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Evaluation of Rapid Methods for the Estimation of Protein in Chickpea (*Cicer arietinum* L.)

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Protein content in chickpea (*Cicer arietinum* L.) was determined by the following rapid procedures: (a) colorimetric estimation of ammonia with phenol–hypochlorite reagents using the Technicon auto-analyzer (TAA) method, (b) dye-binding capacity (DBC) method using acid orange-12 dye, and (c) modified biuret methods of Pinckney (B1) and Johnson and Craney (B2). Results obtained with the above procedures were compared statistically with the standard microKjeldahl (MKJ) procedure. Correlation of MKJ protein values with TAA, DBC, B1 and B2 methods were 0.99, 0.98, 0.96 and 0.95, respectively. Standard errors of estimation obtained by methods B1 (0.99) and B2 (0.95) were high when compared to the TAA (0.55) and DBC (0.69) methods. Possible interference of seed-coat pigments, effect of flour particle size, and time of shaking on protein estimation by the DBC and B1 methods were also studied. Implications of these results are discussed with reference to adapting any of these rapid methods as a routine screening procedure for the estimation of protein in large numbers of chickpea samples in a breeding programme.

1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume crop in the world and is a good source of protein in the human diet in developing countries, particularly India, where energy and protein are obtained mainly from cereals and legumes. It is therefore desirable to improve both the quantity of protein in chickpea and its quality. To achieve this goal, large numbers of samples need to be analysed in order to select lines with the desired protein-quality characteristics. For this purpose there is a need to identify fast analytical methods that give reasonably accurate estimates of quantity and quality of protein.

Nitrogen content in grain samples is usually determined by the Kjeldahl method and an estimate of crude protein is obtained by multiplying the nitrogen content by a factor of 6.25. However, the Kjeldahl method is slow and cumbersome and so is unsuitable for rapid screening purposes. Therefore, these is a need for a rapid, easy to perform, and accurate method for determining protein content in chickpea samples. Among the several methods that are available, the dye-binding capacity (DBC), biuret methods (B1 and B2), and phenol–hypochlorite reaction using a Technicon auto-analyzer (TAA) have been used for the estimation of protein content in other cereals and beans.[1–9] Using the DBC method for protein estimation it is necessary to assume that the proportion of the basic amino acids does not vary for a particular crop under consideration. However, there has not been any comparison of these methods for the determination of protein in a large number of chickpea samples.

One of the objectives of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) is to improve the nutritional quality of chickpea, so there was need to analyse a large number of chickpea cultivars from the breeding programme. Investigations were undertaken in

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the laboratory to compare four methods of protein estimation in order to evaluate the usefulness of rapid methods.

2. Experimental

2.1. Materials

From the germplasm collection, 150 accessions with a wide range of protein content were selected for this study. Whole-seed samples were ground in a Udy mill to pass through a 60-mesh sieve and were dried overnight at 70°C. The analyses were carried out on these dried samples. Samples were divided into low-, medium- and high-protein groups based on crude protein values obtained by the MKJ method. To study the effect of flour particle size on protein, samples of one cultivar (P-1137) were ground in a Wiley mill using 10, 20, 40, 60, 80, and 100-mesh sieves so that all the material passed through the sieve. In order to compare seed coat pigment interference in the biuret and DBC methods, protein content was determined in 'dhal' (decorticated split seed) and in the whole-seed samples. For the preparation of dhal, whole seeds were soaked in distilled water overnight at 5–6°C. Excess water was decanted and seed coats were removed from the seeds manually. Dhal samples were dried at 70°C overnight in an oven before processing for the estimation of protein content.

2.2. Reagents

Reagent dye solution and reference dye solution of acid orange-12 dye were obtained from Udy Analyzer Co, Boulder, Colorado, USA. Reference dye solution (50 ml) was diluted to 1 litre and 1.89 litres of reagent dye solution was diluted to 20 litres. Kjel-tabs (auto tablet), each containing 1.5 g K_2SO_4 and 0.0075 g Se, were obtained from Thompson and Capper Ltd, Runcorn, Cheshire. Sodium hypochlorite was obtained from Reckitt and Coleman Ltd, India. All other reagents used were from BDH England (Analar grade).

2.3. MicroKjeldahl (MKJ) method

The standard microKjeldahl procedure[10] was used for the determination of nitrogen and crude protein was estimated by multiplying the nitrogen content by a factor of 6.25. In the present paper, no distinction has been made between crude protein content ($N \times 6.25$) obtained by MKJ and the values obtained by DBC and biuret methods.

2.4. Colorimetric method using Technicon auto-analyzer (TAA)

In this method, NH_4^+ is estimated colorimetrically in an alkaline medium after reaction with phenol–sodium hypochlorite. A slightly modified automated procedure with the TAA[11] was used. A suitable amount of the sample (70 mg) was weighed and placed in a digestion tube. One Kjel-tab (auto tablet) and 3 ml of sulphuric acid–phosphoric acid mixture [95 parts conc. sulphuric acid, 5 parts of 85% phosphoric acid (v/v)] were added to the digestion tube and a set of 40 tubes was digested at 370°C for 1 h. After cooling, distilled water was added to bring the volume to 75 ml. A suitable aliquot was used for nitrogen estimation in TAA which is capable of analysing 40 samples h^{-1} with a sample-to-wash ratio of 9:1.

2.5. Dye-binding capacity (DBC) method

DBC procedure, using the dye acid orange-12, was followed according to the procedure of Udy.[2] A finely ground sample (320 mg) was weighed and transferred to a plastic bottle and 40 ml of reaction dye solution (acid orange-12, 1.3 mg ml^{-1}) was added. Bottles were stoppered and shaken in a reciprocating shaker for 1 h. The suspension was then filtered and % transmission was recorded against the reference dye solution, using a Udy flowthrough colorimeter.

2.6. Biuret methods (B1 and B2)

Two biuret methods were used for estimation of protein. The procedure B1, as described by Pom-

eranz,[4] for the estimation of protein in soya flour was followed, except that 50% (v/v) propan-2-ol was used in the biuret reagent. The second method was the biuret (B2) method, as described by Johnson and Craney,[5] but 50% (v/v) propan-2-ol was used in the biuret reagent instead of a 60% solution. The use of 50% propan-2-ol in the biuret reagent has been made after our preliminary study on the effect of propan-2-ol concentration on protein extraction and pigment interference. This work is reported in section 3.2.

3. Results and discussion

3.1. Comparison of different methods

Results of correlation coefficients, standard errors of estimation, and regression equations obtained between the MKJ method and other rapid methods evaluated are shown in Table 1. The TAA method was significantly correlated with the MKJ method ($r=0.99$) and DBC method ($r=0.98$). Correlation of MKJ method with the biuret method[4] (B1) was 0.96 and with that of biuret method[5] B2 was 0.95. It was observed that both procedures produced results with higher standard errors of estimation in comparison with the DBC and TAA methods.

Table 1. Statistics for comparing the degree of correlation between TAA, DBC and biuret methods (B1 and B2), respectively with MKJ method for the estimation of crude protein content (N × 6.25)

Method	Correlation coefficient	Standard error of estimation (% protein)	Regression equation
MKJ *vs* TAA	0.989**	0.555	$y=0.291+1.001x$
MKJ *vs* DBC	0.976**	0.688	$y=7.428+0.350x$
MKJ *vs* DBC	0.981**	0.688	$y=1.047+0.671x-0.00376x^2$
MKJ *vs* log DBC	0.980**	0.688	$y=-30.955+33.011 \log x$
MKJ *vs* B1[a]	0.958**	0.993	$y=-6.571+101.223x$
MKJ *vs* B2[b]	0.946**	0.954	$y=-11.810+102.019x$

[a] Modified biuret method of Pinckney (1961).
[b] Modified biuret method of Johnson and Craney (1971).
** Significant at 1% level.

Table 2. Correlation coefficients and standard errors of estimation of different methods of crude protein (N × 6.25) determination in comparison with MKJ method for low-, medium-, and high-protein lines

Method	Correlation coefficient[a]			Standard error of estimation		
	Low	Medium	High	Low	Medium	High
MKJ *vs* TAA	0.842	0.958	0.863	0.565	0.467	0.555
MKJ *vs* DBC[b]	0.773	0.949	0.798	0.585	0.496	0.567
MKJ *vs* DBC[c]	0.779	0.954	0.808	0.585	0.496	0.567
MKJ *vs* B1	0.834	0.789	0.677	0.712	0.649	0.809
MKJ *vs* B2	0.798	0.784	0.732	0.543	0.728	1.024

[a] All values significant at 1% level.
[b] Linear regression equation.
[c] Curvilinear regression equation.

In order to find out the usefulness of these methods in analysing samples with a wide range of protein content, the correlation coefficients and standard errors of estimation between DBC, TAA and MKJ methods were compared for low-, medium- and high-protein lines (Table 2). The MKJ values of medium-protein lines had a significantly higher correlation with DBC and TAA procedures as compared to low- and high-protein lines. On the other hand, correlation between MKJ method and biuret procedures B1 and B2 was higher for low-protein lines as compared to medium- and high-protein lines (Table 2). This table also shows that both the biuret procedures had higher standard errors of estimation for high-protein lines when compared to low- and medium-protein lines.

Correlation studies (Tables 3 and 4) indicated that significant differences in the mean protein content values were not observed between the MKJ and other methods examined in the present investigation. However, it was observed that the mean protein content value for low-protein

Table 3. Mean protein content (N × 6.25) of different groups of chickpea lines as determined by TAA, DBC, and MKJ methods

Method	Mean protein content (%)			
	Low $n=56$	Medium $n=49$	High $n=45$	Total $n=150$
MKJ	17.81 (14.9–19.8)	23.11 (20.2–25.0)	26.47 (25.2–29.6)	22.18 (14.9–29.6)
TAA	17.58 (14.7–19.5)	22.90 (19.4–25.5)	26.03 (24.9–29.5)	21.86 (14.7–29.5)
DBC[a]	18.13 (15.8–20.0)	22.89 (19.0–25.8)	26.44 (24.3–30.6)	22.18 (15.8–30.6)
DBC[b]	17.98 (15.0–20.3)	23.20 (19.0–25.9)	26.27 (24.4–28.9)	22.18 15.0–28.9)
LSD (5%)	0.43	0.61	0.51	0.85

[a] Linear regression equation.
[b] Curvilinear regression equation.
Figures within the parentheses indicate the range of protein content in the samples analysed.

Table 4. Mean protein content (N × 6.25) of different groups of chickpea lines as determined by biuret methods (B1 and B2) and MKJ method

Method	Mean protein content (%)			
	Low $n=42$	Medium $n=49$	High $n=43$	Total $n=134$
MKJ	17.82 (15.2–20.8)	23.07 (21.5–25.0)	26.87 (25.3–29.6)	22.64 (15.2–30.0)
B1[a]	18.12 (14.3–22.2)	23.26 (19.7–25.6)	26.43 (24.4–30.4)	22.67 (14.3–30.4)
B2[b]	18.30 (14.5–21.4)	23.03 (19.3–26.4)	26.45 (25.2–30.5)	22.65 (14.5–30.5)
LSD (5%)	0.70	0.66	0.61	0.90

[a] Modified method of Pinckney (1961).
[b] Modified method of Johnson and Craney (1971).
Figures within the parentheses indicate the range of protein content in the samples analysed.

lines obtained by the DBC method was slightly higher than the MKJ mean protein content value (Table 3). This was also apparent from the relationship between the MKJ and DBC methods as shown in Figure 1. The use of a linear regression equation between DBC and MKJ protein values over-estimated the MKJ protein content in the low-protein lines. However, the use of a new conversion table based on a curvilinear regression equation (Figure 1) between DBC and MKJ protein values improved the results (Table 3). A regression equation between log DBC readings and MKJ protein values was calculated and there was no significant difference between the protein values obtained by using this equation and those obtained by using the curvilinear regression equation.

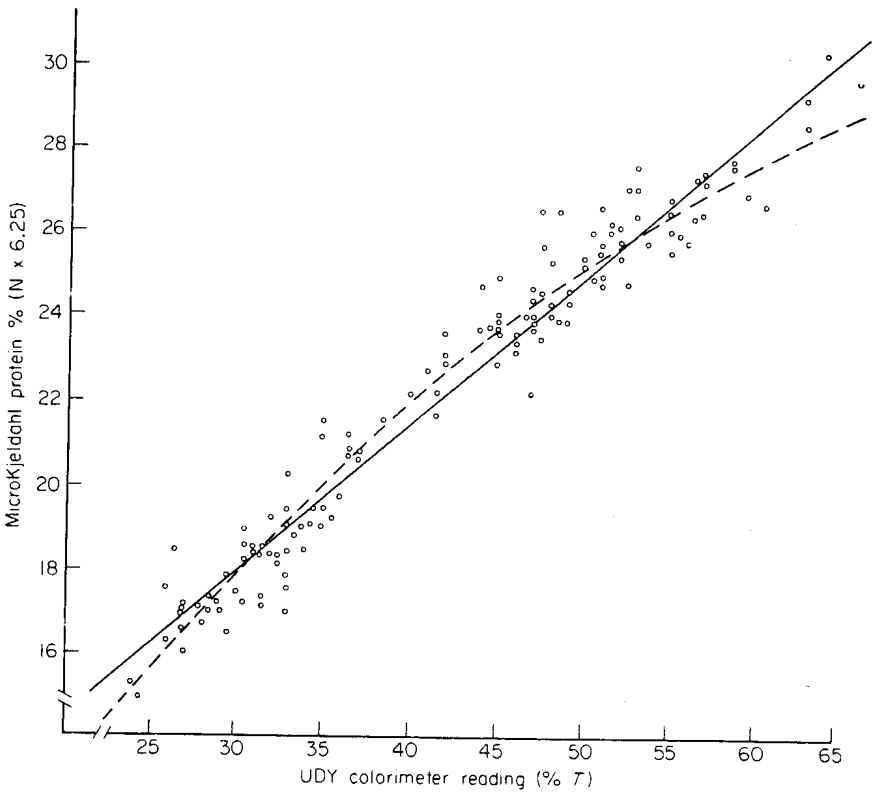

Figure 1. Relationship between dye-binding capacity and microKjeldahl methods. $\hat{y}=7.428+0.350x$; $r=0.976^{**}$. $\hat{y}=1.047+0.671x-0.00376\,x^2$; $r=0.981^{**}$.

Considerable variation in the protein values, particularly in high-protein lines, was observed when the samples were analysed by biuret methods B1 and B2 which was also reflected in the poor correlation obtained between these methods and the MKJ method (Table 2). Although precise reasons cannot be attributed to this variation, one of the shortcomings of the biuret procedures may be due to the poor extraction of protein as result of use of propan-2-ol in the biuret reagent as described in section 3.2.

3.2. Effect of different concentrations of propan-2-ol on protein extraction

In order to study the effect of different concentrations of propan-2-ol on protein extraction, 10 ml of 1M KOH was taken in each of 100-ml volumetric flasks and, after adding 10, 20, 30, 40, 50, and 60 ml of propan-2-ol to the respective flasks, the final volume was made to 100 ml. A suitable amount of the sample (200 mg) was dispersed in 1 ml of propan-2-ol and 40 ml KOH solution, containing a different concentration of propan-2-ol, was added to each sample. Flasks were shaken for 15 min using a mechanical shaker. After centrifugation (3000 *g*) for 10 min supernatants were

Table 5. Effect of different concentrations of propan-2-ol on nitrogen extraction[a]

Concentration of propan-2-ol (v/v)	% Nitrogen extracted
0	84.9
10	80.0
20	74.3
30	70.2
40	61.4
50	56.7
60	49.3

[a] Mean of two determinations.

taken for the estimation of extracted protein by the MKJ method. The amount of N extracted decreases as the concentration of propan-2-ol increases (Table 5) but at a concentration of 40%, or less, the extracts obtained after centrifugation were not clear, indicating the interference of pigments in the extraction procedure.

Earlier workers[12] have reported that the use of 50% propan-2-ol in biuret reagents promoted extraction of all proteins from beans. The work of Johnson and Craney[5] also showed that interfering material was not extracted from cereal seeds when 60% propan-2-ol was used and at the same time this helped to extract all the proteins from the meal. In fact, higher concentrations of propan-2-ol favour the solubility of cereal seed proteins which contain large amounts of alcohol-soluble protein.[13] This is not the case with grain legumes which contain mostly salt-soluble proteins and have very little alcohol-soluble protein. In the present study, although the use of 50% propan-2-ol extracted only 57% of nitrogen (the results were comparable with MKJ values), this may be a fortuitous coincidence. It would seem that incomplete protein extraction and interference of tannins and other pigments, in colorimetric assays are the two main reasons for the unsuitability of the present biuret method for protein estimation in chickpea.

3.3. Effect of shaking and particle size on protein determination in chickpea

Some factors were investigated in establishing conditions for biuret (B1) and DBC methods for protein estimation in chickpea. Increasing shaking time (> 15 min) at room temperature had no measurable effect on absorbance of clarified extracts for biuret method B1. With the DBC method,

Table 6. Effect of particle size on protein determination by four methods[a]

	Particle size (mesh)					
	10	20	40	60	80	100

readings increased considerably up to 1 h of shaking, but beyond that period shaking had no measurable effect on the dye-binding reading (results not reported).

Flour of finer particles was found to give higher protein values by all procedures tested (Table 6). Differences in protein values estimated by the biuret (B1) and DBC methods were greater when compared with MKJ and TAA values. DBC results obtained between 20 and 60-mesh samples were in good agreement with the MKJ method. But in case of the modified biuret method, 40 and 60-mesh samples produced results in good agreement with MKJ values. As it would be impracticable to grind all samples to a very fine particle size, it would be convenient from the point of energy and time consideration to use a particle size of 40–60-mesh for routine screening of large numbers of samples.

3.4. Interference of seed-coat pigments in protein determinations

To study the influence of seed-coat pigments on protein estimation, whole-seed and dhal samples from ten cultivars having different seed-coat colours were analysed by the biuret (B1) procedure and DBC method. The values were compared with MKJ values (Table 7). Seed-coat pigment did

Table 7. Effect of seed-coat pigments on protein determination by DBC, biuret (B1), and MKJ methods[a]

Cultivar	Weight of 100 seeds (g)	Colour	Seed coat (%)	Protein (%)						
				MKJ			DBC		Biuret (B1)	
				Seed coat	Whole-seed	Dhal	Whole-seed	Dhal	Whole-seed	Dhal
NP-34	12.5	White	15.1	3.1	16.3	18.6	16.8	18.9	16.1	18.5
P-3090	21.9	White	14.4	4.0	19.7	22.8	19.7	23.1	19.8	23.4
L-550	20.1	Salmon white	4.5	5.5	18.8	19.5	19.6	20.3	18.8	19.6
K-4	18.1	Salmon white	5.8	5.2	15.6	16.5	16.0	17.0	15.4	16.0
G-130	13.7	Yellow brown	14.5	4.3	20.9	24.6	20.7	25.0	20.7	24.0
BEG-482	12.6	Yellow brown	17.5	3.8	21.0	26.1	21.8	27.2	20.7	25.8
BR-170	12.6	Brown	15.2	3.8	19.7	23.3	20.1	23.3	20.0	24.0
G-24	10.4	Brown	16.1	3.4	16.7	19.6	16.4	19.7	17.1	20.0
Kaka	10.7	Black	16.0	3.7	16.9	20.5	16.4	20.0	16.9	20.1
L-345	10.5	Green	16.0	3.6	22.0	25.2	21.5	24.2	21.6	24.9

[a] Mean of two determinations.

not interfere in the protein determination. Differences in the protein content of whole-seed and dhal samples seemed to be related to differences in seed-coat content of the sample. This observation was confirmed by comparing the results of these two methods with the MKJ method in which seed-coat pigment did not interfere in the estimation of protein content. For example, in the case of BEG-482 (yellow-brown) cultivar, whole-seed and dhal samples differed significantly in their protein contents (5.1%) where the seed-coat was 17.5%, as compared to L-550 (salmon white) cultivar where the difference between whole-seed and dhal protein was small (0.7%) with only 4.5% of seed coat. This indicates that the seed-coat, which is inversely related to seed size,[14] affects the protein content of whole chickpea samples.

4. Conclusions

Many reliable rapid methods are now available for the analysis of protein content in seed. In the present investigation, four methods were compared. Results obtained with a Technicon auto-analyzer were precise and were highly correlated with MKJ values. It is possible to carry out accurate determinations on large numbers of samples within a relatively short time. Therefore, the TAA procedure would be the most suitable method to be used in a breeding programme. As an alternative, where the TAA facility is not available, the DBC procedure can be adapted for the estimation of protein content. The biuret method, due to poor protein extractability, was not as accurate as the TAA or DBC method, but the method may still find use in some programmes, depending mainly on their objectives.

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