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Algal Biomass Constituent Analysis: Method Uncertainties and Investigation of the Underlying Measuring Chemistries

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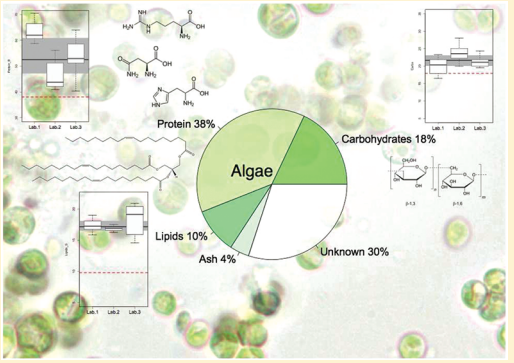
Supporting Information

ABSTRACT: Algal biomass compositional analysis data form the basis of a large number of techno-economic process analysis models that are used to investigate and compare different processes in algal biofuels production. However, the analytical methods used to generate these data are far from standardized. This work investigated the applicability of common methods for rapid chemical analysis of biomass samples with respect to accuracy and precision. This study measured lipids, protein, carbohydrates, ash, and moisture of a single algal biomass sample at 3 institutions by 8 independent researchers over 12 separate workdays. Results show statistically significant differences in the results from a given analytical method among laboratories but not between analysts at individual laboratories, suggesting consistent training is a critical issue for empirical analytical methods. Significantly different results from multiple lipid and protein measurements were found to be due to different measurement chemistries. We identified a set of compositional analysis procedures that are in best agreement with data obtained by more advanced analytical procedures. The methods described here and used for the round robin experiment do not require specialized instrumentation, and with detailed analytical documentation, the differences between laboratories can be markedly reduced.



Algae have the potential to contribute significantly to the biofuels pool of the future; therefore, a need exists to understand the chemical composition of the algal biomass. There are many advantages to algal biomass-based biofuels but also a lot of challenges associated with rendering the process economical, most of which have been part of recent reviews of the literature.[1−3] While a large amount of current algal biofuels research is focused on the biology and engineering aspect of algal biofuels production processes, projects like the Sustainable Algal Biofuels Consortium (SABC) focus on the downstream processing and conversion of algal biomass and thus place an emphasis on accurately and rapidly tracking components throughout the conversion process. Knowing the uncertainty associated with each of the measurements as well as understanding the measurement chemistries will allow for accurate quantification of yields of fuels and fuel intermediates and identify pathways for utilizing the nonlipid components of algal biomass.

The goal of the SABC project is to evaluate biochemical conversion as a potentially viable strategy for converting algal biomass into lipid-based and carbohydrate-based biofuels and to evaluate the fit-for-use properties of those algal derived fuels and fuel intermediates. The SABC approach includes preparing a collection of algal species grown and harvested under different conditions, determining the composition of these algal feedstocks, and exploring multiple routes to convert untreated or pretreated whole algal biomass, oil extracts, and algal residuals into fuels or fuel intermediates. In the processes under investigation, a consistent set of algal biomass analysis procedures is necessary to measure the conversion efficiency of each route.

Similarly, the composition of lignocellulosic feedstocks is important to biomass conversion technology researchers. A recent study specifically investigated the uncertainties of methods commonly used for the analysis of lignocellulosic feedstocks and found that the uncertainty (as measured by the relative standard deviation, RSD, of replicate measurements) of the methods are in the range of 1−3% for the major components (carbohydrates, extractives, ash, lignin, and

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protein) and between 4 and 10% for the component summative mass closure.[4]

Unlike the analysis of feedstocks for cellulosic biofuels, algal biomass characterization is still in its infancy, and it is expected to be more complex and vary more widely based on species and growth conditions. An overview of algal biomass composition in different species has been described previously;[5] however, a detailed study of the precision and accuracy of the methodology used to generate this data has not been carried out. Analysis methods for algae are sourced from the literature and some have their origin in the 1950s.[6−8] Incremental improvements have been made to some methods; however, a detailed comparison of different procedures and investigation of the chemistry for each of the methods has not been carried out.

The objective of this work is to study the applicability of a set of methods for compositional analysis of algal biomass with regards to accuracy and precision, with the aim of providing guidance for standardizing the methodology used for reporting on algal biomass composition between different analysts and institutions. Without agreement in the research community at large, the reporting of algal oil, protein, and carbohydrate content can be inadvertently misleading, often under- or overestimating the actual constituent content. Overestimation of lipid productivity has led to concerns of unnecessary and unhelpful hype surrounding the algal biofuels industry, and standardized accurate methods could help remove the industry from the stigma of poor credibility. In the context of the SABC project, where a rapid quantification of the biomass constituents is important, we have set out to develop a set of procedures to determine algal biomass constituents in a manner that is easily applicable across different laboratories. Standardization of routine measurements ensures reproducibility between collaborators on interlaboratory projects.

To our knowledge, there has not been a report in the literature that specifically addressed the precision and accuracy associated with the algal biomass analytical procedures. Most of the methods researchers are using are empirical in nature; the results depend on the way the methods are run, where small differences in procedures can result in large differences in results. We designed a round robin experiment that allowed us to look at differences between laboratories, researchers within a laboratory, and between different days when an analysis is performed. One biomass sample was selected as standard reference material, so all data from participating analysts and methods can be compared across individual laboratories. For this work, 8 analysts from 3 laboratories (subsequently identified as Lab.1, Lab.2, and Lab.3) executed 5 replicates of each procedure on 2 different days, permitting the evaluation of differences among laboratories, researchers, and experimental analyses.

MATERIALS AND METHODS

Algal Biomass Material. A large quantity (>5 kg) of homogenized, freeze-dried algal biomass was purchased from a commercial source (IGV, http://www.igv-gmbh.com, Germany, August 2008). The biomass sample was grown under nitrogen replete conditions, harvested, frozen, and lyophilized prior to storage. The biomass sample was homogenized to $a <$ 0.1 mm particulate size, and 100 g aliquots were distributed among project participants. The biomass was stored in laboratory freezers (−20 °C) at each of the participating laboratories until ready for analysis.

Description of the Round Robin Procedures: Moisture and Ash Determination (Ash and Moisture). We determined the moisture and ash content in algal biomass according to the published NREL Laboratory Analytical Procedures (LAP, included as Supporting Information).[9] Algal biomass (100 mg) was weighed into ceramic crucibles and dried overnight in a drying oven (105 °C). The next day, the crucibles were cooled to room temperature in a desiccant chamber and weighed to determine moisture content. Ash determination was performed using one of two methods (both described in the LAP); precombustion of oven-dried algal material over a Bunsen burner followed by placement in a muffle furnace (575 °C) until constant weight (Lab.1 and Lab.3) or a placement in a muffle furnace with a temperature ramping program (Lab.2) until constant weight.

Lipid Extraction Using Chloroform−Methanol Soxhlet (Lipids_S). A Soxhlet-based lipid extraction procedure was modified from Guckert et al.[10] and optimized for the isolation of lipids from ground and lyophilized algal biomass. Algal biomass (200 mg) was weighed into single thickness cotton cellulose thimbles and covered with a glass fiber filter. A total of 210 mL of chloroform−methanol (2:1, v/v) was refluxed over the thimble between 12 and 20 h at a siphon rate of 6−8 times per hour using a standard Soxhlet apparatus. The extracts were then quantitatively transferred and brought up to 250 mL with chloroform−methanol (2:1, v/v). The extract was transferred to a separation funnel, and a 0.7−0.75% NaCl (aq) solution was mixed with the aliquot at a final ratio of 8:4:3 chloroform/methanol/NaCl (aq) to remove the nonlipid material from the solvent, similar to the washing procedure described in Folch et al.[11] The resulting biphasic mixture was allowed to settle for 12 h. After settling, the lower (organic) phase containing the lipids was drained into a preweighed round-bottom flask and the solvent was removed using vacuum rotary evaporation at 30−35 °C. The round-bottom flasks were then placed in a 40 °C vacuum oven for further drying. The lipid content was determined gravimetrically.

Lipid Extraction Using DMSO−Ether−Hexane (Lipids_B). A manual extraction procedure was carried out according to Bigogno et al.[12] Algal biomass samples (100 mg) were extracted three times in a screw-cap vial with 5 mL of methanol containing 10% DMSO, by warming to 45 °C for 5 min and stirring at 45 °C for another hour. The mixture was centrifuged, the supernatant removed, and the pellet was re-extracted three times with a mixture of hexane and diethylether (1:1, v/v). Water was added to the combined supernatants to separate the organic phase. The mixture was shaken and then centrifuged for 5 min at 1081 rcf (Lab.1) and 2977 rcf (Lab.2), and the upper phase was collected. The water phase was re-extracted twice with a mixture of diethyl ether−hexane (1:1, v/v). The organic phases were combined and evaporated to dryness under a stream of nitrogen at 30−35 °C followed by freeze-drying. The lipid content was determined gravimetrically.

Carbohydrates by Phenol−Sulfuric Acid (Carbs). Biomass (10 mg) was reconstituted in water (10 mL) to prepare a known sample concentration for each sample (1 mg mL^{-1}). Aliquots of 1 mL sample were reacted with 3 mL of concentrated sulfuric acid (72 wt %) and 1 mL of phenol (5%, w/v) in a water bath. The mixtures were incubated for 5 min at 90 °C. The absorbance at 490 nm was then measured using a spectrophotometer. The absorbance measurements were then compared to a standard curve based on glucose.

Starch Determination (Starch_S and Starch_M). We used two commercially available starch analysis procedures, one based on a modification of the Megazyme procedure (AOAC method 996.11)[13] and one from Sigma.[14] The underlying chemistry of both procedures is similar: measurement of glucose released by the enzymatic hydrolysis of the starch polymer. However, the two methods used different hydrolytic enzyme cocktails and glucose measurement methods. One laboratory used the Sigma starch assay (Lab.2) and two laboratories used the Megazyme starch assay (Lab.1 and Lab.3).

In the Sigma starch assay, 500 mg of biomass was mixed with 20 mL of DMSO and 5 mL of 8 M HCl and incubated for 30 min in a water bath set at 50 °C. The solution was adjusted to a pH 4−5 with 5 M NaOH and brought to a known volume (100 mL) with water. An aliquot of the solubilized sample (1 mL) was incubated with a Starch Assay Reagent (containing amyloglucosidase) for 15 min in a water bath set to 60 °C. After incubation, a known volume of the mixture (1 mL) was incubated with a Glucose Assay Reagent (containing NAD, ATP, hexokinase, glucose-6-phosphatedehydrogenase) for 15 min at room temperature as per the manufacturer's instructions. The absorbance of the final mixture was measured on a spectrophotometer at 340 nm (the maximum absorbance of NADH).

In the Megazyme starch assay, 100 mg of algal biomass was treated with 0.2 mL of 190 proof ethanol and DMSO and then vortexed vigorously. This mixture was then placed in a boiling water bath for 5 min prior to the addition of MOPS buffer and thermostable α-amylase. Samples were incubated in boiling water bath for 6 min while being vortexed every 2 mins. Samples were then incubated in a 50 °C water bath after addition of 0.4 mL of sodium acetate buffer and 0.1 mL of amyloglucosidase. Following the incubation, the samples were centrifuged 5 min for 10 min at 937 rcf. The supernatant was collected and analyzed for glucose. A set of duplicate samples was also analyzed without the addition of enzymes to determine free glucose content, and a triplicate set of pure starch standards were run for quality control purposes.

While two laboratories used the Megazyme enzyme cocktail to release the glucose, both laboratories used different methods to measure the released glucose. Lab.3 used the GOPOD (glucose oxidase peroxidase per the manufacturers specifications) spectrophotometric method for measuring glucose, while Lab.1 used HPLC analysis.

Lab.3 added the GOPOD reagent as per the manufacturers instructions and let the samples incubate at 50 °C for 20 min, after which the absorbance was measured at 510 nm. The GOPOD and water control was used as a blank for the absorbance measurements. Lab.1 analyzed glucose by HPLC, equipped with a Biorad Aminex HPX-87H column, using 0.01 N H_2SO_4 as the mobile phase and refractive index detection (RID) (HPLC analytical conditions were as described in Templeton et al.[4]).

Protein Determination (Protein_N and Protein_B). All three laboratories used two different methods. In the first method, the elemental nitrogen content of the sample was measured with a combustion CHNS-analyzer (Dumas method). This value was converted to the protein content using a literature conversion factor of 4.78.[15]

For the Bio-Rad DC Protein assay, based on the Lowry assay,[8] 1 mg of lyophilized biomass was washed with a phosphate buffered saline solution and solubilized in 1 mL of Reagent A, an alkaline copper tartate solution. The solubilized sample was then diluted in more Reagent A (50 μL of sample in 450 μL of reagent) and reacted with 4 mL of Reagent B, a Folin reagent. The mixture was incubated for 15 min and then measured on a spectrophotometer at an absorbance of 750 nm. The absorbances were compared to a standard curve based on bovine serum albumin (BSA).

Description of Advanced Analytical Procedures.

Fatty Acid Determination. Total fatty acid content of the biomass was included as a reference measurement. One laboratory determined the fatty acid content of the lipid extracts obtained with the two different methods (Lipids_B and Lipids_S). The fatty acid methyl ester (FAME) derivatization technique was adapted from Lepage and Roy[16] and optimized with regards to reaction conditions and extraction time and temperature to compare the two lipid extraction techniques. The procedure consisted of dissolving 10 mg of lyophyilized algal biomass sample in 0.2 mL of chloroform−methanol (2:1, v/v), and subsequent transesterification of the lipids in situ with 0.3 mL of HCl−methanol (5%, wt/v) for 1 h at 80 °C in the presence of 250 μg of tridecanoic acid (C13) methyl ester as an internal standard. The resulting FAMEs were extracted with hexane at room temperature for 1 h and analyzed by gas chromatography−flame ionization detection (GC−FID) (Agilent 6890N; HP5 30 m 0.25 mm i.d. and 0.25 μm film thickness; temperature program 70−300 °C over 23 min at 10 °C min^{-1}). This method was applied to whole algal biomass samples, the dried extracts from both lipid extraction procedures, and the extracted (residual) biomass after lipid extraction.

Carbohydrate Determination by Acid Hydrolysis. One laboratory determined the structural carbohydrates in algal biomass according to the published NREL Laboratory Analytical Procedure (included as Supporting Information).[9] In brief, 100 mg of algal biomass was subjected to a two-stage sulfuric acid hydrolysis (1 h at 30 °C in 72 wt % sulfuric acid, followed by 1 h at 121 °C in 4 wt % sulfuric acid in an autoclave). After hydrolysis, the acid insoluble residue was separated from the hydrolysate using ceramic filtering crucibles. Soluble carbohydrates (glucose, xylose, galactose, arabinose, and mannose) were determined by high-performance liquid chromatography (HPLC analytical conditions were as described in Templeton et al.[4]).

Amino Acid Determination. The amino acid content of algal biomass was included as a reference protein measurement and was determined by an external, commercial laboratory using the standard AOAC 994.12 acid hydrolysis method:[17] hydrolysis in 6 M HCl for 18 h at 110 °C to liberate individual amino acids, followed by HPLC analysis.

Statistical Analysis. We used the statistical program R version 2.13.1 to analyze and plot the data.[18] All tests of statistical significance were performed at the 95% significance level ($p < 0.05$). We applied the Tukey outlier test to the data to remove outliers. This test identifies any sample having a value >1.5 times the interquartile range (IQR) less than the lower hinge or greater than the upper hinge. We applied an analysis of the variance test to the data set in a way that the nested experimental design was conserved. All the raw data and the R scripts used to analyze the data can be found as Supporting Information.

RESULTS AND DISCUSSION

To study the accuracy and precision of algal biomass analytical procedures, a total of nine different analytical methods were

Table 1. Overview of the Censored Data and Summary Statistics[a]

	Moisture	Ash	Starch_M	Starch_S	Carbs	Lipids_S	Lipids_B	Protein_B	Protein_N
mean	4.07	4.17	12.05	7.17	21.58	17.46	11.52	53.61	43.67
SD	0.73	0.18	1.94	0.26	2.57	1.41	2.07	8.22	0.84
SDp. institution	0.32	0.13	0.82	0.66	2.08	1.12	0.52	4.6	0.31
SDp. analyst	0.11	0.14	0.83	0.27	1.81	1.05	0.3	4.06	0.32
SDp. day	0.09	0.14	0.66	0.29	1.68	1.09	0.27	3.72	0.34
N	50	60	40	10	57	42	40	44	20

[a]All data is reported on an oven dry weight (ODW) basis. SD = standard deviation, SDp = Pooled standard deviation, *N* = number of measurements per procedure. Starch_M, Starch_S, Lipids_S, Lipids_B, Protein_B, and Protein_N refer to individual analysis procedures used for the respective measurements, and details can be found in the Materials and Methods.

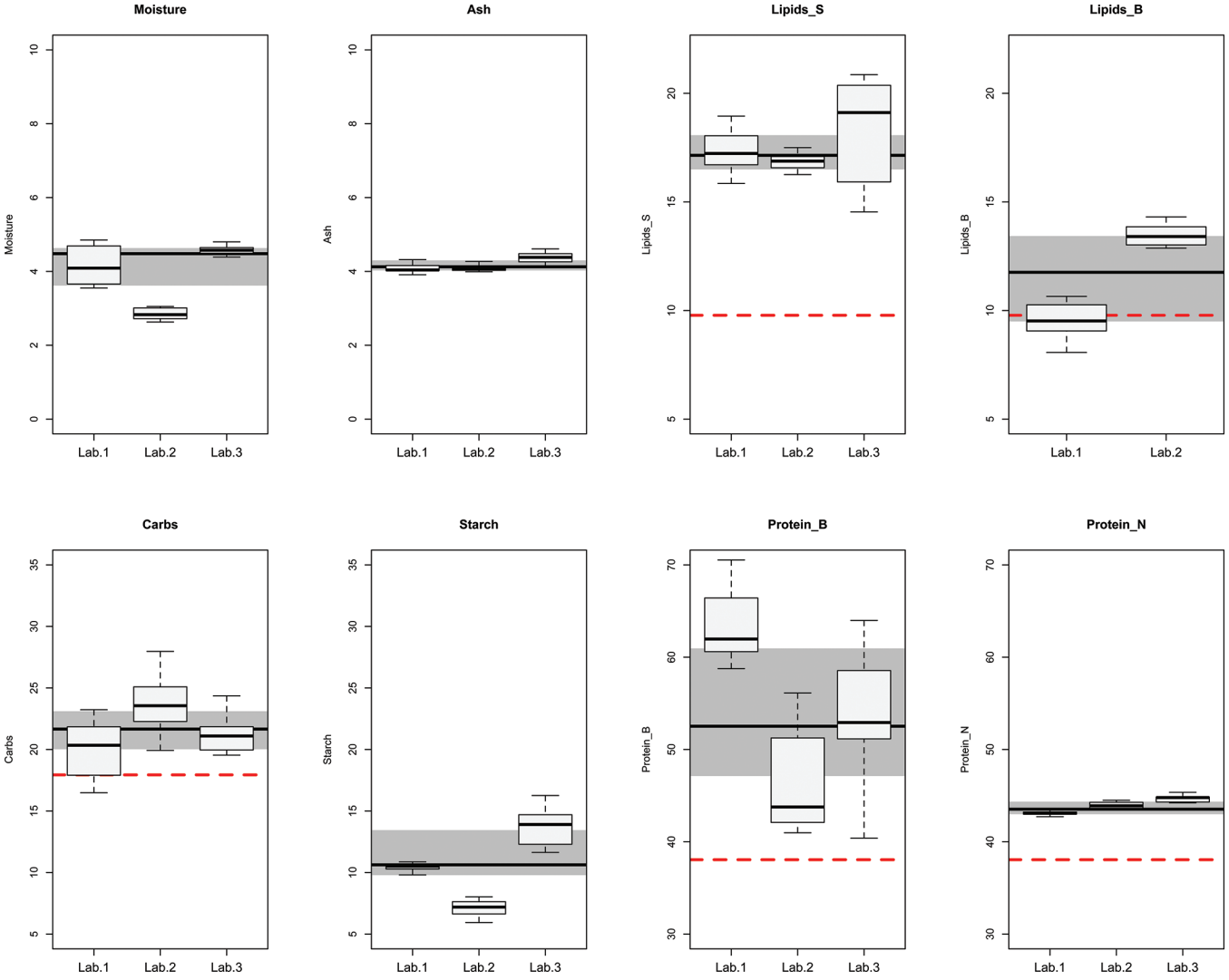

Figure 1. Boxplots for all the procedures investigated in the round robin experiment as an illustration of the variation observed in the data, separated per institution. Moisture, Ash, Lipids_S, (G) Lipids_B, Carbs, Starch (referring to Starch_M for Lab.1 and Lab.3 and Starch_S for Lab.2), Protein_B, and Protein_N. The median value of each data set is shown as a solid horizontal black line, and the interquartile range (IQR) is shown in gray. The dashed red line refers to the reference measurement we used to compare against the empirical methods presented (see text for explanations).

included in a round robin experiment: moisture and ash content, lipids by two different extraction procedures, carbohydrates by two procedures, and starch by two procedures. Methods were carried out as described below; the abbreviated names of the procedure in the round robin experiment are included in parentheses. Two researchers at each laboratory on each of 2 days performed five replicate analyses for 7 of the 8 analytical procedures on a single biomass sample. Not all researchers performed all analyses, so a total of 8 researchers (rather than 6) provided analytical data. For the elemental nitrogen procedure, two laboratories sent the samples to commercial analytical laboratories to perform the measurement and one laboratory had instrumentation to conduct the measurement internally. Of the laboratories that

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Table 2. Significance Analysis of the Variation between and within Laboratories, Analysts, and Days[a]

	institution		analyst		day	
moisture	$<2.2 \times 10^{-16}$	***	$<2.2 \times 10^{-16}$	***	0.000 16	***
ash	1.59×10^{-8}	***	0.992		0.279	
Starch_M	3.34×10^{-14}	***	0.711		0.000 71	***
Starch_S						
Carbs	4.818×10^{-7}	***	0.000 28	***	0.0559	
Lipids_S	0.08377		0.1076		0.9697	
Lipids_B	$<2.2 \times 10^{-16}$	***	2.83×10^{-10}	***	0.0232	*
Protein_B	2.17×10^{-10}	***	0.001 12	**	0.0325	*
Protein_N	0.000 696	***			0.889	

[a]Results are derived from an analysis of variance (ANOVA), and significance levels are indicated as *p*-values. Starch_M, Starch_S, Lipids_S, Lipids_B, Protein_B, and Protein_N refer to individual analysis procedures used for the respective measurements, and details can be found in the Materials and Methods.

used commercial analytical laboratories for the elemental nitrogen analyses, only one set of 5 replicate analyses was obtained per participating laboratory, although one laboratory performed two independent sets of 5 replicates. Each of our laboratories used a different commercial analytical laboratory for elemental nitrogen analysis. The experimental design constitutes a nested experimental design, which allows for statistical significance testing among all the data obtained, within institutions, researchers, and days. The biomass sample used in this study, a *Chlorella* sp., was obtained from a commercial source and is available in sufficient quantities to serve as a standard reference material. In total, 368 analyses were performed on this material in February−May 2011. A summary of the data and associated variation is shown in Table 1, and an illustration of the precision of the data for each of the procedures is shown in Figure 1. The respective measurements can vary significantly between laboratories, and Figure 1 captures the spread in the overall measurement uncertainty and also shows the median value (as a solid black line) with the interquartile range (IQR) for each of the constituents measured (as a gray box). Where appropriate, a reference measurement was included for comparison and is shown as a dashed red line in Figure 1. As will be discussed later, the reference measurements consist of advanced analytical procedures, total fatty acids for lipid quantification, amino acids for protein, and individual sugar measurement after acid hydrolysis for carbohydrate quantification.

Moisture and Ash Determination (Ash and Moisture). We found that the moisture content varied significantly between the different laboratories; this observation can be explained by the environmental differences among the three locations (National Renewable Energy Laboratory in Golden, CO, Arizona State University in Phoenix, AZ and Sandia National Laboratories in Albuquerque, NM) and also between the different days of analysis. Because the moisture content of the algal biomass sample varied over time at each location, a separate moisture measurement was made during the course of all other analyses so that the results of these analyses could be reported on an oven-dry-weight (ODW) basis. In Table 1, all analytical results were corrected for the moisture content and reported on an ODW basis. We report the ash content as a separate measurement because of its relevance in downstream processing and closing the overall component mass balance.

Out of 368 separate analyses, only 5 results were flagged as outliers (3 carbohydrate analyses and 2 lipid analyses using the Soxhlet procedure) using the Tukey outlier test. For all analyses discussed in this paper, the censored data set was used (shown in Table 1.).

Precision and Accuracy of the Methodologies. A measure of precision of the data between different laboratories is the relative standard deviation (RSD) of the replicate measurements, defined as the ratio of the standard deviation of the measurements to the mean value of the measurements. The RSD across all laboratories ranged from ∼2% (for Protein_N) to ∼18% (for Lipids_B) when all the variation (laboratory, analyst, and day) is taken into account. However, the data in the RSD for each individual institution is lower for all 8 measurements. This is likely due to practical differences in the way the procedures were carried out in instrumentation used, which are more likely to be consistent within each laboratory but different between laboratories. Not surprisingly, the RSD is smallest for a single person on a single day. For comparison, the historical precision for methods for compositional analysis of lignocellulosic biomass is in the range of 1−10% of individual measurements.[4]

In order to remove the effect of measurement biases between laboratories, we calculated the pooled standard deviation. For all constituents measured, we found that the variation is smallest within the data from a single analyst and increased when multiple days and laboratories are included. This observation is not surprising in that analysts within one laboratory presumably are trained together and use identical instrumentation, which reduces the overall variation obtained in the data sets.

We performed an analysis of variance (ANOVA) of the data, and the results are shown in Table 2. There are statistically significant differences for all analyses among laboratories. Within each laboratory there were no differences between analysts for Ash, Starch_S, Starch_M, and Lipids_S. However, the significant differences remain between researchers for Carbs, Lipids_B, and Protein_B, suggesting that these may be the least robust of all methods investigated.

The results of the ANOVA must be viewed with some caution, for example, the variability within each lab in ash content was so small that relatively small differences among the laboratories could be detected. These differences, while statistically significant, are so small (mean Lab.1 = 4.079, mean Lab.2 = 4.078, mean Lab.3 = 4.351) that they are not significant in practice.

In addition to differences in the precision associated with the methods, we also found significant differences in the measured values, where multiple procedures were selected for the analysis of one constituent. The mean value of the Lipids_S method

was 34% higher than the mean value of the Lipids_B method, the mean value of the Starch_M method was 41% higher than the mean value of the Starch_S method, and the mean value of the Protein_B method was 22% higher than the mean value of the Protein_N method. It is reasonable to question the accuracy of the procedures and ask what each method is actually measuring and how these differences influence the results.

Differences in Lipid Extraction Methods (Lipids_B and Lipids_S). Lipid quantification is an important part of algal biomass characterization. On the basis of the relatively vague definition of lipids as "molecules not soluble in water but in organic solvents instead",[19] lipid quantification is traditionally carried out through solvent extraction, for which two methods are routinely used in the algal research laboratories included in this study.[10,12] Because algal lipids are a complex mixture of polar and nonpolar molecules, the polarity of the solvent mixtures used will affect the yield of extracted lipids. In this context, a more accurate definition of lipids is "fatty acids and their derivatives". In addition, the extraction process may not remove all lipids and may extract nonlipid components (e.g., chlorophyll and other pigments, proteins, and soluble carbohydrates). A comprehensive comparison of lipid extraction efficiency and comparisons is outside the scope of this work; however, we did study the accuracy and precision of two methods that were used. We based this on the partitioning of the fatty acids between the extracted fraction and the residual biomass to measure the degree of completeness of the extraction process.

The two gravimetric extraction methods studied here provided significantly different results (10.4% for Lipids_B and 16.3% for Lipids_S). While these results are not surprising since the solvent systems used in each method were very different, it reinforces the fact that gravimetric extraction methods are empirical and highly dependent on experimental parameters. To investigate this discrepancy more fully, we measured the fatty acid content as fatty acid methyl esters (FAMEs) of the starting algal biomass material by *in situ* transesterification, a method used more frequently to measure total lipid content in algal biomass by GC.[20] We also measured the FAME in the dried solvent extract by transesterification of the lipids, a common measure of the fuel value of algal lipids. The results are shown in Table 3. Neither the Lipids_S nor the Lipids_B procedure is complete in the extraction of all the lipids as measured by FAME analysis. As the data in Table 3 indicate, the Lipids_S procedure extracts 83% of the fatty acids without washing and 61% with washing, while the Lipids_B method only extracts 46%. The relatively low recovery of the total fatty acids also coincides with an overall lower gravimetric recovery, which gives the appearance of overlap of the gravimetric yield with the reference measurements shown in Figure 1 (dashed red line). However, one has to keep in mind that the data shown for lipids in Figure 1 for Lipids_B and the reference measurement represent different types of analyses and does not necessarily indicate that Lipids_B is a more accurate measure of lipids. The washing step for the Lipids_S procedure was included to remove nonlipid components from the extract; however, data in Table 3 show that this step is also removing fatty acids and thus the washing step should be excluded from future gravimetric lipid measurements. Clearly neither of the gravimetric solvent extraction procedures we tested provided full recovery of all fatty acids that were measured using the *in situ* procedure. If accurate determination of the potential fuel value of algal biomass is important, then FAME analysis of whole biomass algae samples, while more labor- and time-intensive, is necessary. This method has been mentioned previously in the context of a rapid measurement of fatty acids in algae as more accurate relative to extraction-based lipid measurements. However, lipid quantification by solvent extraction is a relatively quick process and does not require advanced instrumentation and thus can be carried out by a large number of laboratories.

Table 3. Fatty Acid Content (% ODW) in the Lipid Extract and Residual Biomass Fractions for Two Lipid Extraction Procedures[a]

method	extract (% DW)	FAME extract	recovery (%)
Lipids_S (unwashed)	17.63 ± 0.34	8.12 ± 0.02	83.0
Lipids_S (washed)	16.33 ± 0.18	6.00 ± 0.02	61.3
Lipids_B	10.40 ± 0.22	4.49 ± 0.01	45.9

[a]The FAME content of the algal biomass was 9.78%, measured via *in situ* derivatization. The recovery is the sum FAME content of the extract and residual biomass divided by the *in situ* FAME content (expressed as %). The FAME content of the extracted and the FAME recovery both depend on the extraction method; only the Lipids_S procedure without washing provides full accounting of the FAMEs.

Differences in Protein Determination Methods (Protein_B and Protein_N). We found large differences between the colorimetric (Protein_B) and nitrogen-ratio (Protein_N) methods we used for total protein measurements: the Protein_B method gave a value of 53.6% while the Protein_N method gave a value of 43.7%. In addition, Figure 1 illustrates the large amount of variation seen in the colorimetric procedure and the more precise data obtained by the elemental nitrogen measurement. The colorimetric procedure is based on the measurement of a color change caused by oxidation of aromatic amino acid residues (mainly tyrosine, tryptophan, and cysteine) by the Folin reagent.[8] The oxidation reaction is susceptible to interferences and is highly dependent on the protein standard used for calibrating the absorbance values, in this work bovine serum albumin (BSA). Measuring elemental nitrogen is based on combustion and is not susceptible to interferences and can be automated.[21] An overall average ratio factor of 4.78 g of algal protein to detected grams of elemental nitrogen has been reported for algal protein quantification.[15] This conversion factor is based on a literature value, which was calculated as an average between 12 strains of algae, grown under a range of different environmental conditions. This factor is a good approximation, but since it is a calculated average, it will vary from the actual protein content in the strain we have investigated.

The colorimetric procedure has been used extensively in the microbiological research community. However, when using this method on algal biomass, one has to make a couple of assumptions with regards to (i) the accessibility of the proteins (and associated aromatic residues) for reaction with the Folin reagent, (ii) the absence of interfering substances in the biomass, and (iii) the applicability of the standard protein used for calibration of the absorbance. The limitations of the Lowry procedure have been reported before.[22−24]

To investigate the accuracy of these two methods (e.g., which of these measurements approximates best the actual protein content), we report the measured amino acid content of the biomass based on chromatographic identification of individual

amino acids after acid hydrolysis (see Table 4).[17] The procedure did not allow the quantification of tryptophan and cysteine because these are notoriously labile during acid hydrolysis. However, taking the lack of these two amino acids into account, we assume that the sum of the reported amino acids, 38%, approximates the actual protein content of the biomass. Comparing the sum of the amino acids with the nitrogen-to-protein conversion value indicates that the latter is a better approximation, with regards to accuracy and precision of the measurement, of the total protein content, compared to the Lowry procedure, which overestimates the protein content by close to 45%. Furthermore, the literature-based conversion factor of 4.78 is an average factor calculated for 12 different strains of algae over a range of different growth conditions. An alternative conversion factor was calculated as an average of 12 strains grown under nitrogen-replete conditions, similar to the growth conditions of our standard reference material. Using this alternative factor of 4.33, we calculated a value of 39% as the total protein content, even closer to the measured 38% amino acid content. Thus, results from the elemental nitrogen measurement followed by conversion using an algae-specific factor provided results more consistent with direct amino acid measurement than did the Lowry procedure. However, we recognize the substantial additional costs involved in amino acid determination relative to the Lowry or nitrogen determination procedure. The nitrogen determination procedure depends strongly on the conversion factor used, but because it is insensitive to non-nitrogenous interferences such as lipids and carbohydrates, it likely could be reliably used for relative protein measurements across a single experiment or for species grown under similar conditions.

Table 4. Amino Acid Content (% ODW)[a]

amino acid	% DW
glutamic acid	4.98
leucine	3.67
lysine	3.65
aspartic acid	3.63
alanine	3.19
arginine	2.78
valine	2.4
phenylalanine	2.17
glycine	2.13
proline	1.98
threonine	1.82
isoleucine	1.6
tyrosine	1.6
serine	1.57
histidine	0.89
total amino acids	38.06

[a]Data are shown as % individual amino acids on a dry weight basis determined as per the AOAC 994.12 method. The amino acids tryptophan and cysteine are labile in this reaction and were not measured in this analysis.

Starch Methods, Variation between Glucose Measurements. For the starch determination, the actual measurement is of the concentration of glucose in solution after enzymatic hydrolysis with α-amylase and/or amyloglucosidase. The glucose measurements were either performed by HPLC (Lab.1) or GOPOD (Lab.3) for the Starch_M procedure and by enzymatic coupling of glucose to 6-phospho-gluconate with the production of NADH (Lab.2) for the Starch_S procedure. This difference in detection of glucose could contribute significantly to the overall quantification. Furthermore, a closer look at the protocols indicated differences in the starch hydrolytic enzymes used. The Starch_S procedure only uses amyloglucosidase, whereas the Starch_M procedure uses both α-amylase and amyloglucosidase. Without further experimentation, it is difficult to conclusively say what causes the significant reduction in total starch measured with the Starch_S procedure, though it is likely that an amylase hydrolysis in addition to the amyloglucosidase is necessary for algal biomass starch measurements.

Because Lab.1 and Lab.3 used different methods of glucose measurements (GOPOD and HPLC) for the Starch_M assay and obtained different values, we investigated whether the assay itself or the glucose measurement was the source of this difference. The glucose concentration from one set of Starch_M samples from Lab.3 was analyzed by both methods. These results showed a small but consistent bias between the HPLC and GOPOD measurements. After correction for this bias, the differences between Lab.1 and Lab.3 are no longer statistically significant, suggesting the glucose measurement rather than the assay itself was the cause of the bias.

Total carbohydrate content measured via a colorimetric phenol sulfuric acid method is widely used. In the method, polysaccharides (carbohydrates) are hydrolyzed into monosaccharides (simple sugars) with concentrated sulfuric acid. The sulfuric acid then dehydrates the monosaccharides further to form furfural and hydroxymethyl furfural. These two compounds then form an aromatic complex with phenol that yields a color change. Although this procedure is rapid and relatively straightforward, it is also highly dependent on the sugar used for the calibration. Not all sugars exhibit a similar colorimetric response; some carbohydrate derivatives do not exhibit any response at all, and thus the carbohydrate content measured by this technique can be an over- or underestimation of the true carbohydrate content.[7]

An alternative carbohydrate quantification procedure that was tested on one set of samples involves a sequential, two-stage acid hydrolysis of sugar polymers with the identification and quantification of the monomers via HPLC. The results from this measurement are shown in Table 5.

Table 5. Individual Sugar Composition after Two-Stage Acid Hydrolysis Followed by HPLC Quantification of Glucose, Xylose, Galactose, Arabinose, and Mannose[a]

sugar	% DW
glucose	11.49 ± 0.10
xylose	0.79 ± 0.05
galactose	4.42 ± 0.08
arabinose	ND
mannose	1.24 ± 0.11
total measured sugars	17.94

[a]Data are shown as the % neutral sugar content after two-stage acid hydrolysis followed by HPLC quantification of glucose, xylose, galactose, arabinose, and mannose. Other sugars were noted in the chromatograms but have not been quantified. ND = not detected.

The estimates of carbohydrates using the phenol-sulfuric acid and two-stage hydrolysis method were 22% and 17%, respectively. Because the two-stage hydrolysis method identifies only the five neutral sugars shown in Table 5 while the phenol-

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sulfuric acid method reacts to a certain extent with all monosaccharides, the results correspond well. The main discrepancy can probably be associated with the relatively high amount of carbohydrates other than glucose (i.e., galactose being the second major monomer) and the difference in response between glucose and galactose in the phenol-sulfuric acid reaction.[7] Furthermore, there is a difference in precision between the Carbs method used in the round robin experiment and the acid hydrolysis procedure. The historical precision of the two-stage hydrolysis procedure is approximately 3% RSD,[4] compared with a 15% RSD for the phenol-sulfuric acid method. The two-stage acid hydrolysis method is more involved and more labor- and time-intensive, and thus the phenol-sulfuric acid Carbs method serves as a reasonable proxy for the carbohydrate determination when keeping the limitations of poor precision in mind.

Mass Balance. The methods used in this cross-laboratory study can be used to estimate a mass closure for the algal biomass by summing ash, lipid, carbohydrate, and protein measurements. We use the Protein_N estimate because it is in better agreement with the amino acid measurement. Using this approach, we can account for 82 or 88% of the total mass, depending on whether the Lipids_B or the Lipids_S measurement is used (Ash = 4.25, Carbs = 22%, Protein_N = 44%, Lipids_S = 17%, or Lipids_B = 11%). Using this summative approach to mass closure, we are implicitly making the assumption that we are not double counting constituents. However, this assumption is not necessarily correct. The Carbs procedure will account for sugars irrespective of where they originated, so fractions of the glycolipids (where carbohydrate moieties are esterified to fatty acids) or glycoproteins could be accounted for in both the Carbs measurement and the Lipids or Protein measurement, respectively. Similarly, both estimates of lipid content are based on a gravimetric lipid extraction yield and this will include nonlipid components (e.g., soluble carbohydrates, hydrophobic proteins).

An alternative way to approach mass closure is to count only clearly identified and quantified constituents (from advanced analytical methodologies), such as total fatty acids (as fatty acid methyl esters), amino acids, monomeric sugars, and ash. When we do this calculation based on currently available information (FAME = 9.8%, monomeric sugars = 17.9%, amino acids = 38.1%, and ash = 4.2%), we can account for 70% of the mass balance. The differences between these approaches are apportioned as follows: ~5% due to protein (38 vs 43%), ~5% due to carbohydrates (22% vs 17%), and 1−7% due to lipids (10% FAME vs 11−18% gravimetric). The unaccounted-for fraction in both mass closure approaches is probably due to the lack of the unidentified carbohydrates (sugar-derivatives such as uronic acids and amino sugars, such as glucosamine, galactosamine, and *N*-acetyl glucosamine) and nucleic acids and unextracted pigments, phospatidic acid associated with the phospholipids, and glycerol moieties from tri-, di-, and monoglycerides. Further work in this area is clearly warranted.

CONCLUSIONS

We applied classical and well-accepted characterization methods to a single algal biomass sample with the objective of finding robust methods that allow rapid measurement of the composition of algal biomass. All the methods we selected are routinely used throughout the algal biomass research community and are empirical in nature, based on classical literature. Different procedures for single analyte measurements are based on significantly different underlying chemistries. We carried out a three-laboratory, 8 analyst, and 24 different day round robin experiment to quantify the precision of these classical procedures for algal biomass analysis. Overall, we found good agreement for some methods and large variability between others. A detailed analysis of the variance associated with the laboratories and analysts pointed to robustness (or lack thereof) of the procedures.

The methods described in this work, especially Ash, Lipids_S, Carbs, Starch_M, and Protein_N, allow for the analysis of algal biomass and do not require specialized instrumentation. However, for accurate mass balance calculations, more advanced analytical methods may be needed. Progress is being made toward a more detailed sugar analysis to include algae-specific carbohydrates, such as uronic acids and amino sugars and lipid quantification methodologies that allow for quantification of the full suite of lipids in algal biomass, including intact phospholipids and glycolipids.

To generate high-quality data on algal biomass, it is important to achieve consensus on methods used. In addition, control measures are needed to ensure the consistency of the generated data. Including a control or reference sample with each batch of analyses provides confidence in the measurements. We were unable to identify the root cause(s) of the differences between the laboratories; we anticipate that with improved procedure documentation, analyst training, and actual method improvements the differences can be reduced.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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