The Pharma Innovation Journal 2023; 12(6): 2621-2626

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(6): 2621-2626

www.thepharmajournal.com
Received: 02-03-2023
Accepted: 04-04-2023

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Estimation of protein of germinated multi-millet puffs using an advanced Kjeldahl titration method

Anurag and Amar P Garg

Abstract
The evaluation of the Kjeldahl digestion method was studied by comparing measured values of total nitrogen, using Advanced Kjeldahl Titration and ordinary Kjeldahl method. The protein content of non-germinated and germinated extruded puff product varied from (07.11% to 10.66%) and (09.45% to 11.98%) as determined by Advanced Kjeldahl Distillation and Digester method (Method 46-13; AACC, 2000) while the protein content of non- germinated and germinated extruded puff varied from (05.11% to 10.96%) and (07.45% to 11.56%) with ordinary Kjeldahl method. Various combinations of millets showed that T3 combination with germinated millets was better in terms protein content as indicator of nutritive value. The total ash content of non- germinated and germinated extruded puff product varied from (04.11 to 04.85) and (04.56 to 06.32). The dietary fiber of non- germinated and germinated extruded puff product varied from (14.66 to 14.98) and (15.66 to 16.25) and the carbohydrates of non-germinated and germinated extruded puff product varied from (66.26 to 67.21) and (66.21 to 67.67). Statistically analysis showed significant difference at (p<0.0001) with respect to each combination. Incorporation of millet and sorghum in rice flour (T_3) results in an incorporation of higher protein content other nutrient composition and overall acceptable sensory properties in the extruded puff product. The proximate composition and functional properties of multi millets puffs were determined using the methods of AOAC. There was significant difference in the proximate compositions of the multi millet puffs (p =0.05) overall acceptable sensory properties.

Keywords: Auto analyzer technicon, total nitrogen, standard Kjeldahl digestion, steam distillation

1. Introduction

UN declared the year 2023 as "International Millet Year" to fight with malnutrition of 2/3rd population of world and to encourage the cultivation of millets as alternate crops as strategic crops under climate change (Garg, 2023, Anurag and Garg 2023). An infinite of proteins could be synthesized from the twenty odd natural amino acids. The proteins form a wide variety of biological molecules to carry out various biological activities in all living organisms such as cell repairing, transportation, enzyme production, biosensors including cell signaling etc. (Mancini, *et al*., 2022) [6]. There are number of ways in which the nitrogen content of sample may be determined. Among these all, Kjeldahl method is one which is most used method for nitrogen determination in food industry. It involves three steps: - the digestion of protein under acid or basic environment, along with distillation followed by titration (Accardo *et al*., 2022) [1]. It has been observed that in the Kjeldahl titration method for nitrogen estimation, first digestion of protein take place in which sulfuric acid alone is used as a digestion medium which helps in digestion of nitrogen present in sample to change it into ammonium sulphate. The amount of sulphuric acid used is one of the most important factors. Further, the use of a catalyst in the digestion process helps in oxidation and thus allows the complete analysis of nitrogen in sample (Saez-Plaza, *et al* 2013) [10]. Secondly, the neutralization process facilitates the conversion of ammonium sulphate into ammonia gas and this process is known as distillation. Titration in acid environment for the detection of nitrogen present in the sample is the final step (Goyal, *et al*., 2022) [4].

Today's digestion systems offer safety both from a personal perspective and from an environmental point of view. The determination of nitrogen content is a frequently conducted analysis practice in food industry and commerce and various organizations have developed their working protocols. India is the fastest-growing countries in technological, economical, and educational advancements. Though India is one of the largest producers of food grains, horticulture products, milk and eggs still it suffered malnutrition problems, mainly and probably because of poor and wrong collection of data/survey and also because of ill and unequal distribution of food (Garg, 2023) [3].

Malnutrition is the main health crisis in developing countries; people experience both physical and mental problems. According to the Global Hunger Index 2022, India ranks 107 out of 121 countries (GHI rank) which is not acceptable to the Government of India as much poor countries like Pakistan, Syria and others have been raked at better position while they are fighting for basic and minimum food in their everyday life. But to address this serious issue, the Government of India has focused on nutritionally rich millets which made the foundation of this study also. Malnutrition is referred to as scarcity, imbalance, or excess intake of food or nutrients (WHO 2022).

Millets are cereal crops generally small-seeded and known for high nutritive value. The grains form a good source of micronutrients, phyto-chemical and have little or no amount of gluten. Gluten free foods are now considered as medicinal foods as they help in management of diabetes. After Covid-19 pandemic, people have become more conscious about the health and nutritional value of food including medicinal/functional foods. Nutrient availability of food depends on various factors like the type of food, raw material, and processing of food. Low nutritional value and non-availability of nutrients from a food source is the major cause of malnutrition in underdeveloped countries. Germinated multi-millet grains with extrusion cooking have been developed widely for the continuous processing of nutritional-enriched food products. During extrusion processing, the raw feed material is exposed to high thermal and shear, and frictional energies to transform it into modified intermediate and finished products (Ilo *et al*. 1996, WHO/UNICEF 2019). Millets also contain calcium, iron, and fibers which help to fortify essential nutrients for healthy growth in children. Millet has more protein than rice and is rich in vitamins A and B, iron, phosphorus, magnesium, and manganese. The usage of millet in infant food and nutrition products is increasing and many manufacturers are expanding their business operations by acquiring smaller firms. Millets are a convenient meal because of their ease of preparation, low cost, and relatively long shelf life. Changing food habits, increasing population, and urbanization have led to increasing consumption of noodles worldwide (Meherunnahar *et al*., 2023) [8]. Pearl millet has high nutritive values in terms of high calcium, iron, phosphorus, and protein contents, etc. Bajra contains total protein (11.6 g/100 g), carbohydrates (61.7 g/100 g), minerals (2.3 g/100 g), and dietary fibers (11.4 g/100 g). Ragi/ Finger millet has a rich amino acid profile with high content of proteins. The most prominent amino acid present in ragi is lysine (Lys), cysteine (Cys), valine, and methionine (Met). Foxtail millet is rich in the source of minerals, vitamins, fiber, starch, and essential amino acids, but methionine and lysine are not present in the foxtail millet in comparison to other cereals and crops. Sorghum is a starchy, gluten-free, high-protein, cholesterol-free source of a variety of essential nutrients, including dietary fiber, iron, phosphorus, and thiamine.

Germinated sorghum was characterized by putrescine-rich and low spermidine levels with higher level tannin free sorghum. Pearl millets, soaking for 24 h, Protein content increased due to the mobilization of stored nitrogen in grains. Fat content and crude fiber increase with sprouting the utilization of energy sources resulting in reduced carbohydrates. Advantage of soaking seeds in water before germination as it resumes the metabolic activity of grain as it absorbs water and reduced the formation of anti-nutritional factors like water-soluble vitamins, soluble carbohydrates and riboflavins (Frias *et al*. 2000; Vidal-Valverde *et al*., 2002) before germination. During germination reserve ingredients are demolished, which are commonly employed for cellular respiration checkups and the synthesis of new cells before developing embryos (Vidal-Valverde *et al*., 2002). Germination enhanced the water absorption capacity, solubility, and oil absorption capacity of flour samples significantly (Yenasew *et al*., 2023) [18].

2. Materials and Methods

2.1 Soaking and Germination Condition of Millets: Soaking and germination is a traditional approach to improve and increase the bioavailability of nutrient components. Before the processes like germination, cooking, canning, and fermentation, grains are hydrated to the level where they obtain the maximum weight due to the absorption of water (Anurag and Garg, 2022) [2]. Four different millets were used in different combinations (Fig. 1). Soaking was made for 12 h while germination was allowed for 72 h at room temperature using standard precautions for adequate moisture content during entire period of germination. The millets were wrapped in a jute bag and placed in a dark cool place.

Fig 1: Germinated millets are used as raw materials for extruded puffs products production

2.2 Preparation of puffs in combinations using millets: The germinated Sorghum millet, Finger millet, Foxtail millet, Pearl millet and Rice grains were selected for blends preparation and each sample was mixed in a different proportion of 11.5:11.5:11.5:11.5:6:4 before extrusion cooking. The blended samples of non- germinated and germinated were conditioned to a moisture content of 20–21% (wt %) by spraying the calculated amount of water-salt mixture and then mixed thoroughly (Table 1, 2)

The blended sample was then allowed to be left for 4h to equilibrate at room temperature prior to extrusion. This pre-conditioning procedure is a necessary step to ensure uniform mixing and proper hydration and to minimize variability and maximize the nutritive status of extruded puff material. The moisture content of each sample was determined by AOAC (2005) method.

Table 1: Standardization and formulation of composite non-germinated multi-millets grain for extruded puff

Raw materials	Composite multi-millets grains samples		
	T_1(NGP)	T_2 (NGP)	T_3 (NGP)
Sorghum millet	11.5	11.5	11.5
Finger millet	11.5	11.5	11.5
Foxtail millet	11.5	11.5	11.5
Pearl millet	11.5	11.5	11.5
Rice	6	4	4
Salt (gm)	2	2	2
Water (ml)	115	115	115

Note: Values stand for the mean of triplicate ± standard deviation. Means with no common letters within a row differed ($p \leq 0.05$). T_1, T_2, T_3(NGP)= Non-Germinated Puffs

Table 2: Standardization and formulation of composite germinated multi-millets grain for extruded puff

Raw materials	Composite multi-millets grains samples		
	T_1 (GP)	T_2 (GP)	T_3 (GP)
Sorghum millet	11.5	11.5	11.5
Finger millet	11.5	11.5	11.5
Foxtail millet	11.5	11.5	11.5
Pearl millet	11.5	11.5	11.5
Rice	6	4	4
Salt (gm)	2	2	2
Water (ml)	115	115	115

Note: Values stand for the mean of triplicate ± standard deviation. Means with no common letters within a row differed ($p \leq 0.05$). T_1, T_2, T_3 (GP)= Germinated Puffs

2.3 Instrumentation
The nitrogen content was estimated using Kjeldahl Distillation and Digester method and the value of nitrogen was converted to crude protein using a factor of 6.25 (Method 46–13; AACC, 2000). Kjeldahl Digester-DG2 is a step included in the quantitative determination of nitrogen content in organic compounds, during which the organic compounds are reacted with hot, concentrated sulphuric acid. Kjeldhal nitrogen analyzer has two main components: Kjeldhal Digester-DG2 and Kjeldhal Distillation KNA-DS2.This system includes a heating mantle and gas venting pipe, required to vent off the toxic gases formed during digestion. The Kjeldahl Digester-DG2 system is manufactured according to the state-of-the-art technology and recognized safety regulation. Its design provides an even distribution of heat waves which leads to better quality digestion. The equipment used was purchased from Spectra Lab and the procedure recommended by the manufacturer was followed with modifications as used in our laboratory in accordance AOAC protocols.

2.4 Sample Preparation for Digestion
An appropriate amount of 0.2-0.3 g of the sample was taken on the butter paper, thimble was made, and added directly in dry and clean digestion vessel to which 5 g of cupric sulphate + 7.5 g of potassium sulphate were added. 15 ml of sulphuric acid for fast digestion followed by 0.5 ml of (30%) hydrogen peroxide. Blank samples (Reagents only) was also prepared. These were then inserted into the vessel in the holes of digestion block at an ambient temperature. Digestion processing of sample was completed when the sample color changed from black to blue. At least 2.5 h were required for the completion of digestion. After digestion, 30ml of distilled water was added to each vessel.

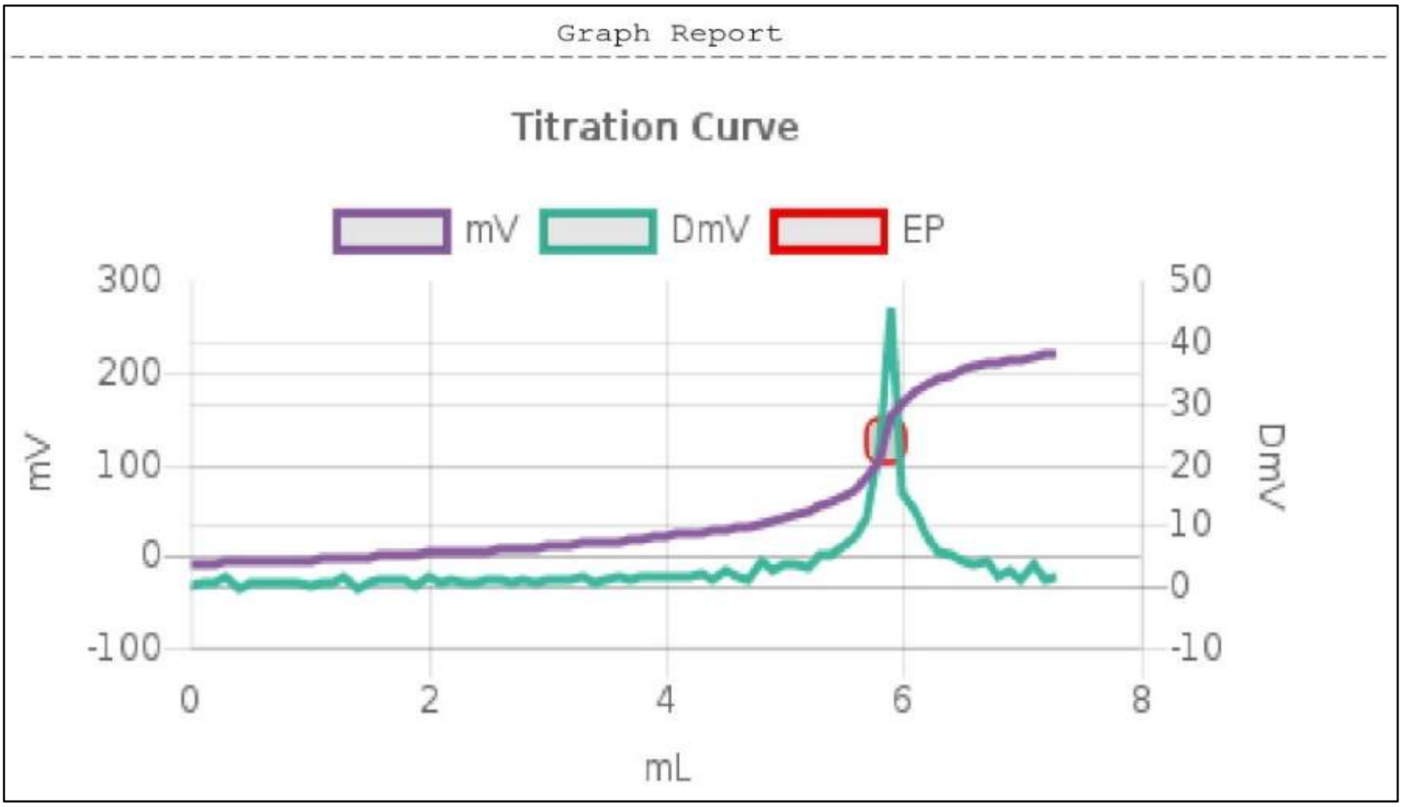
2.5 Kjeldahl Distillation-DS2
The Kjeldahl flask containing the digested sample was placed into Kjeldahl distillation-DS-2 system. Afterwards, the steam distilled with small amounts of sodium hydroxide converted the ammonium salt into ammonia. The released ammonia was trapped in boric acid solution with set values of distillation and reaction times. It provides regulated steam output and conveniently, automatic draining of the sample residues. High-precision pumps ensured constant accurate dosing of selected reagents.

2.5.1 Distillation: Excess base was added to the digestion product to convert NH_4 to NH_3 as indicated in the following equation. The NH_3 is recovered by distilling the reaction product.

Ammonium Sulfate	Heat	Ammonia Gas
$(NH_4)_2SO_4 + 2NaOH$	→	$2NH_3 + Na_2SO_4 + 2H_2O$

Kjeldahl Indicator or ethyl Red Bromcresol Green Indicator was used and titration was made with 0.1N sodium hydroxide solution that produced green color and then added 0.1N Hydrochloric acid solution in a drop wise manner to produce a deep wine-red color. The sample was digested in a digestion unit till it became colorless. Then the sample tubes were kept for cooling and then transferred digested materials into the distillation unit. 40 ml of NaOH (40%) was added into the reaction mixture tube. Released ammonia amount was absorbed in (4%) boric acid solution containing mixed indicator of 10ml bromocresol green and 7 ml of methyl red.

2.6 Titration
The amount of nitrogen in a sample depends on the quantified amount of ammonia ion in the receiving solution. The original pink color of the boric acid solution was turned into green color and at this stage the prepared solution was titrated against 0.1 N HCl until the whole solution color turned into pink color. Titration quantifies the amount of ammonia in the receiving solution. The captured ammonia was carefully measured and excess of a standardized acid solution in the receiving flask keeps the pH solution low and the indicator color does not change until the solution is "back titrated" with base as presented in Fig. 2 and nitrogen content graph report of germinated extruded puffs is presented below.



***Note.** mV- mini volt, DmV- difference mini volt, EP- End Point for T_3(GP)= Germinated Puffs

Fig 2: Titration curve of germinated extruded puffs.

Report analysis of germinated extruded puffs, using of spectra lab auto titration unit for nitrogen content in extruded puffs with specific parameters, showed that the nitrogen content depends upon method used (SVD) step volume dosing with nonpolar electrode on 25 °C with initial (mV) mini volt 47.3 mV which is presented in Fig.2.

$$N = \frac{(\text{Endpoint ml}) * \text{Factor} * \text{Actual Norm} * 100}{\text{Exp Norm} * \text{Sample Weight}}$$

Thus, the percentage crude protein was calibrated according to the following equation.

Nitrogen Factor = N*6.25= % of Protein

2.7 Evaluation of sensory attributes

The assessment of sensory attributes of the developed product was conducted at a Regional public analytical laboratory. The developed product with an added flavor of Maggie masala and dried coated with commercial chat masala was served to a 16-member panel of judges for testing purposes. Judges tested the overall acceptability of developed products as per taste, texture, color, flavor, and appearance and mark them according to the 5-point standard scale (1-poor, 2-fair, 3-good, 4-very good, 5-excellent). The set of results was obtained by the fuzzy logic approach as observed in (Das's 2005) and report.

2.8 Statistical analysis

All the experimental analyses were performed in a triplicate manner and mean values are presented with respective standard deviations. Considered variables were found significant at p<0.001 level using the Graph-pad software vers.2.0.

3. Results and Discussion

Protein content of non-germinated and germinated extruded puff product varied from (07.11% to 10.66%) and (09.45% to 11.98%) respectively as determined by advanced Kjeldahl Distillation and Digester method (Method 46-13; AACC, 2000) while ordinary Kjeldhal method yielded the protein content of same non-germinated and germinated extruded puff as (05.11% to 10.96%) and (07.45% to 11.56%) respectively which clearly suggest that advanced Kjeldahl Distillation and Digester method is better and gives more accurate value of protein content (Table 3). On comparison of control (rice) with millet-based puffs prepared various combinations (given in Table 1, 2) of non-germinated and germinated revealed that protein content of T3 samples (sorghum 11.5:11.5:11.5:11.5:6:4) was better than all other combinations including rice puffs used as control. It suggests that this combination may be used as high nutritive value food. Table 4 shows that the total ash content of non-germinated and germinated extruded puff product varied from (04.11 to 04.85) and (04.56 to 06.32). The dietary fiber of non- germinated and germinated extruded puff product varied from (14.66 to 14.98) and (15.66 to 16.25) and the carbohydrates of non- germinated and germinated extruded puff product varied from (66.26 to 67.21) and (66.21 to 67.67). Statistically studied result had significant difference at (p<0.0001) with respect to each combination. Lower amount of protein content as estimated by other authors may be because of the use of ordinary Kjeldahl method. Similar views were expressed by that nitrogen losses may lead to false analysis of protein in samples. It is concluded that incorporation of millet and sorghum in rice flour (T_3) produced higher protein content in the extruded puff product.

Table 3: Protein (g) evaluation of developed germinated and non germinated extruded puff by advanced Kjeldahl titration and ordinary Kjeldahl method

Raw materials	Advanced Kjeldahl Titration (protein %)		Ordinary Kjeldahl method (protein %)	
	Non-Germinated extruded puff	Germinated extruded puff	Non-Germinated extruded puff	Germinated extruded puff
*T_C	09.45±0.43	10.12±0.23	08.67±0.43	09.12±0.33
T_1	08.86±0.43	10.78±0.12	07.16±0.52	08.12±0.52
T_2	07.11±0.06	09.45±0.29	05.11±0.01	07.45±0.25
T_3	10.66±0.24	11.98±0.34	10.96±0.26	11.56±0.48

***Note:** Values stand for the mean of triplicate ± standard deviation. Means with no common letters within a row differed ($p \leq 0.05$). T_1, T_2, T_3(GP)= Germinated Puffs *Tc- Control, used as rice

Table 4: Nutrient composition of the control (rice based), germinated and non germinated composite flour extruded puff (g/100 g)

Raw materials	T_c	Non-Germinated composite flour extruded puff			Germinated composite flour extruded puff		
		T_1	T_2	T_3	T_1	T_2	T_3
Moisture (%)	03.15±0.43	03.85±0.16	04.34±0.34	04.52±0.21	04.45±0.41	04.22±0.31	04.59±0.61
Total Ash (%)	03.3±0.43	04.11±0.06	04.44±0.41	04.85±0.29	04.56±0.36	05.41±0.56	06.32±0.29
Dietary fibre (%)	13.12±0.43	14.66±0.24	14.33±0.07	14.98±0.34	15.66±0.24	15.95±0.12	16.25±0.14
Carbohydrates (%)	65.17±0.43	66.26±0.41	66.95±0.35	67.21±0.29	66.21±0.25	67.21±0.08	67.67±0.11

***Note:** Values stand for the mean of triplicate ± standard deviation. Means with no common letters within a row differed ($p \leq 0.05$). T_1, T_2, T_3(GP)= Germinated Puffs *Tc- Controlled

3.1 Sensory attributes evaluation of germinated extruded puffs

The sensory assessment of prepared extruded snacks was calibrated according to the significant average score value. The score values of extruded snack products (T1, T2, and T3) were determined using fuzzy logic approach. It indicated the higher overall acceptability of multi-millet-based composite flour snack products. T3 extruded product has more compatibility in terms of appearance (6.01±0.011), color (5.32±0.011), but there no significant change was observed in the flavor (5.14±0.021), texture (5.91±0.050), taste (5.76±0.039) and overall acceptability (5.70±0.031) of three developed products. Further, the developed extruded products were closely comparable to the control. The obtained results were found to be consistent with earlier reported values. All the sensory properties of extruded products are presented in Table 5.

Table 5: Average score values of germinated extruded puffs

Sensory attributes evaluation	Extruded Products		
	T_1	T_2	T_3
Color	5.10±0.02	5.47±0.021	5.32±0.011
Flavor	5.09±0.01	5.11±0.04	5.14±0.021
Texture	4.90±0.03	5.16±0.03	5.91±0.050
Taste	4.50±0.05	5.31±0.01	5.76±0.039
Appearance	4.68±0.03	4.68±0.03	6.01±0.011
Overall acceptability	5.30±0.04	5.15±0.04	5.70±0.031

Note: Values stand for a mean of triplicate±standard deviation. Means with no common letters within a row differed ($p \leq 0.05$ T1- the ratio of preparation raw materials of multi-millets, T2- - the ratio of preparation raw materials of multi-millets, T3- - the ratio of preparation raw materials of multi-millets.

4. Conclusion

The present study concluded that the germinated multi-millet grains, made from millet blends (Sorghum millet, Finger millet, Foxtail millet, Pearl millet) and Rice were better than non-germinated. The formulated grains blends made in the ratio of 11.5:11.5:11.5:11.5:6:4 to produced better qualities of the extruded products. The studied physicochemical properties reflect the best-suited extruded snack products such as having a high expansion ratio, low bulk density, low hardness, low moisture content, and water absorption index with the highest value recommended for all the ingredients, whereas the water solubility index showed a negative correlation. The developed extruded product has overall acceptable sensory properties. Further, the use of low-value cereals with added nutritive quality to produce healthier and multi-nutritive snack food products opens a new dimension in the food industrial sector.

5. Acknowledgements

The facilities provided by Regional Public Food Analyst Laboratory, Food Safety and Drugs Administration; Meerut Uttar Pradesh are gratefully acknowledged.

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