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Laboratory Analytical Procedure (LAP)

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1. Introduction

- 1.1 This Laboratory Analytical Procedure (LAP) uses a two-step sulfuric acid hydrolysis to hydrolyze the polymeric forms of carbohydrates in algal biomass into monomeric subunits. The monomers are then quantified by either high-performance liquid chromatography (HPLC) or a suitable spectrophotometric method.
- 1.2 An optimized hydrolysis procedure is expected to yield complete hydrolysis of all polymeric structural and storage carbohydrates into monomeric sugars. A range of sequential and optimized inorganic acid hydrolysis conditions with respective hydrolysis liquor collection and analysis should be carried out for algal biomass. Here, only a two-step sulfuric acid hydrolysis and quantification of released monosaccharides are presented, and it is noted that not all carbohydrates may be hydrolyzed in this manner. Future updates to this procedure will involve optimization of the carbohydrate hydrolysis procedures.
- 1.3 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 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 *Structural Carbohydrates* – The hydrolysable fraction of monomeric sugars 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* – The fraction of monomeric glucose measured after enzymatic hydrolysis of algal biomass with an enzyme cocktail of α -amylase and amyloglucosidase. This fraction of storage carbohydrates is referred to as starch, but reflects any portion of α -1,4 linked glucose polymers in algal biomass.
- 3.4 *HPLC* – High performance liquid chromatography, which separates compounds based on size exclusion and ligand exchange. Details of a comparison between chromatography columns and conditions for HPLC can be found in reference [3].

4. Significance and Use

- 4.1 This procedure was developed for use on terrestrial feedstock and has not been optimized specifically for microalgal biomass. However, the method has been extensively tested and shown to work for algal biomass with no apparent difficulties.
- 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 For the purpose of describing this procedure, we will refer to HPLC quantification, but care should be taken with respect to monosaccharide resolution and chromatography of complex mixtures of algae-specific monosaccharides when choosing an HPLC system. High-performance anion exchange chromatography (HPAEC) may be the method that gives the best separation of major micro-algae-specific monosaccharides [3], but it is not described here.
- 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 HPLC quantification of complex mixtures of monosaccharides [4]. This is a novel procedure; a manuscript on the development and validation of this procedure is in preparation and will be included as a reference in future versions of this LAP.
- 4.5 This procedure is 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 not be suitable for this procedure as some components of ash may cause side reactions during hydrolysis.
- 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.
- 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 quantification procedure. This quantification is known to be susceptible to significant interferences as well as exhibiting substantially different responses for different monosaccharides. Quantification of total carbohydrates by phenol-sulfuric acid failed to match HPLC-quantification of even simple mixtures of sugars.

6. Apparatus

- 6.1 Analytical balance, accurate to 1 mg or 0.1 mg (e.g., Mettler Toledo XP205 DeltaRange)
- 6.2 Vortex mixer
- 6.3 Water bath, set to $30^{\circ}\text{C} \pm 3^{\circ}\text{C}$
- 6.4 Autoclave, suitable for autoclaving liquids, set to $121^{\circ}\text{C} \pm 3^{\circ}\text{C}$
- 6.5 Spectrophotometer (for spectrophotometric carbohydrate determination), set to measure at 620 nm
- 6.6 HPLC system equipped with refractive index detector and the following column: Shodex sugar SP0810 (Shodex, #F6378105) with de-ashing cartridge holder (Bio-Rad, #125-0139) and micro-guard de-ashing cartridges (Bio-Rad, #125-0118) or equivalent with corresponding guard column

7. Reagents and Materials Needed

- 7.1 Reagents
 - 7.1.1 Sulfuric acid, 72% w/w (specific gravity 1.6338 at 20°C) (Ricca Chemical Company R8191600-1A)
 - 7.1.2 Calcium carbonate, ACS reagent grade (Sigma Aldrich 239216)
 - 7.1.3 Water, 18.2 MegaOhm ($\text{M}\Omega$) water, 0.2 μm filtered
 - 7.1.4 HPLC method:
 - 7.1.4.1 High purity sugar standards: D(+)glucose (Sigma Aldrich G7528), D(+)xylose (Sigma Aldrich X3877), D(+)galactose (Sigma Aldrich G0750), L(+)arabinose (Sigma Aldrich 10839), D(+)mannose (Sigma Aldrich 63579).
 - 7.1.5 MBTH spectrophotometric method:
 - 7.1.5.1 High purity sugar standards: D(+)glucose (Sigma Aldrich G7528)
 - 7.1.5.2 MBTH (Sigma Aldrich 129739)
 - 7.1.5.3 NaOH, ACS reagent grade (Sigma Aldrich 221465)
 - 7.1.5.4 Dithiothreitol, DTT (Sigma Aldrich G0632)
 - 7.1.5.5 Ferric ammonium sulfate dodecahydrate (Fluka 09730)

- 7.1.5.6 Sulfamic acid (Sigma Aldrich 242772)
- 7.1.5.7 Hydrochloric acid (HCl), concentrated (36.5%–38%) (J.T. Baker 9535-03)

7.2 Materials

- 7.2.1 10-mL glass tubes and caps, heavy walled and capable of withstanding high-pressure in an autoclave (VWR, #89079-404). Do not use the caps that are supplied with the vials as they may not allow the samples to vent properly. Rubber stoppers to plug glass tubes (VWR, #15507-144), tear-away aluminum crimp caps (VWR, #16171-851)
- 7.2.2 Adjustable pipet, covering 250 μ L to 10 mL
- 7.2.3 pH paper, range 4–9
- 7.2.4 Cuvettes – glass, quartz, or plastic (spectrophotometric method only)
- 7.2.5 13 x 100-mm glass test tubes or equivalent (spectrophotometric method only)
- 7.2.6 Glass marbles or caps to cover or plug the 13 x 100-mm glass test tubes (7.2.5) (spectrophotometric method only)
- 7.2.7 Disposable syringes (VWR, #BD301073) and compatible 0.2- μ m nylon filters (VWR, #28144-592), if planning to filter as opposed to centrifuging to remove solids
- 7.2.8 1.5-mL capacity HPLC vials (Agilent, #5182-0543) and compatible PTFE/rubber crimp caps (Agilent, #5181-1210) (HPLC method only)
- 7.2.9 Manual or automatic crimpers for caps for glass tubes from 7.2.1 and for HPLC vials from 7.2.8 (if doing the HPLC method)

8. ES&H Considerations and Hazards

- 8.1 Sulfuric acid is corrosive and should be handled with care.
- 8.2 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.
- 8.3 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.
- 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 <1mm.

10. Procedure

- 10.1 Prepare the sample for analysis and hydrolyze
 - 10.1.1 Weigh out all samples in concurrence 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 must be weighed out on the SAME DAY. If not immediately hydrolyzed, seal samples to prevent any changes in moisture content.
 - 10.1.2 Weigh 25 ± 2.5 mg of freeze-dried algal biomass into a labeled and tared 10-mL glass tube. Record the weight of the sample to the nearest 0.1 mg in a lab notebook.
 - 10.1.3 After all the samples have been weighed out, add 250 μ L of 72% (w/w) sulfuric acid to each tube and vortex to thoroughly mix the acid and biomass. Vortex samples carefully to ensure all solids remain at the bottom of the tube and immersed in the acid.
 - 10.1.4 Place tubes in a $30^{\circ}\text{C} \pm 3^{\circ}\text{C}$ 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 5 to 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 MegaOhm ($\text{M}\Omega$) water to each tube. This will bring the sulfuric acid concentration to 4% (w/w).
 - 10.1.6 Place a rubber stopper snugly in the top of the glass tube. Place a tear-away aluminum crimp cap on top of the rubber stopper. Crimp the caps tightly and vortex to mix the contents thoroughly. Tubes may also be inverted to mix, ensure that the solids remain in the liquid, and do not end up stuck to the rubber stopper.
 - 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, open the autoclave door, but allow tubes 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 HPLC or spectrophotometric analysis.
 - 10.2.1 Hydrolyzed samples must either be neutralized the same day or the solids must be separated from the hydrolysate and the hydrolysate (still acidic) may be stored in a refrigerator (4°C) for up to 24 hours before being neutralized. Neutralized and filtered/centrifuged hydrolysate may be stored up to 24 hours in a refrigerator (4°C) before being analyzed. Watch for precipitate formation after the sample has been refrigerated. The sample must be vortexed thoroughly, and then re-filtered or centrifuged to remove the precipitate before analysis by HPLC or spectrophotometer.
 - 10.2.2 Once the samples are cooled to room temperature, vortex each sample and take an aliquot for neutralization. Aliquots are typically about 1–3 mL; do not attempt to neutralize large volumes of hydrolysate at once. The sample does not need to be filtered or centrifuged to remove the solids before aliquoting.
 - 10.2.3 Place the aliquot in a vessel suitable for neutralizing the sample. The vessel must be large enough to contain the sample when it off-gases and bubbles during the neutralization process. Examples might be 25-50 mL Erlenmeyer flasks or 50-mL plastic centrifuge tubes (preferable to remove solids and precipitate from hydrolysate if centrifuging).
 - 10.2.4 Neutralize the aliquot to a pH between 6 and 8 using calcium carbonate. It is very important to neutralize the sample correctly as low and high pHs can ruin an HPLC column or interfere with the spectrophotometric method. The amount of calcium carbonate required to neutralize the sample will depend on several factors, including, but not limited to: aliquot amount and sample buffering capacity. Add calcium carbonate in small amounts and vortex between additions. Allow the sample a short period of time to finish bubbling before checking the pH and/or adding any additional calcium carbonate. DO NOT add large amounts of calcium carbonate at one time, this often leads the sample to froth and turn into a lump of precipitate. A precipitate will form as the neutralization process progresses, this is normal.
 - 10.2.5 For both methods of carbohydrate analysis (HPLC or spectrophotometric), the neutralized sample must be either filtered through a 0.2-µm nylon filter

(use the disposable syringes and compatible filters) or centrifuged to remove ALL solids and precipitate. For the HPLC method, place the filtered or centrifuged hydrolysate in a clean, labeled HPLC vial (1.5-mL vials) and cap tightly with a crimp cap (caps are considered tight when they cannot be turned by hand). For the spectrophotometric method, place the filtered or centrifuged hydrolysate in a clean, labeled vial and cap until needed.

10.3 HPLC analysis for monomeric sugars

- 10.3.1 Prepare a series of calibration standards containing the compounds to be quantified. Refer to Tables 1 and 2 for suggested concentration ranges and sugars. Use at least a four-point calibration, for example, 0.05, 0.25, 1.25, and 4 mg/mL with a 2.5 mg/mL calibration verification standard (CVS) according to the dilution table below:

Table 1: Suggested Dilutions for Calibration Curve Sample Preparations.

Concentration of stock (mg/mL)	Dilution (stock + water)	Final concentration (mg/mL)
10	400 + 600	4
10	125 + 875	1.25
1	250 + 750	0.25
1	50 + 950	0.05
10	250 + 750	~2.5 (CVS)

Table 2: Suggested Standard Concentrations for the HPLC. Choice of monosaccharides based on SP0810 column chromatography and is not an accurate reflection of algal monosaccharides. (For a full list of algae-specific monosaccharides and suggested calibration mixtures, see reference [3]).

Component	Suggested concentration range (mg/mL)
D(+)glucose	0.05-4
D(+)xylose	0.05-4
D(+)galactose	0.05-4
L(+)arabinose	0.05-4
D(+)mannose	0.05-4
CVS	~2.5

10.3.2 Prepare an independent CVS for each calibration set. The CVS should come from a different sugar stock solution than the standards. The concentration of the CVS should fall somewhere in the middle of the chosen calibration range. The CVS should be run immediately after the calibration, after every 10 samples, and at the end of the HPLC run. The CVS is used to verify the quality and stability of the calibration curve throughout the run.

10.3.3 Analyze the calibration standards, CVS, and samples by HPLC using a Shodex sugar SP0810 column equipped with an appropriate guard column (keep the guard column out of the column compartment; it should be kept at room temperature) and a refractive index detector. Be sure to flush both the column and guard column if they are new. Flush them separately into a waste container. DO NOT flush them into the detector or flush the guard column into the sugar separation column. Running at the method temperature and maximum flow rate for approximately 1 hour should be sufficient.

- Sample volume: 50 μ L
- Mobile phase: 18.2 MegaOhm ($M\Omega$) water, 0.2 μ m filtered
- Flow rate: 0.6 mL/min
- Column temperature: 85°C
- Detector temperature: 55°C
- Run time: 50 minutes

NOTE: Run time may be extended if there are late eluting compounds and/or the addition of post-run time may be necessary to elute sugar breakdown products from the column.

10.3.4 Quantify the monomeric sugars by Chemstation (Agilent) or equivalent LC software. Correlation for each calibrated compound should be 0.9999 or better.

10.4 Spectrophotometric analysis for monomeric sugars using MBTH

10.4.1 Preparation of reagents/solutions

- 0.5M NaOH solution – store at room temperature and remake once a precipitate forms
- 3 mg/mL MBTH – store in the dark at 4°C for up to one week
- 1mg/mL DTT – store in the dark at 4°C for up to one week
- Ferric solution: 0.5% ferric ammonium sulfate dodecahydrate, 0.5% sulfamic acid (w/v), and 0.25M HCl. Dissolve 200 mg of ferric ammonium sulfate dodecahydrate and 200 mg of sulfamic acid into 40 mL of 0.25M

HCl. This solution may be stored at room temperature in the dark for up to one month.

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

- MBTH working solution: 1:1 dilution of 3 mg/mL MBTH and 1 mg/mL DTT

10.4.3 Preparation of standards:

- Prepare separate glucose stock solutions for the standards and CVS. The standard and CVS stock 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 at 4°C for up to two weeks.
- 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 x 100 mm or equivalent).

Table 3: Suggested Standard Concentrations for the Spectrophotometric Method.

Concentration of stock (mg/mL)	Dilution (stock + water)	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 μ L CVS glucose stock solution (0.25 mg/mL) and 425 μ L 18.2 MegaOhm (M Ω) water. Prepare the CVS dilution directly into the glass reaction vials.

10.4.4 Reaction

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

- Prepare sample dilutions directly in the glass reaction vials: start with a 1:5 or 1:10 dilution of the filtered/centrifuged neutralized 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 1:10 dilution. The goal is to have the sample absorbance fall in the middle of the calibration curve. A 1:5 dilution would be 100 μ L of filtered/centrifuged neutralized hydrolysate and 400 μ L 18.2 MegaOhm (M Ω) water.
- ALWAYS run a set of standards and a CVS with each batch of samples.
- Add 500 μ L 0.5M NaOH to each glass tube.
- Add 500 μ L MBTH working solution (section 10.4.2) 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. 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 10.4.1).
- 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 MegaOhm (M Ω) 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 MegaOhm (M Ω) 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 1:5, use 100 μ L) and then get a total carbohydrate content by multiplying that concentration by the total volume from the hydrolysis –

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 the HPLC calibration.
- 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 HPLC method).
- 11.5 Calculate the amount of monomeric (structural) sugar in the sample on a percent ODW basis:

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

- 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

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

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

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

where:

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

n = number of samples in set

x_i = measured value from the set

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.
- 14.5 *Sample Size* – 25 ± 2.5 mg for each replicate.
- 14.6 *Sample Storage* – Hydrolysis liquors may be separated from the acid insoluble solids and stored (still acidic) for up to two weeks in a refrigerator (4°C). Neutralized liquors may be stored in a refrigerator (4°C) for up to 4 days.
- 14.7 *Standard Storage* – HPLC 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

16. References

[1] ASTM E1758-01 “Standard Method for the Determination of Carbohydrates by HPLC.” *2003 Annual Book of ASTM Standards*, Volume 11.05. Philadelphia, PA: American Society for Testing and Materials, International.

[2] Van Wyche, S.; Laurens, L.M.L. *Determination of Total Solids and Ash in Algal Biomass*. NREL/TP-5100-60956. Golden, CO: National Renewable Energy Laboratory. 2013. <http://www.nrel.gov/docs/fy14osti/60956.pdf>

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

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