Summative Mass Analysis of Algal Biomass – Integration of Analytical Procedures

Laboratory Analytical Procedure (LAP)

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Technical Report
NREL/TP-5100-60943
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1. Introduction

1.1 This document guides the integration of laboratory analytical procedures (LAPs) to measure algal biomass constituents in an unambiguous manner and ultimately achieve mass balance closure for algal biomass samples. Many of these methods build on years of research in the algal biomass analytical field. By combining the appropriate LAPs, the goal is to break the biomass sample down into constituents that sum as close to 100% by weight as possible. Some of these constituents are individual components, such as individual carbohydrates and lipids as total fatty acids, and some can be groups of compounds, such as extractable lipids. The goal of these analyses is to characterize all of the constituents in the sample, but we recognize that with the current state-of-the-art methods the summative mass accounting does not achieve 100%, and the degree to which biomass can be described is highly dependent on the origin, strain, and cultivation conditions of the algae. This document covers the summative mass balance measurements for algal biomass only. These methods have been extensively applied to a variety of different strains of algae, but as of the publication of this document, the measurements for carbohydrates have not been optimized. The carbohydrate work is ongoing and will be incorporated in future versions after being published.

1.2 This document is designed to help with the complete analysis of the constituents of algal biomass samples by providing the rationale for each of the methods and corresponding calculations. The integrated data reporting and summative calculations with reporting of measurement quality control (QC) should follow the format of a designated spreadsheet to ensure consistent data reporting. A National Renewable Energy Laboratory (NREL) version of the spreadsheet is available upon request.

1.3 This overall mass balance analysis integration takes into account the detailed procedures established and described in the following individual LAPs:

1. *Determination of Total Solids and Ash in Algal Biomass* [1]
2. *Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by* in situ *Transesterification* [2]
3. *Determination of Total Carbohydrates in Algal Biomass* [3]
4. Calculation of N-to-Protein Conversion Factor [4–7]
5. Starch analysis (Megazyme procedure) [8]

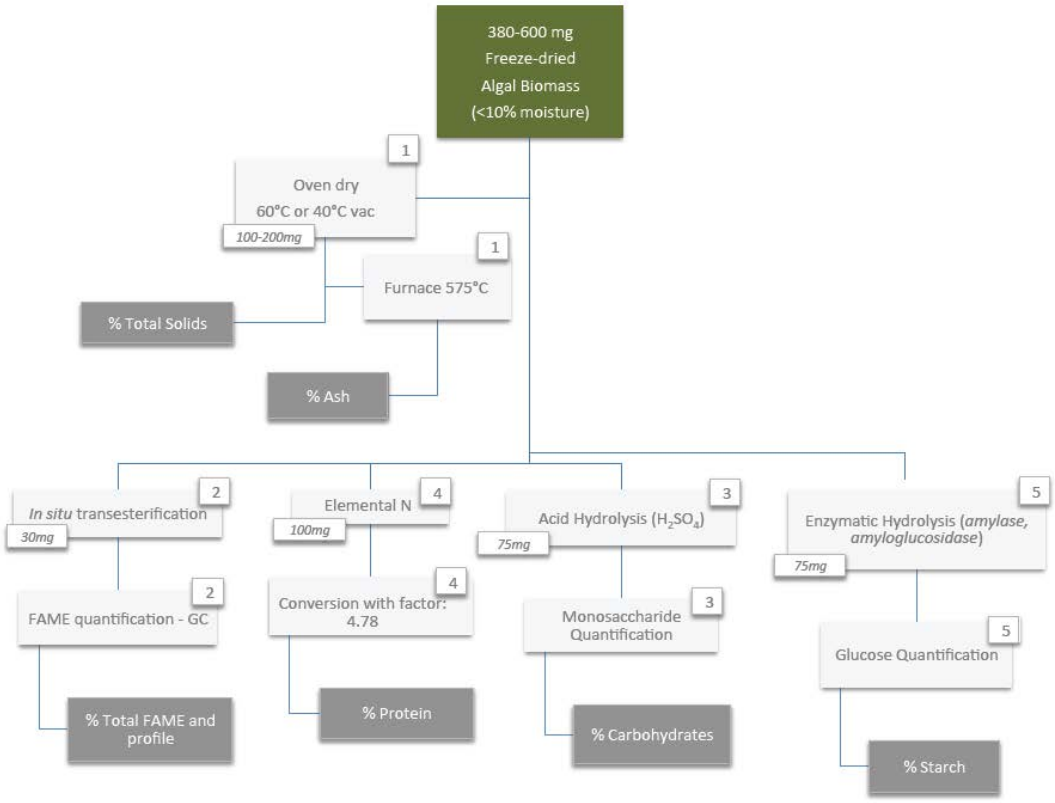

Figure 1. Illustration of summative algal biomass analysis and reference to individual LAPs for analysis details. Numbers in boxes refer to individual LAPs identified in section 1.3.

2. Scope

2.1 This overview of analytical method integration is developed and optimized specifically for microalgal biomass using a combination of the methods for ash, protein, lipids, and carbohydrates (storage and structural). The purpose of the integration LAP is to provide the background and connectivity information on the individual procedures.

3. Terminology

3.1 *Oven Dry Weight (ODW):* The weight of biomass mathematically corrected for the amount of moisture present in the sample.

3.2 *Lipids:* Based on the definition of lipids as "fatty acids and their derivatives" [9], lipid determination as total FAME is an accurate reflection and quantification of the "biofuels potential" of algal biomass. In the context of algal biofuels, the total FAME content is a good approximation; however, it is known that with the presence of polar lipids, such as glyco- and phospholipids, the total FAME content is an underestimation of the lipid content [10]. Alternatively, lipids can be extracted with organic solvents, and gravimetric determination of the extracted fraction can give an approximation of

the lipid content. However, it is necessary to state that an extraction-based determination of lipids can include non-fuel-relevant components, such as pigments, hydrophobic amino acids, and an array of other compounds. The contribution of these compounds to the gravimetric yield will bias high the biofuels potential of the algal biomass. Due to a lack of standardization, extraction procedures will not be included in future work. In addition, extraction-based lipid quantification may also be incomplete, as has been demonstrated in *Chlorella* sp., where the biomass becomes more recalcitrant to extraction under specific cultivation conditions and the majority of the lipids remain associated with the residual biomass [10].

3.3 *Transesterification:* The process of exchanging the organic group of an ester in lipid molecules with the organic group of an alcohol.

3.4 *Fatty Acid Methyl Esters:* The result of the transesterification of lipids, where a methyl group from methanol forms an ester bond with a fatty acid.

3.5 *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.

3.6 *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 and reflects any portion of α-1,4 linked glucose polymer in algal biomass.

3.7 *Protein:* Sum of monomeric soluble and structural amino acids entrained in biomass and measured as either total amino acids by acid hydrolysis or as elemental nitrogen multiplied by a conversion factor calculated based on the ratio of proteinaceous and non-proteinaceous nitrogen in the biomass.

4. Preparation of Algal Biomass prior to Analysis

4.1 Algal biomass samples for compositional analysis are typically frozen and freeze-dried immediately upon harvesting from the culture to preserve the sample and biochemical integrity and avoid degradation of the constituents by residual enzymatic activity.

4.2 The biomass should be finely ground (particle size <100 µm ideally, but less than 1 mm is acceptable) and homogenized prior to analysis, because most of the measurements require the dispensing of small quantities of biomass (~10 to 20 mg), and the aliquot of biomass should accurately reflect the composition of the larger sample.

5. Determination of Total Solids

5.1 Due to the high variability of moisture content in algal biomass feedstocks, all biomass compositional analysis results are reported on a dry weight basis. This allows for

comparison of biomass samples on a consistent basis. At several points in the compositional analysis suite, a "% total solids" determination is required. The LAP *Determination of Total Solids and Ash in Algal Biomass* [1] details appropriate procedures for such determinations. The LAP describes drying by convection oven at 60°C at atmospheric pressure or 40°C vacuum oven. Due to the large fraction of volatiles in algal biomass, moisture determination for algal biomass should not be performed with an infrared balance. Data obtained by this procedure did not correspond with the longer procedure of solids and moisture determination at 60°C at atmospheric pressure or 40°C vacuum.

5.2 The sample aliquot used for drying at 60°C has been exposed to elevated temperatures and oxygen environment and should not be used in further analyses (e.g., lipid degradation can be considerable after atmospheric pressure drying at 60°C), with the exception of determining ash content.

5.3 The sample's ODW should be calculated as follows and carried through to all further biomass measurements:

$$ODW_{sample} = \frac{(Weight_{\ air\ dried\ sample} \times \%\ Total\ Solids)}{100}$$

6. Determination of Ash

6.1 Inorganic materials are present in algal biomass samples. In addition to contributing significantly to total mass closure, inorganic material may interfere with acid hydrolysis. The LAP *Determination of Total Solids and Ash in Algal Biomass* [1] describes two methods for the determination of % ash in biomass. The LAP provides instructions for ash determination using a muffle furnace set to 575°C with prior pre-ignition of the biomass in crucibles over an open flame, and describes the use of a ramping muffle furnace with no pre-ignition.

6.2 Calculation of the sample's ash content should be performed as follows (expressed on an ODW basis):

$$\%Ash = \frac{(Weight_{\ crucible+ash} - Weight_{crucible})}{ODW_{sample}} \times 100$$

7. Determination of Protein

7.1 Conversion of elemental nitrogen to protein

Algal biomass can contain a significant amount of protein (up to 40% ODW); therefore, accurate analysis is imperative for summative mass balance analysis. Measurement of protein in biomass is performed indirectly by measurement of nitrogen content and use of a nitrogen-to-protein conversion multiplier. A factor of 4.78 from the literature [4, 5] is generally applicable and has been confirmed by amino acid determination in

biomass. Ideally, the calculation of a conversion factor for each individual algal strain should be carried out and is typically done by measurement of total amino acid content in the biomass and calculating the conversion factors according to reference [6]. Data from preliminary testing indicated that total nitrogen determinations via combustion or Kjeldahl determination are equal, so either method can be applied.

Protein content through nitrogen-to-protein conversion is calculated as:

$$\%Protein = \%N \times Nfactor$$

where *%N* is elemental nitrogen content determined by combustion or Kjeldahl methods, and *Nfactor* is the specific conversion factor determined for algae (4.78).

7.2 Protein determination as amino acids

There are published methods [7] that explicitly describe a standard amino acid measurement for acid stable amino acids. It may be necessary in some instances to report the concentration of individual amino acids for biomass.

8. Determination of Lipids

8.1 Lipids as FAME by *in situ* transesterification

With this method, lipids are determined as FAME after direct, *in situ* transesterification of algal biomass with an acid catalyst—hydrochloric acid in methanol [2]. This measurement accurately reflects the total biofuels potential of algal biomass and is not influenced by the efficiency of a solvent extraction step. The challenge with accurate lipid quantification as FAME is the utilization of a gas chromatograph (GC) calibration that includes most, if not all fatty acids typically found in algae. The LAP *Determination of Total Lipid as Fatty Acid Methyl Esters (FAME) by* in situ *Transesterification* [2] describes a suggested standard to be used for these analyses and an approach to quantify a selection of unknown fatty acids by transferring response factors from known calibration compounds.

Lipids as total FAME are calculated from the sum of the FAME concentration determined by GC and after normalization for the internal standard present in the reaction as described in the LAP:

$$\%\,Total\ \ FAME = \frac{Total\ \ FAME_{\ C13\ \ normalized}}{ODW_{sample}} \times 100$$

9. Determination of Storage and Structural Carbohydrates

9.1 Determination of starch in algal biomass

Starch determination is relevant for whole biomass analysis because of the often major contribution of storage carbohydrates to the total carbohydrate content and the need to distinguish these carbohydrates from the total acid-soluble carbohydrate determination described in section 9.2. The procedure used to determine starch in algal biomass is substantially similar to and based on the method distributed by Megazyme for the K-TSTA enzyme cocktail mixture of amyloglucosidase and α-amylase and can be found online [8]. The released glucose after enzymatic degradation can be measured using three different methods, two spectrophotometric assays (glucose oxidase-peroxidase [8] and 3-methyl-2-benzotheazolinonehydrazone [MBTH] [11]) and one high-performance liquid chromatograph (HPLC)-based method.

The measured monomeric glucose concentration should be corrected back to anhydrous polymeric starch using a factor of 0.9 (glucose in polymeric glucan [MW = 162] / monomeric glucose [MW = 180] = 0.9), and the measurement should be corrected for the recovery of a QC starch sample included in the assay set as shown in the calculation below:

$$\%\,Starch = \left(\frac{Glucose_{\,(mg/mL)} \times Vol_{\,(mL)} \times 0.9}{\%Recovery_{QC\ Starch} \times ODW_{sample}} \right) \times 100$$

When this measurement is performed in addition to the total carbohydrate determination, the starch content should be subtracted from the total carbohydrate content prior to reporting mass balance analysis data.

9.2 Determination of total carbohydrates in algal biomass

Total carbohydrates in algal biomass are determined as monosaccharides present after inorganic acid hydrolysis of the complete biomass. The hydrolysate is neutralized and filtered and the monosaccharide concentration is quantified either by HPLC or high-performance anion exchange chromatography (HPAEC) [12] or a suitable spectrophotometric method is run on the sample (such as MBTH [11]) as described in the LAP *Determination of Total Carbohydrates in Algal Biomass* [3].

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

10. Calculations

10.1 Each of the respective measurements is reported on an ODW biomass basis and should be summed to provide the summative mass analysis (assuming that the total carbohydrate measure is corrected for starch content as described in section 9.2):

$$Total\ \ Mass_{(\%ODW)} = Ash_{(\%ODW)} + Protein_{(\%ODW)} + Total\ \ FAME_{(\%ODW)} + Starch_{(\%ODW)} + Carbohydrates_{(\%ODW)}$$

10.2 The integrated data reporting and summative calculations with reporting of measurement QC should follow the format of a designated spreadsheet to ensure consistent data reporting.

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

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

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

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. Report Format

11.1 Report the results on the appropriate basis (e.g., ODW) as specified in individual LAPs and in the space provided in the standardized accompanying spreadsheet.

11.2 For the summative mass accounting, a dedicated mass analysis spreadsheet is available that includes the capture of the respective primary data along with QC of the data (e.g., calibration statistics).

11.3 For replicate analyses of the same sample, report the average and the standard deviation for each measurement as detailed in the summary spreadsheet and keep track of historical data using control charts to observe the instrument and analysis trends of a standard biomass sample.

12. Precision and Bias

12.1 Precision and bias of the summative mass analysis will follow the precision of the individual measurements and will need to be determined by a round robin experiment using standard method verification biomass. The targeted relative standard deviation and relative percent difference (RPD) should be less than 10%.

12.2 Improper sample preparation and/or storage may bias the results low due to the degradation of oxygen or heat-sensitive lipids.

12.3 When the respective measurements are summed to investigate the whole biomass analysis, the sum should not exceed 100%. If it is noted that the sum exceeds the maximum, there is either significant interference in the measurement chemistry (possible in the spectrophotometric carbohydrate analyses and the nitrogen conversion) causing the individual measurements to be biased high, or the measurements are not selective and double counting of constituents could be the case. Either case should be investigated in more detail, and summative mass analysis data exceeding 100% should not be reported.

13. Quality Control

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

13.2 *Replicates:* Run all analytical procedures on replicate biomass aliquots as described in the respective LAPs.

13.3 *RPD Criteria:* Refer to respective LAPs for the analytical procedures for quality and precision limits.

13.4 *Sample Size:* A total of between 380 and 600 mg of dried, homogenized biomass is sufficient for mass analysis using the approximate amounts shown in Figure 1. If running analyses in singlets, less biomass is required.

13.5 *Control Charts:* Quality assurance/QC material should be included and periodically control charted to verify reproducibility and highlight trends and bias in the data.

14. Appendices

14.1 Analytical instrumentation needed for all LAPs:

- Analytical balance (0.1 mg accuracy)
- Combustion N-analyzer
- Furnace (575°C) + porcelain crucibles
- Convection drying oven (60°C) or vacuum oven (40°C)
- GC (split/splitless injection, with flame ionization detector or mass spectrometry detection, capillary column with polyethylene glycol-phase—e.g., WAX column)
- HPLC (with refractive index detection and Shodex sugar SP0810 or Dionex PA-1 anion exchange or equivalent column) or spectrophotometer
- Autoclave
- Dry block
- Consumables as detailed in the procedures

14.2 List of revisions/updates:

- Distribution of May 16, 2013, DRAFT version as part of ATP^3 (Algae Testbed Public-Private Partnership) Consortium analytical harmonization effort

- Revision July 26, 2013, updated to remove extractable lipid references and flowchart
- Revision December 2, 2013, updated with NREL-relevant text and updated references for publication
- Revision December 29, 2015, updated methods and references

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