf.

[3] D.W. Templeton, M. Quinn, S. Van Wychen, D. Hyman, and L.M.L. Laurens. 2012. "Separation and quantification of microalgal carbohydrates." *J. Chrom. A* (1270): 225–234.

[4] S. Van Wychen, W. Long, S.K. Black, and L.M.L. Laurens. 2017. "MBTH: A novel approach to rapid, spectrophotometric quantitation of total algal carbohydrates." *Anal. Biochem.* (518): 90–93.

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