Determination Of Plant Proteins Via The Kjeldahl Method And Amino Acid Analysis: A Comparative Study.

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Abstract: The amount of protein in most foods is usually determined by multiplying its Kjeldahl nitrogen content by a factor of 6.25. The reliability of this method in quantitating plant proteins was investigated. Ten lesser known plant leaf samples of nutritional significance among certain populations in Nigeria were used for this study. Protein contents of the plant samples were determined via the kjeldahl method using the conventional nitrogen to protein (N:P) conversion factor 6.25 (i.e. total nitrogen × 6.25) and by summation of amino acid residues (considered more accurate and taken here as the actual protein content). From data of total amino acid and total nitrogen, specific N:P conversion factors were calculated for each sample. The N:P factors ranged from 3.24 to 5.39, with an overall average of 4.64. Protein contents were also calculated using this new factor. Comparison of the calculated protein contents showed that the traditional conversion factor of 6.25 overestimated the actual protein content of the samples. The degree of overestimation ranged from 16%-93%. Protein contents calculated with our adjusted factor (4.64) gave results that are in good agreement with the actual protein content. Our results indicate that calculation of protein content by N × 6.25 is highly unsuitable for plant samples.

Index Terms: Plants, protein, Total Nitrogen (TN), Amino acids, Kjeldahl, nitrogen-to-protein(N:P), conversion factors,

1 INTRODUCTION

The protein content of foods is mostly been determined on the basis of total nitrogen content. The Kjeldahl method is almost universally applied to determine nitrogen content, total nitrogen is then multiplied by a factor to arrive at the protein content. This approach is based on the assumption that nearly all of the nitrogen in the diet is present as amino acids in proteins. On the basis of early determinations, the average nitrogen (N) content of proteins was found to be about 16 percent, which led to use of the calculation N x 6.25 (100/16 = 6.25) to convert nitrogen content into protein content. The use of a single factor, 6.25 is confounded by two considerations; First, not all nitrogen in foods is found in proteins, nitrogen is also contained in other compounds, such as amides, free amino acids, peptides, nucleic acids, nitrogenous lipids, ammonium salts, nucleotides, nitrates, creatine, choline and secondary compounds [1], [2], where it is referred to as non-protein nitrogen (NPN). Secondly, the nitrogen content of specific amino acids (as a percentage of weight) varies according to the molecular weight of the amino acid and the number of nitrogen atoms it contains (from one to four, depending on the amino acid in question). Amino acid analysis is considered a more scientifically correct way of quantifying proteins. Protein content is calculated as the sum of the amino acid residues (total amino acid minus the mass of water i.e. 18g /mol of amino acid). This is sometimes referred to as the "true protein". The advantage of this approach is that it requires no assumptions about, or knowledge of, either the NPN content of the food or the relative proportions of specific amino acids - thus removing the problems associated with the use of total N x a conversion factor.

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protein nitrogenaceous substances such as pigments (chlorophyll and phycoerythrin), nucleic acids, free amino acids and inorganic nitrogen (nitrate, nitrite and ammonia) [3], [4] ,[5]. Non-protein nitrogen has been shown to be relatively high in two types of foods (leaves and fruits) [6], [7], [8], [4], [9]. Many studies have consistently demonstrated that plants are generally rich in non-protein nitrogen compounds leading to potentially large errors when using the formula total nitrogen multiplied by 6.25. It is against this background that this study was carried out to investigate and compare the protein content of plant samples quantified via the Kjeldahl method and amino acid analysis. Ten (10) lesser known plants were used for the study. The specific objectives of this work are to: (i) Compare the results of two methods (Kjedahl method and amino acid analysis) in quantifying proteins in plants (ii) Determine the nitrogen to protein(N:P) conversion for each vegetable. iii) Propose a conversion factor which can be used for routinely converting total nitrogen to protein in similar samples. iv) Determine protein concentrations based on the adjusted N:P factor and compare the results .

2 MATERIALS AND METHODS

2.1 Collection and preparation of Plant samples:

Young tender leaves of *Hibiscus cannabinus, Haematostaphis barteri, Sesamum indicum, Balanites aegyptiaca, Cassia tora, Celtis integrifolia Anona senegalensiss, Ceiba petandra, Ficus ingens and Solanum melongena* were collected randomly from the wild and farmlands in Adamawa state, Nigeria. The samples were identified by a Taxonomist. Several plants of each species were combined to get representative samples. The samples were washed with distilled water, cut into small pieces, air dried (away from sunlight) and ground into fine powder using porcelain mortar and pestle.

2.2 Determination of Total Nitrogen (Kjeldahl method)

2 g of powdered sample was digested in a Kjeldahl digestion flask by boiling with 20 ml of concentrated H_2SO_4 and a Kjeldahl digestion tablet (catalyst) until the mixture was clear. The digest was filtered into a 250 ml volumetric flask and the solution made up to mark with distilled water and connected

for distillation. Ammonia was steam distilled from of the digest to which had been added 50 ml of 45% sodium hydroxide solution. 150ml of the distillate was collected in a conical flask containing 100ml 0.1N HCl and methyl red indicator. The ammonia that distilled into the receiving conical flask reacted with the acid and the excess acid in the flask was estimated by back titration against 2.0M NaOH with colour change from red to yellow (end point). Determinations were made on all reagents alone (blank determinations).

%Nitrogen was calculated as follows:

$$\frac{[(\text{ml standard acid} \times \text{N of acid}) - (\text{ml blank} \times \text{N of base})] - (\text{ml std base} \times \text{N of base}) \times 1.4007}{\text{Weight of sample in grams}}$$

Where N=normality

2.3 Amino acid analysis

Amino acid analysis was carried out according to the method described by Sparkman *et al* [10]. Each sample was defatted by soxhlet extraction with chloroform, methanol mixture (2:1). 1.0g of each defatted sample was acid hydrolyzed with 7.0 mL of 6 N HCl in vacuum-sealed hydrolysis vials at 110°C for 22 hours. Norleucine was added to the HCl as an internal standard. The tubes were cooled after hydrolysis, opened, and placed in a desiccator containing NaOH pellets under vacuum until dry. The residue was then dissolved in 5ml of acetate buffer (pH 2.0) filtered through a Millipore membrane (0.22 µm pore size) and analyzed for amino acids by loading into the Amino acid analyzer (TSM).

2.4 Calculation of protein contents and nitrogen-to-protein (N:P) conversion factors.

Nitrogen Protein 1 (NP1) was estimated by multiplying the total Nitrogen (TN) by 6.25 [11]. Amino acid protein (AAP) was calculated as the sum of individual amino acid residues (the molecular weight of each amino acid less the molecular weight of H_2O (i.e. 18 g in 1M of each amino acid)). The residue for each amino acid was calculated by multiplying the amino acid value with a corresponding conversion factor, then making a summation of all amino acid residue values for each sample [12].Nitrogen protein 2 (NP2) was calculated as total nitrogen (TN) multiplied by the adjusted (new) N:P factor. The N:P factors were determined for each vegetable as the ratio of amino acid protein (AAP) to total nitrogen (TN) of the sample: N:P factor = AAP/TN [9], [13] ,[14], [15]. The mean of the Calculated N:P factors was taken as the adjusted N:P factor.

3 Results and Discussions

The nitrogen content and amino acid residues of the samples are presented in tables 1 and 2 respectively. Table 3 presents the protein contents of the samples: Nitrogen protein 1(NP1) calculated as total nitrogen × 6.25, amino acid protein (AAP) calculated as sum of the amino acid residues and nitrogen protein 2 (NP2) calculated as total nitrogen × 4.64(i.e. the new N:P factor). In this, study we take the actual concentrations of protein in the samples as the values calculated from the sum of amino acid residues (AAP). This method is considered more scientifically correct and has been widely accepted for accurately determining protein concentration. Results obtained reveal that determination of protein by the traditional method (i.e. Total nitrogen× 6.25), significantly overestimated the protein contents of the plant samples. The degree of overestimation ranged from 16% in *Ficus ingens* to 93% in *Cassia tora*. The notably high overestimation observed for *C.Tora* indicates very high concentrations of non-protein nitrogen, probably transient stocks of inorganic nitrogen. High over estimations were also obtained for S. indicum (55%) and *C. intergrifolia* (45%). These values reflects the amounts of non- protein nitrogen in the samples. Nitrogen content of the samples ranged from 0.42% to 2.97% (Table 1). The values for total amino acid residue (i.e. true protein content) ranged from 2.14% to 12.39% (Table 2). We observe that higher nitrogen content did not result to higher true protein content. The highest N content was obtained for *S.indicum* while *B. aegyptiaca* had highest value for true protein content. Among the samples, such disparities were also observed for *C.intergrifolia, A.senegalensis,C. Petandra* and *F. ingens*. These findings adds further credence to the fact that not all the nitrogen in plant samples are from protein and that plants contain variable amounts of non-protein nitrogen. Non-protein N content is said to vary from plant to plant, between different tissues on the same plant, and even in the same tissue at different stages of growth and development [17], [18], [6].

Table 1. Nitrogen content of the plant leaf samples

(Expressed as percentage of dry matter)

Plant samples	Total Nitrogen
Hibiscus cannabinus	2.21
Haematostaphis barteri	1.98
Sesamum indicum	2.97
Balanites aegyptiaca	2.54
Cassia tora	1.86
Celtis integrifolia	1.31
Anona senegalensis	1.12
Ceiba petandra	1.04
Ficus ingens	0.98
Solanum melongena	0.42

Table 2. Amino acid residues of the samples

(Expressed in percentage)

Amino Acids	H.C	H.B	S.I	B.A	C.T	C.I	A.S	C.P	F.I	S.M
Aspartic acid	1.21	1.16	1.40	1.36	0.70	0.69	0.71	0.65	0.54	0.28
Threonine	0.55	0.38	0.61	0.49	0.24	0.26	0.23	0.15	0.19	0.10
Serine	0.24	0.30	0.34	0.33	0.26	0.19	0.27	0.19	0.29	0.07
Glutamic acid	1.95	1.67	1.76	1.91	0.90	0.9	0.80	0.63	0.70	0.31
Proline	0.42	0.35	0.50	0.31	0.24	0.19	0.23	0.20	0.29	0.11
Glycine	0.11	0.16	0.13	1.46	0.26	0.27	0.28	0.25	0.21	0.09
Alanine	0.29	0.36	0.34	0.36	0.34	0.34	0.26	0.25	0.33	0.13
Valine	0.65	0.55	0.80	0.69	0.34	0.38	0.26	0.27	0.36	0.09
Methionine	0.16	0.15	0.19	0.13	0.08	0.07	0.08	0.06	0.11	0.03
Isoleucine	0.49•	0.52	0.64	0.60	0.28	0.28	0.27	0.30	0.36	0.10
Leucine	1.22	0.98	1.49	1.08	0.57	0.64	0.72	0.59	0.45	0.22
Tyrosine	0.55	0.52	0.59	0.57	0.29	0.28	0.19	0.23	0.27	0.12
Phenylalanine	0.81	0.69	0.89	0.86	0.45	0.36	0.41	0.36	0.34	0.11
Histidine	0.42	0.36	0.46	0.50	0.19	0.15	0.20	0.13	0.15	0.08
Lysine	0.69	0.53	0.86	0.79	0.44	0.28	0.38	0.24	0.26	0.11
Arginine	0.90	0.92	0.84	0.75	0.36	0.22	0.45	0.34	0.38	0.18
Cysteine	0.14	0.13	0.14	0.20	0.08	0.17	0.06	0.05	0.05	0.01
Total	10.8	9.73	11.98	12.39	6.02	5.67	5.79	4.89	5.28	2.14

Table 3. Calculated Protein contents and nitrogen to protein (N:P) factors of the samples. NP1, AAP and NP2 are expressed as percentages (%) of the dry matter. N:P factors have no units.

Plant samples	Nitrogen protein (NPI)	Aminoacid protein (AAP)	N:P factors	Nitrogen protein (NP2)
H. cannabinus	13.78	I0.80	4.65	10.25
H. barteri	12.40	9.73	4.91	9.91
S.indicum	18.59	11.98	4.03	13.78
B.aegyptiaca	15.86	12.39	4.88	11.79
C. tora	11.63	6.02	3.24	8.63
C. integrifolia	8.20	5.67	4.33	6.07
A.senegalensis	7.00	5.79	5.17	5.19
C. petandra	6.52	4.89	4.70	4.83
F. ingens	6.13	5.28	5.39	4.54
S. melongena	2.63	2.14	5.10	1.95
			4.64(average)	

Calculated N:P factors for all the plant leaf samples were lower than the conventionally used 6.25. The values calculated ranged from 3.24 to 5.39 (Table 3). The average N:P conversion factor, was 4.64. Protein contents calculated using this factor gave values that are in better agreement with the true protein as compared to the values obtained with 6.25 conversion factor. This finding suggests the suitability of our N:P conversion factor for calculating plant leaf proteins. Figures 1 & 2 present a comparison of the calculated protein contents in the samples. Significant differences can be seen between NP1 (protein content calculated with 6.25) and the true protein content (AAP). For AAP and NP2 values, it can be seen that only *C.tora* showed significant difference. This can be attributed to the presence of high amounts of non-protein nitrogen in *C.tora* leaf samples. Comparison of the present results with data available in literature is difficult because of the lack of previous data on the samples of study. However, several studies on other plant samples have proposed different but closely related N:P conversion factors for different plant species; Sosulski and Imafidon [19] suggested the factor 5.72 to calculate the protein concentrations of maize. Yeoh and Truong [20] determined the factor 4.48 for cassava roots. Tokoro *et al* [21] carried out a study on 12 kinds of vegetables and suggested an N:P conversion factor of 4.7. Pirjo *et al* [22] proposed a factor of 5.33 for vegetables, fruits and berries. Sriperm *et al* [23] calculated N:P factors ranging from 5.37-5.68 for different feedstuff. A conversion factor of 5.64 was reported for wild fruits[9]. A study carried out on 90 plant species reported a range of 3.28- 5.16 as N:P factors for leaf proteins and suggested the factor 4.43 for plants in general [24]. In a study by Fujihara *et al* [5] N:P conversion factors of vegetables ranging from 2.99 to 5.84 and averaging 4.39 was reported. In this present study on lesser known plant species, we reports an average conversion factor of 4.64. This value falls within the range of values reported for different plant samples by previous investigators. The variations in N:P factors reported by different authors for similar samples may be as a result of several factors such as; Statistical power (sample size), sample source; samples collected from nitrogen rich soil may give high percentage nitrogen which may introduce errors, fertilizer and other nitrogen sources have been shown to result in increased N content. Variation of the

concentrations of nitrogen-rich amino acids may also markedly influence the establishment of N-Prot factors [19]. It is worthy to note that this present work and the previous studies reviewed on plant leaf samples all established N:P factors less than the conventionally used 6.25. This strongly indicates that the use of 6.25 N:P conversion factor is highly inadequate for such food groups. Jones [16] suggested that N x 6.25 be abandoned and replaced by N x a factor specific for the food in question. Over the years, many other authors agree with this and proposed different N:P factors for various food types. Despite these caveats, most of the scientific community have continually ignored this alternative methodology and have continue to use the 6.25 conversion factor for determining protein contents of all kinds of samples leading to gross overestimation of the protein content of most food types. This study indicates that it is necessary to always consider the existence of non-protein nitrogen when converting nitrogen to protein especially in samples of plant origin.

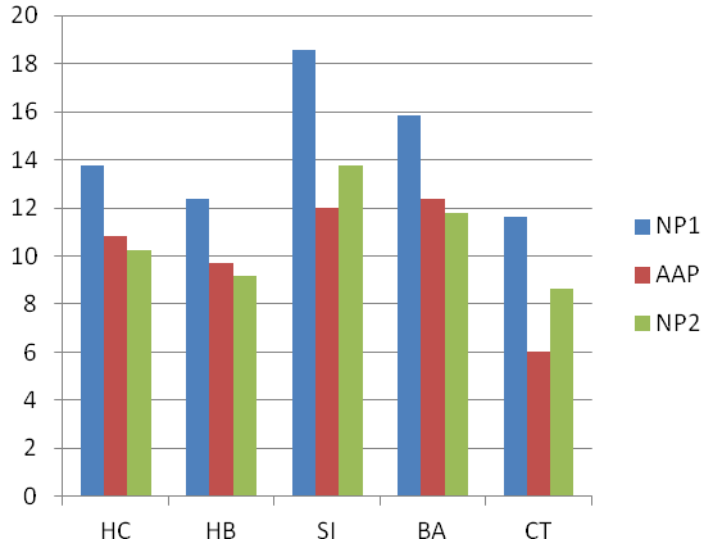

Fig. 1 Comparison of calculated protein contents *for H.cannabinus* (HC), *H. Barteri* (HB), *S. Indicum*(SI), *B.aegyptiaca*(BA) *and C.Tora*(CT)

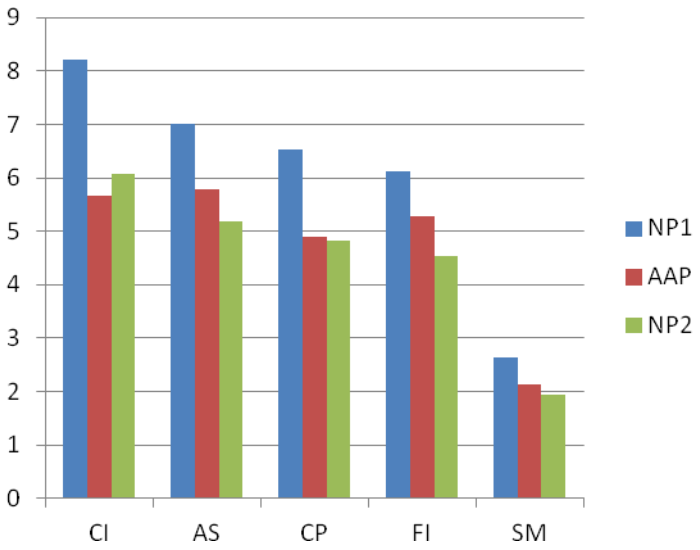

Fig. 2 Comparison of calculated protein contents *for C.intergrifolia (*CI), *A.senegalensis* (AS), *C.Petandra (*CP), *F.Ingens* (FI) *and S.Melongena*(SM*).*

4 CONCLUSION/RECOMENDATIONS

Although the Kjeldahl method is satisfactory for determining total nitrogen, it is imprecise for determining total protein content. The calculation of protein content by N × 6.25 is highly unsuitable for plant samples due to the presence high amounts of non-protein nitrogen. Our adjusted N:P factor (4.64) seem reliable for converting nitrogen to protein in plant leaf samples. We emphasize that the most accurate method of determining protein content is by amino acid analysis. This method is however costly and not readily available to many researchers. In response to these considerations we recommend that protein content be calculated as total nitrogen multiplied by a specific N:P factor for the food type. The accuracy of this method however, depends on the establishment of N:P factors specific to individual food groups. We therefore, strongly recommend that the appropriate organizations re- evaluate and update the methods for protein determination in line with current knowledge and research findings.

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