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Methods for the Estimation of Protein in Pigeonpea [*Cajanus cajan* (L.) Millsp.] and the Relationship Between Whole Grain and Dhal Protein Contents

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Protein determinations of 172 grain and dhal (decorticated dry split seed) pigeonpea samples were carried out using three methods: (a) micro-Kjeldahl (MKJ), (b) colorimetric estimation of NH_4^+ with phenol hypochlorite reagents using the Technicon Auto-Analyser (TAA), and (c) dye-binding capacity (DBC) method using Acid Orange 12 dye. Protein percentages determined by the TAA and MKJ methods were highly correlated for whole-grain (0·948**) and dhal (0·967**) samples. The DBC method gave reliable results for dhal samples only. In the DBC procedure, higher protein percentages were recorded with smaller flour particles and longer mixing time, but different temperatures and durations of heating had no effect. Positive and highly significant correlations were obtained between the protein values of whole-grain and dhal samples in all the methods. Small grains gave a lower correlation between whole-grain and dhal protein content due to the observed negative correlation between grain size and percentage of seed-coat.

1. Introduction

Plant breeding techniques to improve the nutritional quality of pigeonpea have received considerable attention.[1,2] An essential step in the improvement of grain quality is the identification of high protein genetic material. As this requires the determination of protein content of large numbers of samples, a rapid and accurate method of analysis is needed. Several rapid methods for the estimation of protein have been recommended for screening large numbers of samples in cereals and other grain legumes.[3–9] Recently the evaluation of rapid methods for the estimation of protein content in chickpea has been carried out to identify a suitable procedure.[10] Analysis by rapid methods is achieved usually at the expense of some accuracy, so this aspect needs careful examination.

In India, pigeonpea is consumed mainly in the form of dhal (decorticated dry split seed). However, preparation of dhal from whole grain is a tedious and time-consuming process, and is a rate-limiting step in screening large numbers of samples. Earlier workers have reported the levels of protein in the whole-grain and dhal samples of some cultivars of pigeonpea.[11] However, it is desirable to study the relationship between the protein content of whole-grain and dhal samples to determine if the whole-grain samples could be analysed quickly, avoiding the dhal preparation steps. As far as is known, no such comparisons for pigeonpea are available in the literature.

The present study was undertaken to examine whether rapid methods, such as the Technicon Auto-Analyser (TAA) and the dye-binding capacity (DBC) can be used instead of the slower but usually more accurate micro-Kjeldahl (MKJ) method, and whether protein analysis of a whole grain sample can be used to satisfactorily assess the protein content of dhal. Further, in view of certain other factors in the estimation of protein content by the DBC method in soya bean,[4] the effects of different temperatures and durations of heating and mixing in pigeonpea by the DBC procedure were studied.

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2. Experimental

2.1. Materials

Seed samples from a breeders' trial comparing seven early, 14 medium, and 22 late cultivars in a randomised block design with four replicates and samples from 83 germplasm lines were used. They were grown in an experimental farm at Patancheru, near Hyderabad, during the 1977–78 and 1978–79 rainy seasons, respectively. The weight of 100 seeds was determined for each cultivar and whole-grain and dhal samples were analysed. Dhal samples were prepared by soaking the whole seeds in distilled water overnight at 5°C. Excess water was decanted and seed-coats were removed from the seeds manually. The whole grain, seed-coat, and dhal fractions were dried at 70°C overnight in an oven and then weighed. Samples were ground in a Udy cyclone mill to pass through a 60-mesh sieve.

2.2. Methods of analysis

Protein contents in whole-grain and dhal samples were determined by three different methods and the results were determined on a dry weight basis. The protein content of dried seed coat was determined by the MKJ method only. The germplasm lines were analysed for protein content by the TAA procedure. The relationship between flour particle size and protein content was examined in the cultivars HY-3C and Gwalior-3. Whole-grain and dhal samples were ground in a Wiley mill using 20, 40, and 60 mesh sieves so that all the materials passed through the sieve. Protein was estimated by the DBC procedure.

2.2.1. Micro-Kjeldahl (MKJ) method

Nitrogen content was determined by a standard MKJ method[12] and crude protein content was obtained by multiplying the nitrogen content by a factor of 6.25.

2.2.2. Colorimetric method using Technicon Auto-Analyser (TAA)

A slightly modified automated procedure of TAA[13] was used to estimate the NH_4^+ in digested samples as described earlier.[10] The nitrogen value thus obtained was converted into crude protein content by multiplying with a factor of 6.25. Using this procedure, two persons can analyse about 100 samples a day, which includes the time taken for preparing the reagents and the washing of glassware.

2.2.3. Dye-binding capacity (DBC) method

The DBC method, using the dye Acid Orange-12, was followed according to the procedure described by Udy[5] for the estimation of protein content. Using this procedure, two persons can analyse about 160 samples a day including the preparation of reagents and the washing of filters and bottles.

3. Results and discussion

3.1. Protein content of pigeonpea fractions

The ranges and means of the protein contents of the different seed components of the 43 cultivars from the breeders' trial determined by the MKJ method are presented in Table 1. Seed-coat content ranged between 13.2 and 18.9% and 100-grain weight varied from 6.3 to 13.9 in these cultivars. A negative and highly significant correlation (−0.801**) was obtained between the grain weight and seed-coat content. Protein content varied between 17.9 and 24.3% for whole grain and between 21.1 and 28.1% for dhal samples. On an average, dhal protein was found to be 3.1 units higher than the whole grain protein content. However, these observations are in contrast with the findings of another study[11] in which the mean dhal protein content of 20 samples was found to be lower than that of the whole-grain samples. Differences between the calculated and the observed dhal protein values existed but were not statistically significant. The calculated mean values for dhal protein content were less than the observed values. The protein values of whole seed might have been underestimated because of the presence of seed-coat. No significant correlation between

Table 1. Ranges and means of components of pigeonpea[a]

Constituent	Minimum	Maximum	Mean	Correlation with 100-grain weight
100-grain wt (g)	6.3	13.9	9.9	
Seed-coat (%)	13.2	18.9	15.5	−0.801**
Protein content (%)[b]				
Seed-coat	4.5	6.4	5.4	0.021
Whole grain	17.9	24.3	21.2	0.156
Dhal component				
Determined[b]	21.1	28.1	24.3	0.131
Calculated[c]	19.9	27.6	23.6	
Calculated[d]	20.8	28.5	24.2	

[a] Based on an analysis of 43 cultivars.
[b] MKJ values.
[c] Using the equation: $Pd = Pw \times 100 - Psc \times Sc/100 - Sc$.
[d] Using linear multiple regression equation (see text).
** Significant at 1% level.

protein content and grain weight for these cultivars was observed. This was also confirmed when 83 germplasm lines with a wide range in 100-grain weight (4.9 to 21.1 g) were analysed for protein content by the TAA method.

3.2. Comparison of different methods of protein estimation

The protein content values obtained by the TAA and DBC methods were compared with those of the MKJ method using the values for the 43 cultivars from the breeders' trial. Table 2 illustrates the correlation coefficients and standard error of the estimates between MKJ, TAA, and DBC

Table 2. Comparison of methods of protein estimation for whole-grain and dhal samples

Method	Correlation coefficient	Standard error of estimate (% protein)	Regression equation
Whole-grain protein			
MKJ against TAA	0.948**	0.525	$Y = 0.943 + 0.947X$
MKJ against DBC	0.874**	0.828	$Y = 0.987 + 0.968X$
Dhal protein			
MKJ against TAA	0.967**	0.612	$Y = 3.409 + 0.868X$
MKJ against DBC	0.943**	0.703	$Y = 2.198 + 0.923X$

** Significant at 1% level.

methods. The MKJ procedure was found to be positively and significantly correlated with TAA procedure for the whole grain (0.948**) and dhal (0.967**) protein.

Correlation of values of the MKJ method with those of the DBC method was 0.874** for whole-grain and 0.943** for dhal samples. Also, the standard error of estimates was higher for whole-grain (0.828) compared to dhal samples (0.703). This difference could be due to interference of seed-coat pigments in the DBC method. When whole-grain and dhal samples of about equal protein content were analysed, it was observed that seed-coat absorbed some of the dye, resulting in higher DBC values (percentage transmission) in the case of whole grain samples.

3.2.1. Factors that affect the protein estimation by the DBC method

The effects of duration of mixing, flour particle size and temperature on protein values of whole-

Table 3. Effect of flour particle size and time of mixing on protein estimation by DBC method[a]

	Particle mesh size[b]			Time of mixing[c] (min)				
Cultivar	20	40	60	15	30	60	90	120
Whole grain								
HY-3C	18.5	19.9	20.3	19.8	20.1	19.7	19.9	19.9
Gwalior-3	19.2	22.7	23.1	22.5	22.8	23.1	23.4	23.4
Dhal								
HY-3C	22.0	24.0	24.3	23.7	23.8	23.8	24.0	24.1
Gwalior-3	23.6	27.0	27.4	26.5	26.8	27.1	27.5	27.6

[a] Average of two estimations.
[b] Mixed for 60 min.
[c] 60-mesh samples.

grain and dhal samples of two cultivars estimated by the DBC method were investigated. It was found that the smaller sized flour particle (20-mesh) sample had a lower protein content compared with a 40-mesh sample (Table 3), indicating the effects of interaction of finely ground materials. Different durations of mixing did not significantly affect the protein values although the protein percentage increased with longer mixing time (Table 3). Such variation among the cultivars might also affect the correlation between the MKJ and the DBC methods. However, for routine screening it was observed that the DBC results of 40 and 60 mesh samples were similar.

It has been reported previously that the DBC values were affected when samples of soya bean and wheat products were heated at 130°C for 20 h.[4] This was attributed to the substantial loss of available lysine during heating although heating for 10 h had only a slight effect on protein values. To test, this, whole-grain and dhal samples of three cultivars were dried at 70, 100 and 130°C for 24, 15, and 2 h, respectively, and DBC values were obtained on these samples. Moisture (percentage) lost due to various treatments was determined, and protein values obtained on undried samples were appropriately corrected as estimated values (Table 4). When determined by DBC method, only a slight variation in protein values was observed due to heating.

Table 4. Effect of heating on protein estimation by DBC method[a]

			Protein (%)					
			Sample treatment					
		Fresh weight	70°C for 24 h		100°C for 15 h		130°C for 2 h	
Cultivar	Component	basis	Observed	Estimated	Observed	Estimated	Observed	Estimated
HY-3C	Whole grain	21.8	23.0	23.2	23.0	23.3	23.0	23.5
	Dhal	24.0	25.2	25.4	25.1	25.7	25.5	25.9
ST-1	Whole grain	23.0	25.4	24.6	25.0	24.8	25.0	25.0
	Dhal	25.5	27.2	27.1	26.8	27.3	27.2	27.5
Sharda	Whole grain	22.8	24.1	24.3	23.9	24.5	24.2	24.6
	Dhal	24.8	26.6	26.3	26.2	26.6	26.3	26.9

[a] Observed values were obtained on dried samples. Estimated values were obtained by applying the moisture corrrection to protein values obtained in undried samples.

3.3. Relationship between whole grain and dhal protein contents

A positive and significant correlation ($r = 0.865^{**}$) was observed between the whole-grain and dhal protein contents determined by the MKJ method (Table 5), while the TAA and DBC methods

Table 5. Correlation coefficient and standard error of estimate between whole-grain and dhal protein content obtained by MKJ, TAA and DBC methods[a]

Method	Correlation between whole-grain and dhal protein	Standard error of estimate (% protein)	Regression equation
MKJ	0.865**	0.871	$Y=5.807+0.866X$
TAA	0.891**	0.646	$Y=5.593+0.882X$
DBC	0.771**	1.033	$Y=7.302+0.799X$

[a] Based on 43 cultivars.

exhibited correlation coefficients of 0.891** and 0.771**, respectively. The relatively lower correlation coefficient obtained by the DBC method could be due to the interference of seed-coat pigments in the whole-grain samples.

The relationship between whole-seed and dhal protein content can be affected by the percentage of seed-coat, its protein content, and grain size, The effects of seed-coat and its protein content were examined for the 43 cultivars by calculating the expected protein content of dhal according to the equation:

$$Pd=\frac{Pw\times 100-Psc\times Sc}{100-Sc}$$

where Pd, Pw, and Psc are percentages of dhal, whole-grain, and seed-coat protein, respectively, and Sc represents the percentage of seed-coat in the whole-grain sample. The minimum, maximum, and mean values are reported in Table 1. The calculated dhal protein percentages differed from the observed values by 0.5–8.7%.

The difference between whole-grain and dhal samples varied between 2.9 and 3.7 units. Whole-grain and dhal protein values showed a higher correlation coefficient ($r=0.927$**) for the medium group compared to that of low and high groups, thus indicating a variability in relationship among the different groups as reported in Table 6. Also, the correlation coefficient ($r=0.869$**) of all the

Table 6. Relationship between the protein content of whole-grain and dhal samples in 83 germplasm accessions analysed by the Technicon Auto-Analyser

Group	100 grain weight (g)	Protein (%)		Unit difference between whole seed and dhal protein	Correlation coefficient[a]
		Whole grain	Dhal		
Low ($n=28$)	7.0 (4.9–8.2)	21.3 (19.5–22.8)	25.0 (21.7–27.8)	3.7 (2.2–5.4)	0.794**
Medium ($n=27$)	9.6 (8.5–11.0)	21.5 (19.4–23.6)	24.9 (22.4–27.3)	3.4 (2.5–4.6)	0.927**
High ($n=28$)	14.2 (11.1–21.1)	20.8 (18.2–24.1)	23.7 (20.3–27.5)	2.9 (1.6–3.2)	0.879**
Total ($n=83$)	10.3 (4.9–21.1)	21.2 (18.2–24.1)	24.6 (20.3–27.5)	3.3 (1.6–5.4)	0.869**

[a] Between whole grain and dhal protein contents.
** Significant at 1% level.
Mean values and ranges are shown in parentheses.

three groups together was considerably lower. In the case of the medium group about 86% variation in dhal protein content appeared to be associated with the variation in whole-seed protein content. In the case of the low group, only 63% of variation in dhal protein was associated with the variation

in the whole-grain protein, and this might be due to the observed negative correlation between grain size, and percentage of seed-coat.

In an attempt to determine if the correlations could be improved by the use of variables such as percentage of seed-coat and protein percentage in seed-coat, the following linear multiple regression equation was obtained: $Y=0.915+1.135x_1-0.222x_2+0.187x_3$, where x_1, x_2, and x_3 represent the percentage of whole-grain protein, seed-coat content and seed-coat protein, respectively. A correlation coefficient of 0.916** was obtained between the whole-grain and dhal protein content. As expected, a slight improvement in the coefficient between these variables was achieved. Using this equation dhal protein content was calculated for 43 cultivars, and the minimum, maximum, and mean values are reported in Table 1. The calculated dhal protein percentages varied from −1.4 to 3.7 from the observed values. However, this equation will find little use in a screening programme as it involves the estimation of other components also. Therefore, it appears that for preliminary ranking of germplasm accessions and breeding material, whole-grain samples could be analysed using the rapid procedure of TAA if large numbers of samples are involved. However, in a selection procedure for high-protein lines involving smaller number of samples, analysis of dhal samples is preferable.

4. Conclusions

Rapid procedure of TAA for protein analysis could be used for both the whole grain and dhal samples, while the DBC procedure seems to be better-suited to analyse dhal samples only. Considering the cost and simplicity of the DBC method in relation to the TAA method, analysis of whole-grain samples by the DBC method is suggested where large number of samples (germplasm) are involved and where ranking of cultivars for their protein content is more important rather than the absolute amount. Small grains gave a lower correlation between whole-grain and dhal protein content and overall only 76% of the variation in dhal protein could be attributed to the variation in whole-grain protein content.

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