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Research Article

Anti -Nutritional and Amino Acid of Flour Blends From Water Yam, Yellow Maize and African Yam Bean

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Abstract: Anti-nutritional and amino acid composition of water yam yellow maize and African yam bean flour mixtures were evaluated in this study. The flour samples processing followed the standard unit operation for dry –milling prior to the determinations. Four blends of composite flours were prepared by homogenously mixing Water yam (WY), Yellow maize (YM) and African yam bean flour (AYB) in different ratios. Anti -nutritional contents result indicates that phytate content ranged from 0.44 to 1.99 %; trypsin inhibitor ranged from 0.12 to 1.01mg/g and tannin content ranged from 0.64% to 2.09 %. There were significant ($p < 0.05$) differences in the anti –nutritional c contents amongst the flour samples. Sample DIN (60% WY: 10%YM: 30% AYB) was significantly ($p < 0.05$) lower than other samples in all the tested parameters. The amino acid composition of the flour blend was also evaluated. The result indicated that amino acid composition of flour sample DIN was significantly ($p < 0.05$) higher than other flour samples except for cystein content of flour sample EJO (100 % WY). All the flour samples showed superiority in amino acid content when compared with FAO/1991 reference amino acid requirement for adult consumers.
Keywords: anti-nutritional, amino acid, flour.

1.0 INTRODUCTION

The problem of adequate nutrition in Nigeria is much similar to those of other developing countries especially due to low protein intake which has been attributed to the increasingly high cost of animal sources such as beef, mutton, game (bush meat) and sea food. This protein malnutrition which a major public health problem is seen more among vulnerable groups such as children, expectant, lactating mothers (Akinyele, 1987).

There is now a renewed interest on the utilization of plant proteins which is both cheap and is readily available for consumption usually in composite flour form.

Composite flour technology refers to the process of mixing various flours from tubers with cereals or legumes with or without addition of wheat flour in proper proportions to make economic use of local cultivated crops to produce high quality food products. Some studies were reported on the use of cereal-tuber- legume combination for the production of various products (Akubor and Ukwuru, 2005). It can be deduced from these reports that the qualities of product depend on the proportional composition of the composites and flour properties. Yam makes a significant contribution in the diets of Nigerians. The tubers were found with large starch and some protein and essential amino acids. The same report showed that yam species and grains contain some toxic compounds and can impact serious health complications (Anthony 2004). In like manner a very important plant protein source, the African yam bean an underutilized crop species which is one of the components of the composite crop has been reported to have a lot of anti-nutritional properties.

The health implication may be bio-unavailability of the food nutrient, vomiting and diarrhea when large amount are ingested without proper processing or if eaten raw. In this study an attempt was made to understand the anti- nutrient and amino acid

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profile of flour mixture from water yam, yellow maize and African yam bean.

2.0 MATERIALS AND METHODS

The water yam was identified as TDA 297 and bought at national root crop research institute (NRCI), Umudike, Abia State, Nigeria. The yellow maize and the cream colour African yam bean were identified and bought at national institute of horticulture (NIHOT) Mbato sub zone, Okigwe, imo state. Xanthan gum (G 1253, sigma –aldrich USA) was procured from pharmaceutical shop in Onitsha, Dangote iodized table salt was purchased from a food shop in Eke Market in Afikpo, Ebonyi State, Nigeria.

2.1 Preparation of Raw Materials

2.1.1 Water Yam Flour

Water yam was washed, peeled manually under water containing 0.20% solution of sodium metabisulphate. Slicing of the water yam (3mm x 5mm) was done with a stainless knife, the transferred into another container of the same concentration of sodium metabisuphate and allowed for 5 min. The sliced water yam were removed and allowed to drain for 1hr under air current and dried at 60^{o}C for 6hrs in a chirana type air convention oven (HS201A). Dried chips were cooled for 2hr at room temperature under air current and milled using Brabender roller mill (Model 3511A). The flour sample was sieved through 0.50mm mesh size, packaged and sealed in polyethylene bag for further use.

2.1.2 African Yam Bean Flour

The cream coloured African yam bean seeds were sorted and cleaned in an aspirator (Model: OB 123 Bindapst Hungary) located at the food processing laboratory of federal polytechnic, Mubi. Cleaned seeds were soaked for hr at room temperature. The seeds were sundried for 4 days at (30^o$\pm$ 2^{o}C) and milled with Brabender roller mill (Model 3511A) to pass through screen with 0.50mm openings, resulting coarse meal were re-milled into fine flour. The flour was stored in an air tight plastic container at room temperature for further use.

2.1.3 Yellow Maize Flour

The yellow maize grain were sorted, and cleaned in an aspirator (Model OB 125 Bindapst Hungary) located at the food processing laboratory at federal polytechnic, Mubi to eliminate contaminants. The cleaned maize grains were conditioned at 40^{o}C for 30min in a stainless steel container. The seeds were sundried for 4days at (30^o$\pm$2^{o}C) and then cracked and milled with Brabender roller mill (Model 3511A). The seed coats were removed to obtain the maize flour to pass through a screen with 0.50mm openings and resulting coarse meal were re-milled into fine flour. The flour was stored in an air tight plastic container at room temperature for further use.

2.2 Flour Blending Ratio

The flours from the water yam, yellow maize and African yam bean (AYB) were blended in the ratio as shown in (Table 1) prior to preliminary study of the flour samples.

Table 1: Flour blending ratio

CODED SAMPLES	WY (%)	YM (%)	AYB (%)	Total (%)
AFK	30	40	30	100
BGL	40	30	30	100
CHM	50	20	30	100
DIN	60	10	30	100
EJO	100	0	0	100

Sample EJO = Control (100%) Water Yam
WY = Water Yam, YM = Yellow Maize, AYB = African Yam Bean

2.3 Analytical Procedure

The following analyses were carried out in triplicate of water yam, yellow maize and African yam bean flour blend.

2.3.1 Anti -Nutritional Contents

Phytate content was quantified according to young and Greaves 1940; wheeler and Ferrel (1971) methods. Trypsin inhibitor was quantified according to the method of kakade *et al.*, (1974) as modified by Smith *et al.*, (1980). Tannin content was determined using (Pearson, 1976) method.

2.3.2 Amino acid profile analysis of flour

Amino acid content of the raw flour blends were determined in duplicate according to the methods described by previous researchers (Benitez, 1989; and Obatolu, 2002). The amino acid profile of the samples was determined in two steps: hydrolysis of the protein to constituent amino acid followed by quantitative analysis of the amino acids in the hydrolysate.

The sample were dried to constant weight, defatted, hydrolysed, evaporated in a rotary evaporator and the loaded at the technicon sequential multisample (TSM) amino acid analyzer (NY, USA) located at the Department of Biochemistry, University of Jos, Nigeria. To defat the samples, 2g of each dried sample were weighed into extraction thimble and the fat was extracted with chloroform / methanol mixture (2:1 ratio) using soxhlet extraction apparatus as described by AOAC (1995). The extraction lasted for 8hrs.

Hydrolysis of samples was done using 30-50mg of the defatted raw flour blends (RFB) and corresponding extrudates, which were weighed into glass ampoule (this was avoid possible oxidation of some amino acids during hydrolysis). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105±5°C for 22hrs. The ampoule was allowed to cool before they were broken open at the tip and the content was filtered through a glass filter paper. The filtrate was then evaporated to dryness at 40°C under vacuum in a rotary evaporator. The residue was dissolved with 5ml of acetate buffer (pH 2.0)n and stored in plastic specimen bottles which were kept in a deep freezer.

5-10µl of the hydrolysate from each sample was loaded into the technicon sequential multisample (TSM) amino acid analyzer. The sample were dispensed into the cartridge of the amino acid analyzer. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. Each analysis lasted for 76 minutes. Amino acids were quantitatively determined against the standard in a technicon auto analyzer chart (no. 011 – 9648 – 01, Technicon Instruments Co., Tarrytown, New York).

2.4 Statistical Analysis

The experimental design was a 3 x 3 factorial in complete Randomized Design (CRD) where the three flour sources and their combination ratios were the two factors under consideration. Data generated from the study were subjected to Analysis of Variance (ANOVA) and means separated using FLsd 0.05 with SPSS version 22.0.

3.0 RESULTS

3.1 Anti – nutritional content of Water yam, Yellow maize and African yam bean flour blends.

Table 2: Anti –nutritional factors of water yam, yellow maize and African yam bean flour blends

Sample	Phytate (%)	Trypsin inhibitor (mg/g)	Tannin (%)
AFK	$1.73^{b}\underline{+}$ 0.15	$9.20^{c}\underline{+}$ 0.01	$2.00^{a}\underline{+}$ 0.20
BGL	$1.30^{c}\underline{+}$ 0.01	$9.88^{b}\underline{+}$ 0.01	$1.68^{b}\underline{+}$ 0.01
CHM	$1.99^{a}\underline{+}$ 0.00	$1.01^{a}\underline{+}$ 0.01	$2.09^{a}\underline{+}$ 0.00
DIN	$0.44^{e}\underline{+}$ 0.20	$0.12^{c}\underline{+}$ 0.00	$0.64^{d}\underline{+}$ 0.01
EJO	$1.04^{d}\underline{+}$ 0.01	$0.99^{a}\underline{+}$ 0.00	$1.40^{c}\underline{+}$ 0.10

Values are means of triplicate determination + standard deviation.

Means with the Same Superscript within the Column Are Not Statistically (P>0.05) Different From Each Other

Keys

WY = Water Yellow: YM = Yellow Maize: AYB = African yam bean

AFK = 30% WY: 40% YM:30% AYB

BGL = 40% WY: 30% YM: 30% AYB

CHM = 50% WY: 20% YM: 30% AYB

DIN = 60% WY: 10% YM: 30% AYB

EJO = 100% WY

The Result Of The Anti – Nutritional Content Of The Raw Flour Blends Are Shown In Table 2.

The phytate content of the flour blends ranged from 0.44 to 1.99%, with sample CHM (50% WY: 20% YM: 30% AYB) having the highest phytate content, while sample DIN (60% WY: 10% YM: 30% AYB) had the least phytate content. The phytate content of the flour samples were statistically ($p < 0.05$) different from one another. Trypisin inhibitor content values ranged from 0.12 to 1.01mg/g, with flour sample CHM having the highest value, while flour sample DIN had the least trypins inhibitor. Flour samples AFK (30% WY: 40% YM: 30% AYB) and DIN and flour samples CHM and EJO (100% WY) respectively were statistically ($p>0.05$) not different from each other but differed ($p<0.05$) statistically from flour sample BGL. Addition of yellow maize up to 30% and African yam bean reduced the trypisin inhibitor. The tannin content values ranged from 0.64 to 2.09%, with sample CHM having the highest value, while flour sample DIN had the least tannin content. Samples AFK and CHM were not statistically ($p>0.05$) different from each other but differed ($p<0.05$) from other flour samples. Flour sample EJO was statistically ($p<0.05$) lower than all other flour sample. Addition of yellow maize and African yam bean flour might have reduced the tannin content in the composite flour.

3.2 Amino Acid Profile of Water Yam, Yellow Maize and African Yam Bean Flour Blends.

TABLE 3: Amino acid profile of Water yam, Yellow maize and African yam bean flour blends.

Amino acid	AFK	BJK	CHM	DIN	EJO	FAO(1991)	Adult
Lysine	4.05^{b}	3.00^{b}	2.33^{b}	8.66^{a}	3.66^{b}	5.80	1.60
Valine	5.33^{b}	4.00^{bc}	3.66^{bc}	7.33^{a}	3.33^{c}	3.50	1.30
Methionine	1.33^{b}	2.00^{b}	1.33^{b}	3.33^{a}	1.33^{b}	2.2	1.70
Phenylalanine	5.33^{ab}	5.66^{ab}	5.00^{ab}	7.33^{a}	4.66^{c}	5.43	1.90
Histidine	3.66^{cd}	3.33^{d}	4.66^{bc}	6.33^{a}	5.00^{b}	1.90	1.60
Tryptophan	2.00^{a}	2.00^{a}	2.00^{a}	2.66^{a}	1.66^{a}	1.1	-
Leucine	6.66^{b}	7.66^{ab}	7.33^{ab}	8.33^{a}	4.00^{c}	6.60	1.90
Proline	3.66^{ab}	3.66^{ab}	3.00^{b}	5.33^{a}	2.00^{b}	-	-
Glycine	4.33^{ab}	3.33^{ab}	4.33^{ab}	5.66^{a}	3.33^{ab}	-	-
Arginine	5.33^{b}	4.66^{bc}	4.00^{bc}	7.66^{a}	3.00^{b}	2.0	-
Tyrosine	3.66^{ab}	4.00^{bc}	3.00^{ab}	4.66^{a}	2.33^{b}	-	-
Alanine	5.00^{a}	3.66^{b}	4.66^{ab}	5.50^{a}	3.66^{b}	-	-
Aspartic acid	11.00^{ab}	11.33^{ab}	10.33^{ab}	13.33^{a}	9.00^{b}	-	-
Serine	5.00^{a}	4.33^{a}	5.66^{a}	6.33^{a}	4.66^{a}	-	-
Glutamic acid	14.00^{a}	14.33^{b}	12.00^{c}	18.00^{a}	11.00^{c}	-	-
Threonine	5.00^{a}	4.00^{a}	3.00^{bc}	5.66^{a}	2.00^{c}	3.40	0.90
Isoleucine	2.66^{abc}	3.33^{ab}	1.66^{c}	4.00^{a}	2.00^{bc}	2.80	1.30
Cystein	2.00^{a}	1.33^{a}	2.00^{a}	1.33^{a}	2.33^{a}	2.50	-

Values are mean of triplicate determination ± standard deviation. Means with the same superscript within the column are not significantly ($p > 0.05$) different from each other.
AFK = 60 % WY: 40% YM: 30 % AYB, BGL =40 %WY: 30 %YM: 30 % AYB
CHM = 50 % WY: 20 % YM: 30 % AYB, DIN =60 %: 10 %YM: 30 %AYB
EJO= 100 % WY

The Result Of The Amino Acid Profile Of Water Yam, Yellow Maize And African Yam Bean Flour Blends Are Shown In Table 3.

The lysine content of the flour blends ranged from 2.33 to 8.66mg/100g, with flour sample DIN (60 % WY: 10 % WY: 30 % AYB) having the highest value, while flour sample CHM (50 %WY: 20 % WY: 30 % AYB) had the least value. Samples AFK (30 % WY: 40 %: 30 % AYB), BGL (40 % WY: 40 % YM: 30 % AYB), CHM (50 %WY: 20 % WY: 30 % AYB) and EJO (100 % WY) were not significantly ($p>0.05$) different from one another but were significantly ($p<0.05$) lower than sample DIN in lysine content. Addition of 10% yellow maize and 30% of African yam bean flours might have increased the lysine content of the flour blend. The valine content of the flour blends ranged from 3.33 to 7.33mg/100g, with sample DIN having the highest value, while sample EJO had the least valine content. Flour samples AFK, BJK and CHM were not significantly ($p>0.05$) different from one another, but were significantly ($p<0.05$) lower than flour sample DIN. Addition of 10% yellow maize and 30% African yam bean flours might have increased the valine content of the flour sample. The methionine content of the flour samples ranged from 1.33 to 3.33mg/100g, with sample DIN having the highest value, while samples AFK, CHM and EJO had the least value. Flour sample EJO was significantly ($p<0.05$) lower than all other flour samples. The values for phenylalanine ranged from 4.66 to 7.33mg/100g, with sample DIN having the highest, while sample EJO had the least value. The phenylalanine content of EJO flour sample was significantly ($p<0.05$) lower than other flour samples. Inclusion of 10% yellow maize and 30% Africa yam bean flours might have increased the phenylalanine content of flour sample. The histidine content ranged from 3.33 to 6.33mg/100g, with flour sample DIN having the highest value, while sample BGL had the least value. Flour sample DIN was significantly ($p<0.05$) higher than all other flour samples in histidine content.The tryptophan content ranged from 1.66 to 2.66mg/100g, with sample DIN having the highest value, while sample EJO had the least value in tryptophan content There was no significant ($P>0.05$) difference in tryptophan content of the flour blends. The leucine content ranged from 4.00 to 8.33mg/100g, with sample DIN having the highest value, while sample EJO had the least value. The leucine content of EJO flour sample was sigificantly ($p<0.05$) lower than all other flour samples. The proline content of the flour ranged from 2.00 to 5.33mg/100g with sample DIN having the highest value, while sample EJO had the least value. Flour samples AFK, BGL and DIN were not significantly ($p>0.05$) different from one another. The glycine content of the flour sample ranged from 3.33mg/g to 5.66mg/100g, with sample DIN having the highest value, while samples, BGL and EJO had the least values. Flour samples AFK, CHM, DIN and EJO were not significantly ($p>0.05$) different from one another. The arginine content of the flour sample, ranged from 3.00 to 7.66mg/100g with flour sample DIN having the highest value, while the flour sample EJO having the least value. Flour sample DIN was significantly ($p<0.05$) higher than all other flour samples. Inclusion of 10% yellow maize and 30% African yam bean flours might have increased the arginine content of the flour sample. The tyrosine content of the flour samples ranged from 2.33 to 4.66mg/100g with sample DIN having the highest value, while flour sample EJO had the least value. Flour samples AFK, BGL, CHM and DIN were not significantly ($P>0.05$) different from one another but were significantly ($p<0.05$) higher than flour sample EJO. The alanine content of the flour samples ranged from 3.66 to 5.50mg/100g, with flour sample DIN having the highest value, while flour samples BGL and EJO had the least value. All the flour samples did not differ ($p<0.05$) significantly from one another in alanine content. The aspartic acid content of the flour samples ranged from 9.00 to 13.33mg/100g, with flour sample DIN having the highest value, while flour sample EJO had the least value. Sample AFK, BGL, CHM and DIN were not significantly ($p>0.05$) different from one another but were all significantly ($p<0.05$) higher than flour sample EJO. The serine content of the flour ranged from 4.66 to 6.33mg/100g, with flour sample DIN having the highest value, while flour sample BGL had the least value. All the flour samples did not differ ($p>0.05$) significantly in the serine content. The glutamic acid content of the flour samples ranged from 11.00 to 18.00mg/100g, with flour sample DIN having the highest value, while flour sample EJO had the least value. Flour sample DIN was sigificantly ($p<0.05$) higher than all other flour samples. Inclusion of 10% yellow maize and 30% African yam bean in the flour blend increased the glutamic acid content of the flour. The threonine content of the flour samples ranged from 2.00 to 5.66mg/100g, with flour sample DIN having the highest value, while flour sample EJO had the least value. Flour Samples AFK, BGL and DIN were not significantly ($p>0.05$) different from one another but differed ($P<0.05$) significantly from flour sample EJO. The isoleucine content of the flour samples ranged from 1.66 to 4.00mg/100g, with flour sample DIN having the highest value, while flour sample CHM had the least value. Flour samples AFK, BGL and DIN did not differ ($P>0.05$) significantly from each other, but differed ($P<0.05$) significantly from flour sample CHM. The cystein content of the flour samples ranged from 1.33 to 2.33mg/100g, with flour sample EJO having the highest value, while flour sample BJK had the least value. All the flour samples did not differ ($p>0.05$) significantly in cystein content. Generally, flour sample DIN appeared to be superior in amino acid content compared to all other flour samples and that of reference standard (FAO/WHO/1991) except for cystein content.

4.0 DISCUSSION

4.1 Anti-Nutrient Composition of Raw Flour

4.1. 1. Raw Flour Phytate Content

The phytate content of the raw flour and their blends are shown in Table 2. The phytate content ranged from 0.44 – 1.73% in this study. Earlier reported values of phytate content of water yam were lower than the value observed in this study (Polycarb *et al.*, 2013; Shajeela *et al.*, 2011). Phytic acid is an important storage form of phosphorus. It is insoluble and cannot be absorbed in the human intestines. Phytic acid has 12 irreplaceable hydrogen atoms with which it could form insoluble salts with metals such as calcium, iron, zinc and magnesium, the formation of form insoluble salts with metals such as unavailable for absorption into the body. Phytic acid can also affect digestibility by chelating with calcium or by binding with substrate or proteolytic enzyme and making trace element unavailable to the consumers, stunted growth and ricketing in young animals (Osagie, 1998) therefore, the low level of phytate in the four sample DIN (60% WY10% YM:30% AYB) is nutritionally advantageous and making trace element available to the consumer, however, the local methods of food processing used in Nigeria minimized the concerns posed by metal chelation and protein –binding action brought about by the phytate naturally present in food materials of plant origin (Osagie, 1998). Moreso, the use of exogenous phytases to enhance phosphorus digestibility is now common practice in countries where the contribution of agriculture to environment pollution is a pollution is a concern (Ukachukwu, 2015).

4.1.2. Raw Flour Trypsin Inhibitor

The trypsin inhibitor content of the water yam flour and the blends are shown in Table 2. The trypsin inhibitor content of flours in this study ranged from 0.12 – 1.01mg/g. the value observed in this study for water yam flour was lower than earlier report by Shaajeela *et al.*, (2011), the variation could be as a resulted of varietal and agronomical differences. Tryspin inhibitors have low molecular weight (20,000 – 25,000) with relatively few disulphide bonds but possessing a specificity which is primarily directed towards trypsin. Boiling for sufficient time makes the tuber soft enough and inactivates all the trypsin inhibitors (Bradbury and Holloway, 1988). However, heat treatment needs to be conducted under closely controlled conditions to avoid reducing the availability of amino acids, particularly lysine (Ukachukwu 2015).

4.1.3 Raw Flour Tannin Content:

The tannin content of the raw water yam flour and the blends are shown in Table 2. The tannin content of flour in this study ranged from 0.64 – 2.00%, the value observed for water yam in this work was lower than the earlier reported value (Shaajeela *et al.*, 2011). Onwuka (2005) reported that the presence of tannins can cause browning or other pigmentation problem in both fresh and processed foods. Phenolics and tannin are water soluble components (Uzogaraa *et al.*, 1990) and as such can be eliminated by soaking followed by cooking (Singh, 1988; Murugesan and Ananthalakshmi, 1991; Kataria *et al.*, 1989; Singh and Singh, 1992; Shanthakumari *et al.*, 2008). Polyphenols (condensed tannins) are fairly high in cowpeas. Tannins decrease protein quality by decreasing digestibility and palatability. They also bind iron, making it unavailable (Aletor and Adeogun, 1995) and other evidence suggests that condensed tannins may cleave DNA in the presence of copper ions (Shirahata *et al.*, 1998). It has been suggested that tannins play a major role in the plant's defense against fungi and insects (Osagie, 1998). Since tannins are mostly located at the seed coats (Sing, 1988) dehulluing which was one of the unit operation in flour making in this study could have aided in tannin reduction in this study.

4.2 Amino Acid Compositions of Raw Flour

The amino acid of the raw water yam flour and the blends are shown in Table3.

The amino acid analysis of food products is an important index of its protein quality and can produce useful information on the nutritional quality and authenticity of food products and the sources of raw materials used in food manufacture (Alozie, *et al.*, 2009). The results obtained in this study showed that all the samples contained all the amino acids that are found in plant proteins. In this study the predominant amino acid was glutamic acid while the least amino acid was cystein. Adeoti *et al.*, (2013) observed a result of similar trend for maize Tuwo – Cirina forda flour blend. Glutamic acid contributed the highest amount of total non-essential amino acids. In this study, the values obtained for the amino acid content of the samples are comparable to their corresponding protein content and this implies that the amount of non-protein nitrogenous materials in this blends is significant (Wung and Cheung, 2000).There was an improvement in the protein content of the composite flour. This is most probable due to the addition of African yam bean to the flour blends. Amino acids are important components for healing and protein synthesis processes; any deficience in these essential amino acids will hinder the recovery process (Zuraini, *et al.*,, 2006). According to Witte *et al.*, (2002), glycine together with other essential amino acids such as alanine, arginine, and phenylalanine form a polypeptide that will promote and tissues healing. Yam and maize on their own are not balanced foods. Nutritional deficiency diseases occur within poorer regions where cereal and root and tuber crops are consumed alone as staple foods and a major health issues, especially micronutrient deficiencies, can lead to several health consequences including impaired cognitive and physical development (Baah *et al.*, 2009).

5.0 CONCLUSION

Addition of African yam bean and yellow maize flour to water yam flour could increase the anti – nutrient as well as the amino acid of the composite flour. However anti nutritional contents in processed foods are usually reduced and a times eliminated by several unit operations especially by heat treatments. Flour sample DIN (60%WY:10% YM:30% AYB) was generally considered as the best blend that could be used for value added products based on its reduced anti-nutritional content and enhanced amino acid profile as compared with FAO/WHO/1991) reference amino acid for both children and adult consumers.

Competing Interests
Authors have declared that no competing interest exists.

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