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Methods of Protein Estimation and the Influence of Heat Stress on Rice Grain Protein

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ABSTRACT

Rice is an important staple food crop of the world and contains 6-7 % of protein. Protein is estimated as total nitrogen content by widely accepted Kjeldhal method and converted by using an appropriate correction factor. Protein estimated by extraction buffers is superior to traditional analytical methods. Buffer containing 50 mM Tris Hcl (p^H-7.5), 2 % SDS, 0.6 % 2-mercaptoethanol and 4M urea reported highest total protein in rice without mentioning incubation time and its validation. Thus an experiment was conducted to standardize the incubation time required for dissolution of sample in the above buffer. Grains from eighteen rice varieties that were cultivated under normal and high temperatures were taken for the study and were incubated for seven different time treatments viz., 0, 30, 60, 90, 120, 150 and 180 minutes and the extracted protein was quantified following Lowry method. Further, the efficiency of this buffer was compared with 1N NaOH solution and Kjeldahl method. Experimental reports indicated that incubation period does not have any impact on total protein content. At high temperature, seven genotypes viz., GSR-328, Tellahamsa, Sita, Akdhan, Dhaniyadhan, Pantdhan-16 and GSR-330 recorded almost similar total protein content under both temperature conditions whereas less variation in protein content was observed in rest of the genotypes under normal and high temperature conditions. The other two methods yielded more protein content than the buffer and this was expected with Kjeldahl method whereas the reasons for higher protein content observed with 1N NaOH solution needs to be identified.

Key words: *Heat stress, Incubation time, Kjeldahl, NaOH, Protein extraction, Rice grain*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops, and staple food for almost half of the world's population. Rice grain contains approximately, carbohydrates 76.2 %, lipids 0-3.2 %, proteins 6-7 %, fibre 3.6 %, energy 367 kcal and fractions of several vitamins and minerals[26]. Among the several nutrients, proteins are one of the major groups of food components in rice.

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Among the cereal proteins, rice protein is valued for its nutrition and quality as it is rich in lysine, which is an important essential amino acid[1] for growth and development. The hypoallergenic property and the high nutritive quality make rice protein a competitive protein ingredient in the food market [13]. However, the use of rice protein in food systems is now limited due to its unavailability and unknown functional properties.

Total protein content in rice grain ranges from 4.3-18.2 % which is quite low compared to the legume crops[19]. Milled rice or rice endosperm contains 3.8–8.8% albumin, 9.6–10.8% globulin, 2.6–3.3% prolamin and 66–78% glutelin[2]. Though glutelin is the major storage protein in rice, it is highly insoluble in water due to its high molecular weight, heterogeneity and disulfide bonds[10 & 14] which makes the extraction of total protein difficult from the grain. In cereals, protein was initially estimated by total nitrogen content like Kjeldahl[15], Dumas[7] method etc., and later shifted to protein extraction followed by estimation. Several reports suggested that acidic ($p^H > 3$) and alkaline ($p^H > 10$) solutions are efficient in extracting glutelin / total protein from the grain[23]. Based on this, researchers worked on different solutions and buffers to extract the proteins *viz.*, trichloroacetic acid (TCA) precipitation[5], TCA / Acetone precipitation and fractionation[11], no precipitation fractionation[9] and extraction of different classes of proteins using respective buffers[25]. Among the methods, protein estimation by total nitrogen appears to be widely followed[15]. However, in Kjeldahl method there is a chance of interference of non-protein nitrogen which may result in error / false representation in total protein content. Thus, it is essential to exploit several other methods of extraction so that a precise estimate of total protein content is possible. Extraction of protein using buffers is a notable alternative to pre-existing methods. Though various buffers were employed for rice protein extraction, the buffer which yielded more total protein content[25] and its component classes was used in the present study. Methods that estimate the extracted protein suggested for specific incubation time, 6hrs[2] using Burrel shaker based on total nitrogen determination, 1-2hrs[3] by vortexing using TCA/acetone extraction and Santos *et al.,* 2013 using Tris-HCl method, 1hr[10] by Orbit shaker using Osborne's protein fractionation method, 15-30min[20] using phenol extraction method and 10min[8] using phenol extraction method, after the addition of respective buffer to the tissue appears important for complete digestion and extraction of proteins (Table 3). However, none of these studies mentioned any justification for the respective incubation time. Since the incubation time of 60min suggested by the best protein extraction buffer[25] for the extraction of total protein occupies almost half of the total time required for executing this protocol, it is essential to unravel the significance of this incubation time in protein extraction. Therefore, the present article focuses on determining the precise protein content of rice grain samples using this buffer at various incubation times. Further, protein content of the same samples was also determined by other two popular methods, Kjeldahl and 1N NaOH methods, and the possible reasons for the variations in the estimated protein content among these three methods and the need for fixed incubation time were discussed.

MATERIAL AND METHODS

Eighteen rice accessions cultivated at Indian Institute of Rice Research farm and the same set of samples which were cultivated under polythene sheet where an average of 5°C temperature higher than the ambient conditions prevailed were selected for the study (Table 1). The harvested paddy was stored for three months at room temperature and cleaned thoroughly from dirt or inert matter. Paddy was de-hulled (Mini lab rice huller, M/S Krishi International), polished (Mini lab rice polisher, model no. K-710, M/S Krishi International) and the polished grains were ground to a fine powder using mortar and pestle and protein content in each ground sample was determined by the following three methods.

Protein extraction:

The method[25] which reported higher total protein content in rice grains was used for the extraction of total protein in the present study. The extracted protein was quantified using Lowry method [16] with Bovine Serum Albumin (BSA) as standard protein. A weighed sample of 25mg was used for protein extraction to which 1ml of sample buffer (50 mM Tris Hcl (p^H-7.5), 2 % SDS (w/v), 0.6 % 2-mercaptoethanol (v/v) and 4M urea) was added, samples were vortexed for proper mixing and kept for a series of incubation times *viz.*, 0min, 30min, 60min, 90min, 120min, 150min and 180min on rotator at 60 rpm. This was performed to know the level of variation on total protein content with different time intervals of incubation. At the end of each incubation time, samples were centrifuged (10000 rpm, 4°c for 20 min), supernatant was transferred to fresh eppendorff tubes, acetone was added to the supernatant and tubes were kept for overnight incubation at 4°C. After incubation, samples were centrifuged (10000 rpm, 15°c for 20 min) and supernatant was decanted. The pellet was dried to eliminate acetone, 1 ml of 2 % SDS in 1N NaOH was added and kept in water bath maintained at 40°c for 1hr or till the precipitate was dissolved completely. Appropriate volume of this solution was used to determine protein by Lowry method[16].

NaOH extraction method:

In this method, 50mg of ground rice sample was taken into 15ml screw- capped tube, 0.5ml of ethanol (to wet the sample and to avoid clump formation) was added and made sure no precipitate is formed at the bottom of the tube. To this, 4.5ml of 1N NaOH was added and incubated for 15min in a boiling water bath to ensure complete dissolution of sample. After 15min, tubes were taken out from boiling water bath and allowed to cool down to the room temperature. From this solution, an appropriate volume was taken for estimation of protein content using Lowry method.

Kjeldahl method:

This method estimates the total protein content by analysing total nitrogen in the sample. 0.25g of the ground rice sample was taken into digestion tubes, 3ml of concentrated H_2SO_4 was added to oxidise the organic substance and to release reduced nitrogen in the form of $(NH_4)_2SO_4$. A mixture of potassium sulphate, copper sulphate and titanium dioxide was added to the above solution to increase the boiling point and to act as catalyst for speeding up the reaction. After adding all these chemicals, samples were kept for digestion for 2hrs till dark coloured solution turns to colourless and clear solution. Further distillation was carried out with 15ml of NaOH which converts ammonium sulphate to ammonia indicating amount of nitrogen present in the sample. The end of the distillation unit condenser is dipped into boric acid solution (violet colour). The ammonia in the sample reacts with boric acid changing the colour of the solution from purple to green; a sample volume of nearly 50ml was collected and back titrated with 0.05N HCl till the green colour turns to light pink. Titre value was used for calculation of amount of nitrogen present in the sample with blank value (titration without sample) taken as 0.3.

$$\text{Amount of nitrogen present in the sample} = \frac{(\text{Titre value-Blank value}) \times 0.07}{\text{Weight of the sample}}$$

Here, blank value is taken as 0.3.

Total protein content = Amount of nitrogen in the sample × 5.95

(Here, 5.95 is used as a correction factor for total protein content in case of rice[17].

Statistical analysis:

Analysis of Variance (ANOVA) was carried out to understand the presence of variation in the material for the concerned trait with respect to time intervals within genotype and overall variation among the genotypes. Correlation studies[22] were also made to compare the level of association between

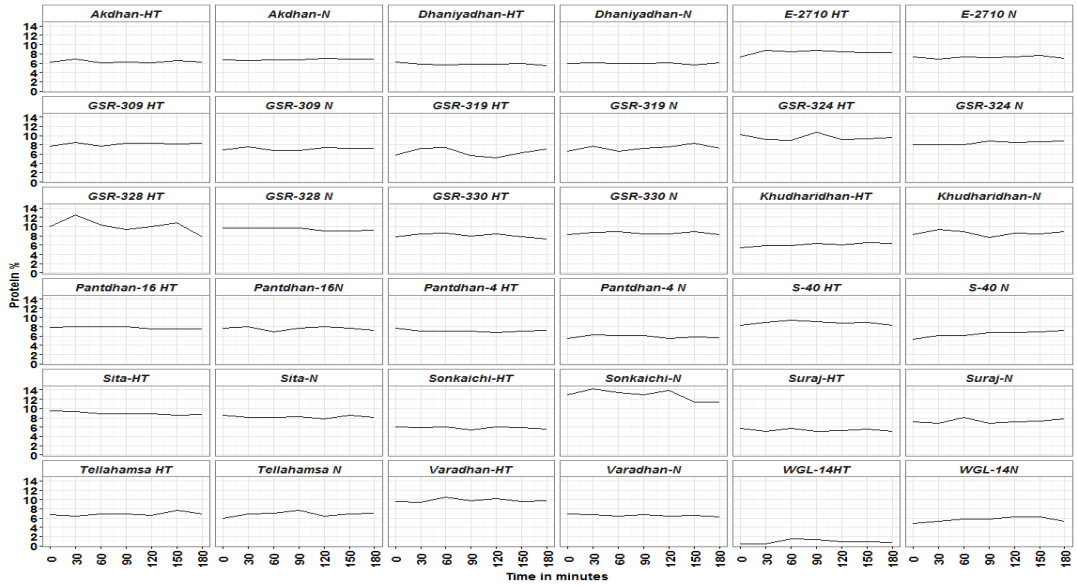
protein content estimated using three methods *viz.*, buffer extraction method, NaOH method and Kjeldahl method.

RESULT

Analysis of Variance (ANOVA) revealed the presence of significant variation among the genotypes for protein content estimated after extraction whereas significant variation was not observed in protein content in relation to incubation time (Table 2) *viz.*, 0min, 30min, 60min, 90min, 120min, 150min and 180min under both the temperature conditions, suggesting that for total protein extraction, incubation is not necessary after addition of the extraction buffer, however, thorough mixing (vortex) of the sample in buffer is required. Among the genotypes grown under normal temperature conditions, Sonkaichi recorded highest protein content (12.89 %) followed by GSR-328 (9.44 %), Khudaridhan (8.56 %) and GSR-330 (8.55 %) whereas WGL-14 (5.63 %), Pantdhan-4 (5.84 %) and Dhaniyadhan (5.96 %) recorded lower protein content in their grains (Table 3). To analyse the efficiency of protein extraction using buffer, protein content was also estimated in the same samples using 1N NaOH solution and Kjeldahl method separately. The estimated protein content values with 1N NaOH solution were presented in Table 4. The protein content ranged from 5.81(E 2710) to 10.69 (GSR 330). The mean protein value of all the genotypes was higher compared to buffer extraction method, though some entries recorded higher in buffer extraction were recorded low values in 1N NaOH solution and vice versa (Table 4). According to the classification given by Silveira et al.[21], which was based on electrophoresis, none of the genotype recorded higher protein content, whereas most of the genotypes *viz*., GSR-330 (10.69 %), GSR-324 (10.44 %), Suraj (10.33 %), Varadhan (10.10 %), Sonkaichi (10.08 %), WGL-14 (9.83 %), Khudaridhan (9.65 %), GSR-328 (9.56 %), GSR-319 (9.36 %) and GSR-309 (9.23 %) recorded medium protein content. However, Pantdhan 16 (8.71 %), S 40 (8.17 %), Akdhan (8.03 %), Tellahamsa (7.99 %), Dhaniyadhan (7.99 %), Sita (7.54 %), Pantdhan 4 (6.12 %) and E 2710 (5.81 %) recorded low protein content. In most of the genotypes, increased protein content was observed using NaOH method [24] compared to buffer extraction method. This can be due to extraction of untapped protein component by NaOH compared to buffer used for extraction and this further emphasize for the improvement of extraction buffer. As expected, Kjeldahl method also recorded higher protein content (Table 4) compared to buffer extraction method because the total nitrogen present in the sample not from protein component alone. Protein content using Kjeldahl method ranged from 7.66 (GSR-330 and Tellahamsa) to 13.83 (Varadhan) with a mean value of 10.74. Grain samples from plants grown under high temperature conditions were ground and total protein content was estimated using buffer extraction method. Results revealed that GSR-328 (9.85 %) recorded highest protein content followed by Varadhan (9.66 %), GSR-324 (9.59 %), Sita (8.73 %) and S 40 (8.69 %). However, WGL-14 (1.23 %) recorded lowest protein content followed by Suraj (5.39 %), Dhaniyadhan (5.79 %), Khudaridhan (6.04 %) and GSR-319 (6.29 %). Results were mentioned in Table 3.Considering both the temperature conditions *viz*., samples grown in normal and high temperature grown conditions, among the varieties, WGL-14 recorded lowest (5.63 and 1.23 %) and GSR-328 recorded highest (second best in normal conditions) protein content (9.44 and 9.85 %).Among the eighteen genotypes, seven (GSR-328, Tellahamsa, Sita, Akdhan, Dhaniyadhan, Pantdhan-16 and GSR-330) recorded almost similar protein content under normal and high temperature conditions (Table 3). However, a marked decrease (1-3%) in protein content from normal to high temperature condition was observed among WGL-14, Sonkaichi, GSR-13, Suraj and Khudaridhan genotypes indicating the susceptibility of these genotypes to high temperature in terms of protein content[4, 6 & 27]. Increase in protein content under heat condition in comparison to normal temperature was observed in Pantdhan-4, Varadhan, S 40, E 2710, GSR-309 and GSR-324. This can be

attributed to a metabolic response of protein/s possibly Heat Shock Proteins[4, 12, 18 & 28]. Variation in total protein content as well as individual proteins at high temperature in comparison with normal cultivation conditions is an interesting area which needs to be deciphered using proteomic studies and identification of responsible proteins may further help in developing temperature tolerant varieties. However, remaining genotypes *viz.*, GSR-328, Tellahamsa, Sita, Akdhan, Dhaniyadhan, Pantdhan-16 and GSR-330 exhibited stable performance in both the environments. Though above mentioned genotypes recorded stable protein content in both the environments, they can't be categorised in to temperature tolerant genotypes, simply based on total protein content.Correlation studies among three methods of protein extraction *viz.*, buffer extraction method, NaOH method and Kjeldahl method revealed no significant association among the methods for the protein content in the genotypes used in the study (Fig 2). Though metabolic convergence exists among all the living species, molecular nature of the nutrients vary from plants to animals and among their species. For example, plants possess the capacity to uptake nitrogen in the form of nitrate and reduce it into ammonia which is inturn converted to organic nitrogen. Whereas animal metabolism needs nitrogen in the form of organic molecules as raw nitrogen source and they can only exchange nitrogen among molecules, except, during urea cycle where ammonia emanated from the oxidative deamination of glutamic acid is fixed to synthesize carbamoyl phosphate. Further, among the various organic forms of nitrogen sources, protein or standard amino acid fraction is desirable over nucleotides which upon oxidation produce uric acid and the accumulated uric acid can crystallize with sodium ions leading to joint pains otherwise designated as dietary gout. Abnormal increase in uric acid levels is one of the important drawbacks of single cell protein. Hence, there is a need to determine the availability of safe fractions like protein and amino acids in the food components than the total nitrogen value which also includes non-protein nitrogen followed by identifying the high protein containing,genotypes.

Fig. 1: Protein content of eighteen genotypes at different incubation time intervals



(Where, N- Normal Temperature; HT- High Temperature)

Table 1: List of genotypes taken for the protein content analysis

1. WGL-14	6. Sita	11. Sonkaichi	16. GSR 309
2. Khudharidhan	7. Varadhan	12. Dhaniyadhan	17. GSR 324
3. GSR 328	8. Akdhan	13. Suraj	18. GSR 330
4. Tellahamsa	9. GSR 319	14. E 2710	
5. Pantdhan 4	10. S 40	15. Pantdhan 16	

Table 2: Analysis of Variance (ANOVA) for protein content of different genotypes at different time intervals using buffer extraction method and to compare variation among three different protein extraction methods *viz.*, buffer extraction method, NaOH method and Kjeldahl method

Source of variation	Degrees of freedom	Mean Sum of Squares
Among the genotypes		
Genotype	35	25.71**
Residual	216	0.24
Within a genotypes at different time intervals		
Time intervals	6	0.48^{ns}
Residual	245	3.88
Among the genotypes		
Genotype	17	7.57^{ns}
Residual	36	7.08
Within a genotype using three methods		
Methods	2	7.41^{ns}
Residual	51	7.23

Table 3: Protein content of given genotypes at normal and heat conditions

Genotype	Time (min.)	Protein content (%) at normal condition	Protein content (%) at heat condition	Genotype	Time (min.)	Protein content (%) at normal condition	Protein content (%) at heat condition
WGL-14	0	4.85	0.48	Tellahamsa	0	6.00	6.71
	30	5.27	0.49		30	6.82	6.48
	60	5.74	1.49		60	6.99	6.86
	90	5.81	1.33		90	7.72	6.91
	120	6.22	0.91		120	6.48	6.49
	150	6.22	0.82		150	6.88	7.61
	180	5.34	0.78		180	7.10	6.96
	Mean	5.63	1.23		**Mean**	6.85	6.98
Khudharidhan	0	8.19	5.44	Pantdhan-4	0	5.46	7.67
	30	9.43	5.94		30	6.34	7.08
	60	8.82	5.84		60	6.05	7.06
	90	7.63	6.30		90	6.05	7.04
	120	8.64	6.10		120	5.57	6.81
	150	8.46	6.50		150	5.81	7.04
	180	8.97	6.32		180	5.64	7.18
	Mean	8.56	6.04		**Mean**	5.84	7.09
GSR-328	0	9.66	9.94	Sita	0	8.62	9.50
	30	9.76	12.46		30	8.06	9.30
	60	9.63	10.26		60	8.03	8.92
	90	9.68	9.29		90	8.21	8.91
	120	9.09	9.95		120	7.82	8.79
	150	9.03	10.71		150	8.63	8.48
	180	9.22	7.81		180	8.08	8.75

	Mean	9.44	9.85		**Mean**	8.20	8.73
Varadhan	0	6.82	9.58	Sonkaichi	0	12.93	5.97
	30	6.67	9.37		30	14.16	5.82
	60	6.43	10.48		60	13.43	6.05
	90	6.69	9.73		90	12.90	5.44
	120	6.47	10.15		120	13.97	6.00
	150	6.54	9.57		150	11.46	5.82
	180	6.31	9.69		180	11.37	5.48
	Mean	6.56	9.66		**Mean**	12.89	6.20
Akdhan	0	6.76	6.31	Dhaniyadhan	0	5.98	6.19
	30	6.54	6.93		30	6.12	5.83
	60	6.66	6.04		60	5.97	5.60
	90	6.78	6.29		90	5.92	5.71
	120	7.01	6.09		120	6.11	5.70
	150	6.91	6.53		150	5.55	6.00
	180	6.93	6.28		180	6.05	5.52
	Mean	6.80	6.84		**Mean**	5.96	5.79
GSR-319	0	6.69	5.83	Suraj	0	7.14	5.79
	30	7.79	7.19		30	6.76	5.11
	60	6.70	7.38		60	8.02	5.67
	90	7.29	5.75		90	6.86	5.09
	120	7.59	5.28		120	7.09	5.29
	150	8.38	6.30		150	7.31	5.63
	180	7.20	7.17		180	7.79	5.14
	Mean	7.38	6.29		**Mean**	7.28	5.39

S-40	0	5.40	8.40	E-2710	0	7.39	7.38
	30	6.08	8.94		30	6.91	8.77
	60	6.09	9.38		60	7.28	8.48
	90	6.68	9.19		90	7.26	8.77
	120	6.69	8.83		120	7.35	8.45
	150	6.99	8.93		150	7.59	8.27
	180	7.18	8.32		180	7.09	8.31
	Mean	6.44	8.69		**Mean**	7.27	8.35

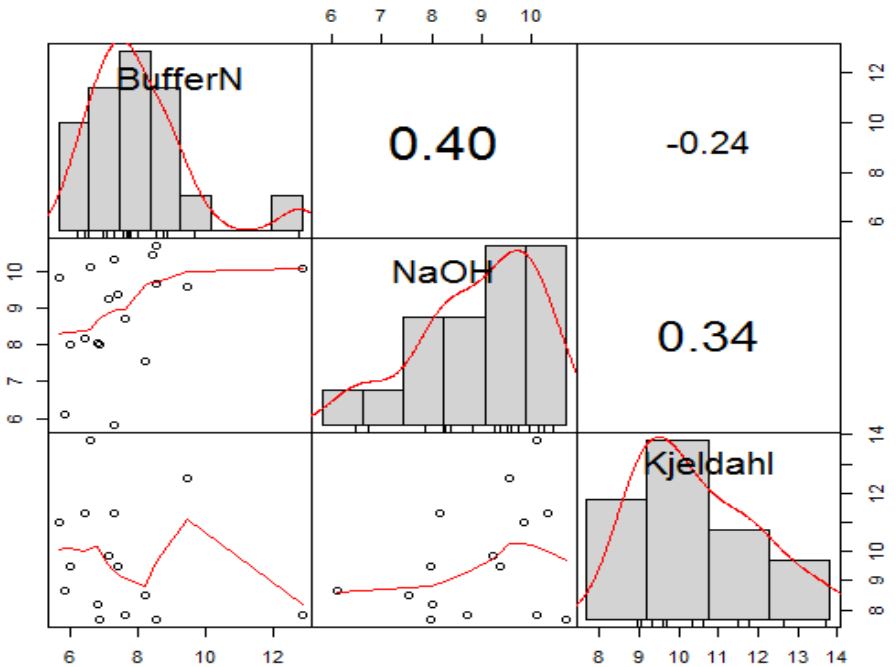
GSR-309	0	6.92	7.65	Pantdhan-16	0	7.64	7.78
	30	7.55	8.57		30	8.06	7.98
	60	6.81	7.66		60	6.98	8.09
	90	6.73	8.29		90	7.63	7.94
	120	7.38	8.31		120	7.96	7.49
	150	7.31	8.18		150	7.71	7.57
	180	7.24	8.32		180	7.15	7.52
	Mean	7.13	8.14		**Mean**	7.59	7.77

GSR-324	0	8.01	10.20	GSR-330	0	8.21	7.73
	30	8.03	9.19		30	8.70	8.50
	60	8.05	8.97		60	8.96	8.51
	90	8.86	10.67		90	8.45	7.88
	120	8.47	9.17		120	8.46	8.41
	150	8.74	9.33		150	8.87	7.74
	180	8.87	9.65		180	8.25	7.29
	Mean	8.43	9.59		**Mean**	8.55	8.01

Table 4: Comparison of protein content values of genotypes obtained using three methods *viz.*, Buffer extraction method, NaOH method and Kjeldahl method

S.No	Sample name	Buffer extraction method (Mean)		NaOH method	Kjeldahl method
		Normal temp.	Heat		
1	WGL-14	5.63	1.23	9.83	11.00
2	Khudharidhan	8.56	6.04	9.65	--
3	GSR 328	9.44	9.85	9.56	12.50
4	Tellahamsa	6.85	6.98	7.99	7.66
5	Pantdhan 4	5.84	7.09	6.12	8.66
6	Sita	8.20	8.73	7.54	8.50
7	Varadhan	6.56	9.66	10.10	13.83
8	Akdhan	6.80	6.84	8.03	8.16
9	GSR 319	7.38	6.29	9.36	9.50
10	S 40	6.44	8.69	8.17	11.33
11	Sonkaichi	12.89	6.20	10.08	7.83
12	Dhaniyadhan	5.96	5.79	7.99	9.50
13	Suraj	7.28	5.39	10.33	11.33
14	E 2710	7.27	8.35	5.81	--
15	Pantdhan 16	7.59	7.77	8.71	7.83
16	GSR 309	7.13	8.14	9.23	9.83
17	GSR 324	8.43	9.59	10.44	--
18	GSR 330	8.55	8.01	10.69	7.66

Fig. 2: Correlation among different methods of protein analysis *viz.*, Buffer extraction method, NaOH method and Kjeldahl method for same set of genotypes



CONCLUSION

The present study revealed that, incubation time is not required for the extraction of total proteins with the buffer. Upon comparison of buffer extraction method with NaOH method and Kjeldahl method, despite higher values obtained by the later two methods the variation was not statistically significant. Since the buffer extraction method is based on extraction of total protein content, instead of total nitrogen in other method can be credited as better method for extraction and quantification of total protein in rice grain. Among the genotypes grown at high temperature conditions, some (five) recorded lower values while most of the genotypes recorded similar (seven) to higher (six) total protein content to the genotypes grown at normal temperature conditions., thus further study is required to understand the effect of temperature stress on total protein content of rice grain.

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