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Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel

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ABSTRACT

Accurately determining protein content is important in the valorization of algal biomass in food, feed and fuel markets. Conversion of elemental nitrogen to protein is a well-accepted and widely practiced method, but depends on developing an applicable nitrogen-to-protein conversion factor. The most complete method to determine this factor takes six different hydrolyses of the subject material and these are not always carried out in reported literature studies. We report new data for conservative conversion factors determined from 21 algae samples along with over 50 amino acid profiles from the literature, representing distinct cultivation conditions for fresh and marine algae. We find that the amino acid profile among different algae samples is consistent, however the large variability between strains in non-protein nitrogen (up to 54% in microalgae) causes variability in the calculated conversion factor. We include our calculated novel nitrogen-to-protein conversion factors for model and commercially relevant biofuel algal strains and compare these with the literature.

1. Introduction

The protein content of algae has strong influence in determining potential food, feed, co-product and fuel uses for algal biomass [1,2]. For example, in the context of an algal biofuel production pathway, the value of the residual biomass (after oil removal) depends on its compositional characteristics and affects the overall process economics [3–5]. Research to develop economically viable algal biofuel or bioproduct pathways is urgently needed and future deployment of successful processes will depend on reducing production costs and finding value from all biomass components [6–10]. As part of the overall technoeconomic modeling and process optimization there is a need to accurately track algal biomass components in and out of different unit operations and this includes accurate quantification of the protein content [11,12]. Good component mass balance accounting gives added confidence that each of the components has been accurately measured. The protein content of microalgae can range from 7% to 40% [1,2,13,14] and can change dramatically over the course of its lifecycle [15]. One source of biomass that is currently commercialized for food supplement sale thanks to the high protein content (reported to be >50–60% of the biomass) is *Spirulina* [16]. Thanks to this high protein content the biomass can contribute to human diet supplementation [17]. Protein content determination in *Spirulina* in commercial preparations is often based on the common determination using a 6.25 factor [18] and a reassessment of the protein versus non-protein nitrogen determination in mass cultivated biomass is needed but has not been carried out for this organism [16,19].

In order to develop viable algal bioproduct processes and to assess multiple process conditions, protein analysis methods need to balance analyte specificity, precision and accuracy with method robustness, ease of use and low cost. Analytical methods to determine protein, for food labeling purposes, have been reviewed by Moore et al. [20] and include 1) copper or dye binding spectroscopic techniques, 2) UV or IR techniques, 3) amino acid (AA) analysis hydrolysis methods, and 4) elemental nitrogen analysis which is converted to protein using a nitrogen-to-protein conversion factor.

The use of spectrophotometric methods can be useful for generating relative protein data, but can be less useful for determining absolute protein values, which are needed for component balance calculations. In the case of the Lowry spectrophotometric procedure, the color development is based on the reduction of the Folin reagent (Cu^{2+} to Cu^{+}) by aromatic residues and peptide bonds in protein, after which the Cu^{+} is chelated by bicinchoninic acid (BCA) to form the detected color [21–23]. Because the Folin reagent will react with other reducing substances in solution, this assay is susceptible to algae species- and growth condition-specific interferences which often cause a high bias [15,24]. All spectrophotometric methods depend on complete extraction of all proteins from the biomass matrix, but it is difficult to completely solubilize all the cytosolic, structural, membrane bound or other protein types found in algae in order to expose them to the colorimetric reagent external to the cell. In addition, the choice of a standard protein for calibration

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is critical, since its response must be similar to that of the released sample proteins. This poses an additional uncertainty in that a typical standard protein may or may not represent the average protein composition found in algal strains. A large discrepancy between the Lowry protein assay and a nitrogen-to-protein factor-based calculation was observed for three strains harvested at different stages of nutrient deprivation and shown to be highly dependent on the physiological and biochemical status of the cells [15].

The most direct method for protein determination is by acid hydrolysis (often a 6M HCl digestion for 24 h) followed by HPLC amino acid analysis [25]. This method has the advantage of breaking down the biological matrix and does not depend on selective removal of protein from the biomass. Free amino acids can also be detected in the hydrolysate along with AAs hydrolyzed from proteins. However, for complete AA analysis multiple (up to six) hydrolyses for each sample are needed to completely quantify the chemically diverse amino acids found in proteins [26]. In addition to the typical 24 h HCl hydrolysis, separate hydrolyses are needed for Trp and for the sulfur containing AAs (Met and Cys). Two additional hydrolysis timepoints (12 h and 48 h) are run to account for AAs (Thr, Ser and Tyr) that are partially degraded during hydrolysis plus a separate 2 h ammonia hydrolysis is run to determine the NH_3 released from Gln and Asn. Direct amino acid quantification after several hydrolyses per sample is a useful method, it is also expensive and time consuming and thus less applicable for screening or processing a large number of samples. A combined method can harness the completeness and specificity of the direct AA analysis with a simpler, higher throughput nitrogen analysis method (%N) by using an appropriate nitrogen-to-protein conversion factor.

Calculating protein using a nitrogen-to-protein conversion factor is not subject to spectral interferences or protein extraction efficiency differences since the entire sample is consumed during the %N analysis. The %N analytical methods, either combustion (Dumas method) or Kjeldahl, are simple, fast and inexpensive compared to hydrolysis followed by AA analysis. They can be run on multiple samples and can be easily adapted to process monitoring or timepoint analyses. The %N methods have the disadvantage of not being specific for protein nitrogen but rather they measure the *total* nitrogen found in the sample. Algae have many nitrogen containing components such as chlorophyll, nucleic acids (DNA/RNA) and amino sugars (e.g. glucosamine, galactosamine) in addition to protein. This non-protein nitrogen (NPN) needs to be properly accounted for within the nitrogen-to-protein conversion factor. In their determinations of algal protein conversion factors Lourenço et al. [13,14] quantitated the major NPN classes (chlorophyll, nucleic acids and inorganic N) in algae samples and the NPN accounted for about 15–30% (with some above 40%) of the total nitrogen in the algae. These authors were able to close the nitrogen balance to around 90–95% including the protein nitrogen.

Jones [27] described differences in nitrogen content of food and how nitrogen-to-protein factors would need to be adjusted for different foods. Tkachuk [28,29] determined factors for wheat plus cereals and oilseeds. Several cereals were analyzed for nitrogen and amino acid content by Mossé and colleagues [30,31]. Mossé suggests a method to determine a useful nitrogen-to-protein conversion factor [26] for use in food and feed nutritional analysis, and describes how to determine upper (k_A) and lower (k_P) limits for this factor and ultimately suggests combining these into a single, averaged factor (k). These factors can be easily defined mathematically, as in Eqs. (1) and (2), though there are many practical, analytical and computational pitfalls to avoid when calculating these factors.

$$k_A = (\sum E_i)/(\sum D_i) \quad (1)$$

$$k_P = \sum E_i/N. \quad (2)$$

The term $\sum E_i$ is the sum of the amino acid residues or the anhydrous amino acids (AAA), accounting for the mass loss during polymerization into proteins. The term $\sum D_i$ is the sum of the nitrogen content of each of the AA residues including ammonia released during hydrolysis. The term N refers to the %N found in the samples by combustion or Kjeldahl methods and includes both protein and non-protein nitrogen (NPN) found in the sample.

The first factor, k_A, is calculated by determining the sum of anhydrous amino acids (AAA) divided by the sum of the %N found within these AAAs. However, the k_A factor assumes all nitrogen measured comes from protein (i.e. NPN = 0) and this is true only for purified protein samples. For biomass samples, k_A will over-predict protein values due to the presence of NPN [26]. The second factor, k_P, is estimated by the sum of AAA divided by the total %N, which includes any NPN found in the sample. For biomass samples using k_P to calculate protein assumes the NPN content is similar as in the calibration samples. As a practical matter k_A is an upper bound to the conversion factor and k_P is a lower bound. Mossé makes the argument that the best conversion factor (k) for protein in real samples is an average of k_A and k_P.

The key to using %N as a predictor of protein content is to have access to a useful nitrogen-to-protein conversion factor for the samples being analyzed. A common method to determine crude protein utilizes the historical conversion factor of 6.25 times the %N value. This factor, which Yamaguchi [32] traces back to the year 1839, tends to overestimate protein in most biomass and even food applications and has been criticized by several authors [33–36]. For food and feed applications, specific nitrogen-to-protein conversion factors have been previously reported. Diniz et al. [37] determined nitrogen-to-protein conversion factors (k_P) of 5.39 to 5.98 for nine species of fish from Brazilian coastal waters. Sriperm et al. [36] calculated all three different types of conversion factors for various feedstuffs and determined k_A values of 5.68 for corn, 5.64 for soybean meal, 5.74 for corn dry distillers grain (DDGS), 5.45 for poultry by-product meal and 5.37 for meat and bone meal. Nitrogen-to-protein conversion factors for microalgae have been reported recently and an overall average k_i factor of 4.78 was reported and is often used [13,14,38,39]. The general trend for reported and specifically calculated factor appears to be much lower than the traditional 6.25 factor. Other authors have previously mentioned the difficulties in evaluating a conversion factor and related this to additional evidence that the cell wall of algae plays an important role in protein quantification [40].

Upon thorough review of the literature on food, feed, and algae applications of nitrogen-to-protein conversion factors, there appear to be inconsistencies between factors (k_A, k_P or k) reported for calculating protein content measurements. In addition to a review and literature data mining study, we report new data for all three k factors analyzed from 21 algae samples, representing distinct cultivation stages and fresh and marine microalgal strains. This allows us to compare the amino acid profile of microalgae between strains and investigate the origins of the nitrogen-to-protein conversion factors. We recalculate k factors from literature reports, where primary data are also reported, and revise them to be on a consistent and comparable basis. We compare the effects of including different analytical tests on the calculations for the k factors. We report on differences in literature reported k values and make recommendations on the best approaches to produce and utilize the k factors for protein determination in algal bioprocess research.

2. Materials and methods

2.1. Sample selection

A total of 21 algal biomass samples were selected to represent a range of different types of algae that are relevant to ongoing outdoor cultivation and biomass production scenarios. Cultivation conditions have been described before [15]. In brief, biomass from three strains, *Scenedesmus* sp. (LRB-AP 0401), *Chlorella* sp. (LRB-AZ 1201) and *Nannochloropsis* sp. was provided by Arizona State University and