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Effect of Blending Ratio on the Nutritional Value of Millet and Guinea Corn using Mixture Design

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Abstract:- This study focused on immunonutrition which is referring to boosting immune system response through diet. In this study, the effect ofthe blending ratio of millet and guinea corn on their nutritional value was investigated for vitamin K, vitamin D, and Zinc mineralswhich are considered to help bolster immunity and their relevance in the fight against covid-19.The proximate analysis result showed that guinea corn has a higher value of ash, carbohydrate, and crude protein contents whilemillet on the other hand has a higher moisture content, calorific value, crude lipids, and crude fiber contents as compared to that of guinea corn. Using Design Expert for experimental design, a mixture D-Optimal design was employed. The analysis of variance (ANOVA) for the yield of vitamin K and D, and Zinc mineral were statistically significant at "prob > F" less than 0.05. Optimization of the analysis revealed that a blend in the ratio of 55.2 to 44.8 would be the best blend that maximizes vitamin K, D, and Zinc minerals with desirability of 65.1 %. Although cereals are not a significant source of vitamin K and D the blends from the study, if processed into food, would be able to provide an additional quantity of the vitamins and minerals needed to boost our immune system.

Keywords:- *Immunonutrition, nutritional value, immune-boosting,Covid-19, cereals.*

I. INTRODUCTION

Nutrition is an important part of human existence. Proper food and good nutrition are necessary for survival, physical growth, mental development, performance, and productivity throughout the entire lifespan. Nutrition deficiency may sometimes lead to either a recognized disease of unknown etiology or obscure signs and symptoms in an individual. For instance, there were claims that deficiency in vitamins plays a part in the production of renal lithiasis (Higgins, 1935) and certain muscular dystrophies (Bicknell, 1940). Nutrition deficiencies and excesses affect the components of our immune system. The immune system helps the body to fight against infection. It plays a major role in the body's ability to fight off any infection, lower the risk of developing tumors, degenerative diseases as well as autoimmune disorders. A defective immune system can result in allergic disease, immunodeficiency, and autoimmune disorders. However, an immunodeficiency disorder that is caused by several factors including poor dietary intake renders the body defenseless and becomes highly prone to illness from invading pathogens or antigens. Today, it is widely accepted that an adequate nutritional status is paramount for the development, maintenance, and expression of the immune response (Maggini,S et al; 2017). Micronutrients such as vitamins and nutritionally essential micro-nutrients such as zinc (Zn) influence and support every stage of the immune response in which their deficiency can affect both innate and adaptive immunity thereby causing immunosuppression and thus increasing the susceptibility to infection. Different approaches can be employed to offer children and adults improved food with low-cost and locally available food formulations with an improved nutritive value. One study established that porridges prepared from extruded millet and press-dried cowpea had a high nutritional quality with acceptable properties of weaning foods (Almeida-Dominguez et al., 1993). It was reported that when millet was mixed with amaranth and buckwheat to make extruded breakfast cereal products instead of the mixture of wheat and maize flour, nutritional and physical quality of the cereal products were altered. In addition to this, all of the extruded products with the inclusion of pseudocereals exhibited an enormous reduction in readily digestible carbohydrates in contrast to the control product during *in vitro* glycemic profiling (Brennan MA et al., 2012).Millet and red guinea corn were chosen for this study owing to their nutritive value, availability in the local market, cheapness, and being part of the most neglected cereals. Hence, this study was to evaluate the nutritive value of a mixture of millet and guinea corn in various proportions with a view to incorporating this into our daily meals

II. MATERIALS AND METHODS

The millet and red guinea corn grains used in this study were purchased from the midwifery market, a local market, in Asaba, Delta State, Nigeria.

- Pre-Treatment of Raw Material
  The millet and red guinea corn grains were sorted to remove dirt and other extraneous materials, and then washed in clean water and sun-dried for one week.

- Analysis of Samples
  The analyses were carried out at Central Research and Diagnostic Laboratory, Tanke, Ilorin, Kwara State, Nigeria. The proximate analysis was done on the samples as well asthe determination of vitamins (D and K) and Zinc (Zn) mineral content of each of the blends. The vitamins were determined using high-performance liquid chromatography (HPLC).

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- Proximate Analysis
  The methods of the Association of Official Analytical Chemists (AOAC 2005) was used to determine the amount of moisture, ash, fat, protein, crude fiber, and carbohydrate contents of the pure samples. The carbohydrate was determined by difference.

- Preparation of sample for HPLC analysis
- *For vitamin D*
- Apparatus

  - Column- Waters HSS T3 (2.1x150mm, 1.7µm particle size; Waters Corp, Milford MA, USA)
  - Liquid Chromatography- Waters Acquity UPLC Binary solvents, manager capable of 15000 psi
  - Detector- Waters Quattro Premier XE, Xevo Triple Quadrupole Mass Spectrometer
  - Injector- Waters Acquity Sample Manager with Integrated Column Oven

- Chemicals and Reagents
  Methanol (HPLCgrade), Acetone (HPLC grade), Acetronitrile (HPLC grade), Solvents-(1) Pentane (HPLC grade), ethyl ether, potassium hydroxide (ACS grade), acetic acid-Glacial (99.9%), pyrogallic acid (ACS grade), Butylated hydroxyltoluene (BHT) 99.8%.

  Isotope standard- isotope vitamin D_3

- Sample Preparation
  10g of the sample was measured directly into a 50mL centrifuge tube (glass) and this was inoculatedusing stable isotope-labeled internal standard. It was then saponified at 75°C for 30 minutes using potassium hydroxide (KOH) in order break up the product matrix and enhance analyte extraction. Pentane-ether was used as a solvent for the extraction of Vitamin D. The Vitamin D was collected and dried under Nitrogen. The dried extract was reconstituted with acetonitrile-water (70:30) and was then filtered before analysis.

- Instrument and Parameters
  To prevent interfering compounds, Vitamin D was separated using UPLC and quantitated using tandem mass spectrometry. An isocratic mobile phase with a 12minutes run time was used. The flow rate was 0.3mL/min with a mobile phase consisting of 0.1% acetic acid in 75% acetonitrile/ 25% methanol (MeOH). A 10µL sample solution was injected for the measurement. After the completion of the analysis, the result was automatically printed out.

- For vitamin K
- Standards, Reagents, and Samples
  Vitamin K_1 (Phylloquinone) in-stock standard, Absolute ethanol 200 proof (EtOH), glacial acetate (99.9%), monobasic potassium phosphate, zinc chloride, zinc powder (98+ %, <10 microns), sodium acetate, sodium dodecyl sulphate (SDS), candida rugosa lipase, and screw-cap test tubes (3.8 x 200mm), Also, screw caps with Teflon liners, HPLC grade of Methanol (MeOH), acetonitrile (MeCN), isooctane, and isopropanol.

- Sample Preparation
  Samples were done using a modification of Association of Official Analystical Chemist (AOAC) official method 999.15. A 5g solid sample was weighed and transfrerred into a 3.8 x 200mm screw cap test tube. 15mL deionized water was added and mixed thoroughly until it completely dissolved. Then, a 5mL pH 8.0 buffer solution was added. This was thoroughly mixed by hand. 1g candida rugosa lipase was then added. The resulting solution was incubated for 2 hours at 37°C in an incubator-genie attached to the rotating plate with the speed set at 14. The plate of the incubator was adjusted to be able hold 3.8mm tubes by attaching 8 clips on each side and tubes were perfectly fitted into the thermostatic chamber. A 10 mL of Absolute ethanol (EtOH) was added at room temperature. This was followed by vortex mixing for 15 seconds after which 1g of potassium carbonate was added. The tubes were rotated for 60 minutes in a Rugged Rotator with the speed set at 40%. The rotating speed was set to a maximum value as soon as a thick emulsion was observed. This allowed the sample to move from one end to the other during the rotation. A 50mL of isooctane was then added to each of the samples, and they were further rotated for additional 60 minutes. The samples were then left for 10 minutes in the refrigerator to separate the 2 layers and centrifuged for 10 minutes at 1500rpm. Finally, a 2mL portion of the organic layer was measured out and transferred into an autosampler vial for direct HPLC analysis.

- HPLC Analyses
  The following equipments/apparatus were used during the analysis.
  - Acquity Fluorescence Detector
  - Acquity Ultra-Perfomance Liquid Chromatography (LC) (Wasters Corp., Milford,MA) equipped with a long column heater/chiller
  - Acclaim C30 column (250×3.0 mm internal diameter, 3 µm particle size, Dionex, Sunnyvale, CA), and
  - A 20×4 mm stainless steel post-column reactor packed with zinc powder.

The fluorescence detector has the following settings:
- Excitation wavelength set at 243nm
- Photomultiplier gain at 1.0, and
- Emission wavelength was at 430nm.

The mobile phase was prepared by dissolving 0.82g of sodium acetate, 2.74g of zinc chloride, and 0.6 mL of glacial acetic acid in 1L MeOH. The solution was stirred for a few minutes and 1L of MeCN was then added. The flow rate was 0.5mL/min while the post-column reactor and the chromatographic column were maintained at 15°C. A20 µL of the sample was measured out as injection volume for separation. Before each set of the analysis, Helium was used to degas the mobile phase for at least 1 hour before use. The post-column reactor was tightly dry-packed using metallic zinc and was conditioned for 1 hour before use. At the completion of the analysis, the result was printed out automatically.

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- Zinc Mineral Analysis
  The method employed for zinc (Zn) mineral analysis was that decribed by the Association of Official Analytical Chemists (AOAC 2005). The prepared samples were ashed in an oven at 550^{o}C and the resulting ash was boiled with 10mL of 20% hydrochloric acid. It was then filtered into a 100mL standard flask. This was filled up to the mark with deionized water. Atomic Absorption Spectrophotometer (AAS PG instrument model 990FG) was used to determine the amount of zinc mineral from the resulting solution.

- Experimental Design
  The experimental design and statistical analysis were done using Design Expert Software (version 10.0.1). The samples are labeled sample A (pure millet) and sample B (pure red guinea corn). The pure samples were mixed in the following ratio; sample C 80:20, sample D 70:30, sample E 60:40, sample F 50:50, and sample G 40:60. The amount of vitamin K and D were determined in each sample using high-performance liquid chromatography (HPLC) equipment. The amount of Zinc (Zn) mineral in each sample was also determined.

A mixture design, D-Optimal, was used to study the effects of the mixture of the independent variables (blends) on the dependent variables (responses) using the Scheffe quadratic mixture model. Mixture designs are used when the response changes as a function of the relative proportions of the components. All components must be entered in the same units of measurement and each run must sum to the same total.A total of fifteen (15) experimental runs were performed according to the experimental design configured for the two mixture components with ten (10) replicates. Three (3) dependent variables (responses) were considered to evaluate the effect of the blending ratio of the pure samples. The experimental design is shown in Table 1.

	Component 1	Component 2	Response 1	Response 2	Response 3
Experimental Run	A:Millet (g)	B:Red Guinea Corn (g)	Vitamin K1 (µg)	Vitamin D3 (%)	Zinc (ppm)
1	80	20			
2	60	40			
3	60	40			
4	70	30			
5	40	60			
6	40	60			
7	40	60			
8	50	50			
9	40	60			
10	60	40			
11	60	40			
12	80	20			
13	80	20			
14	80	20			
15	40	60			

Table 1: Mixture design layout for two components

Where $40 \leq A \leq 80$ and
$20 \leq B \leq 60$.

Tables for the analysis of variance (ANOVA) were generated. The significance of all the terms was statistically evaluated at prob>F less than 0.05. The predicted coefficient of determination (R^2), Adj-R^2, and lack of fit were also evaluated to study the accuracy of the final model. The resulting analysis was then optimized to identify the blends that fully maximize the responses. The resulting analysis was optimized using a numerical solution.

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III. RESULTS AND DISCUSSION

A. *Proximate Analysis Results*

The result of the proximate analysis done on the pure samples is displayed in Table 2.

	Sample	
Proximate Analysis	Millet (A)	Red Guinea Corn (B)
Moisture (%)	19.93	17.2758
Ash %	1.2426	2.0748
CHO (%)	50.094	55.59307
Calorific Value (kJ/100g)	1654.6	1633.99
Crude Fiber (%)	2.1566	1.13604
Crude Lipids (%)	17.818	14.577
Crude Protein (%)	8.7595	9.34326

Table 2: Proximate Analysis Results

From the table above, millet has a moisture content of 19.9295% which is slightly higher than that of red guinea corn at 17.2758%. The ash content of 2.0748% observed with red guinea corn was higher as compared to 1.2426% observed in millet. Red guinea corn has 55.59307% of carbohydrates while millet has 50.09353%. A calorific value of 1654,593 kJ/100g was observed in millet which was higher than 1633.99 kJ/100g in red guinea corn. The crude fiber was higher in millet (2.15659%) as compared to that of red guinea corn (1.13604%). Crude lipids content was higher in millet (17.8182%) as compared to the one observed with red guinea corn (14.577%). Red guinea corn was higher in crude protein contents (9.34326%) compared to that millet of 8.75954%).

IV. RESULTS OF VITAMINS AND ZINC MINERAL DETERMINATION

Table 3 displayed the result of the vitamins and zinc mineral determination from the laboratory for various blending ratios of millet and guinea corn according to the mixture design for two components.

	Component 1	Component 2	Response 1	Response 2	Response 3
Experimental Run	A:Millet (g)	B:Red Guinea Corn (g)	Vitamin K1 (µg)	Vitamin D3 (%)	Zinc (mg)
1	80	20	18.9149	56.9381	0.434
2	60	40	17.7315	80.6472	0.991
3	60	40	17.7015	79.884	1.099
4	70	30	18.045	74.286	0.559
5	40	60	16.982	66.27	0.969
6	40	60	16.982	66.27	0.969
7	40	60	16.98	66.27	0.969
8	50	50	17.522	76.53	1.386
9	40	60	16.982	66.27	0.969
10	60	40	17.7315	81.098	0.969
11	60	40	17.7015	80.6472	1.099
12	80	20	18.892	56.9381	0.44
13	80	20	18.9148	56.9381	0.48
14	80	20	18.9149	56.9381	0.54
15	40	60	16.98	66.27	0.969

Table 3: Mixture design layout and results

A. *Effect of blending ratio of millet and red guinea corn on the yield of vitamin K*

The vitamin K in the blends was observed to range from 16.980 µg to 18.9149 µg with sample C (80:20) giving the highest yield while the lowest was sample G (40:60). It was further observed that as the quantity of the red guinea corn increases in the blends, the vitamin K reduces.

The analysis of variance (ANOVA) for the cubic model regression on the effect of blending ratio on the yield of vitamin K showed that the model has an F-value of 21393.53. The value of "prob>F" was less than 0.05 which implies that the model is significant (Table 4). The model terms are significant at a confidence level of 95%. In this case, A, B, AB, and AB (A-B) are significant model terms. The "predicted R^2" of 0.9997 was in reasonable agreement with the "adjusted R^2" of 0.9998 which indicates a good

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explanation of the variability by the selected model. The lack of fit was found to be insignificant which implies that the model is a good fit. The Coefficient of Variation, CV % (0.064) gives the precision and reliability of the experiment carried out (where a lower value of CV % indicates a better precision and reliability of the experiments carried out). The adequate precision of 325.622 indicates an adequate signal which means that the model can be used to navigate the design space.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	8.44	3	2.81	21393.5	< 0.0001	significant
[1]Linear Mixture	*8.19*	*1*	*8.19*	*62304.9*	*< 0.0001*	
AB	*0.16*	*1*	*0.16*	*1204.43*	*< 0.0001*	
AB(A-B)	*0.092*	*1*	*0.092*	*700.31*	*< 0.0001*	
Residual	1.45E-03	11	1.32E-04			
Lack of Fit	*1.49E-04*	*1*	*1.49E-04*	*1.15*	*0.3086*	*not significant*
Pure Error	*1.30E-03*	*10*	*1.30E-04*			
Cor Total	8.44	14				
Std. Dev.	0.011		R-Squared	0.9998		
Mean	17.8		Adj R-Squared	0.9998		
C.V. %	0.064		Pred R-Squared	0.9997		
PRESS	2.92E-03		Adeq Precision	325.622		
-2 Log Likelihood	-96.13		BIC	-88.01		
			AICc	-87.95		

Table 4: ANOVA for the cubic model for vitamin K

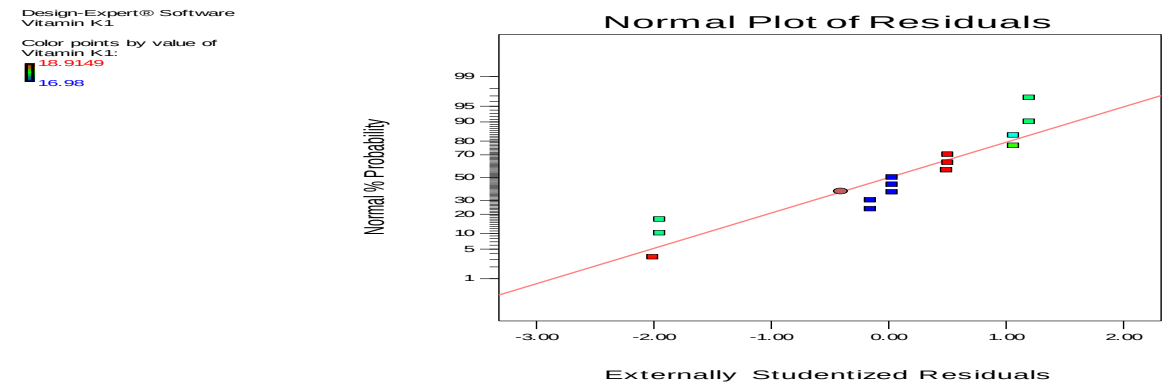

Fig. 1: Normal plots of residuals for vitamin K

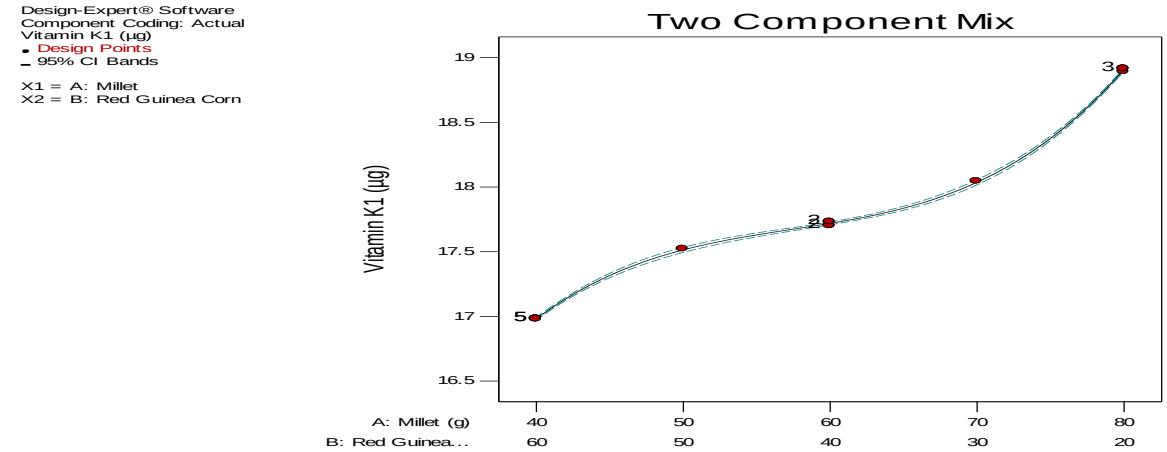

Fig. 2: Two-component mix plots for vitamin K

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B. Effect of blending ratio of millet and red guinea corn on the yield of vitamin D

The vitamin D in the blends was observed to range from 56.9381 % to 80.6472 % with sample E (60:40) giving the highest yield while the lowest yield was sample C (80:20). It was also observed that as the millet quantity in the blend increases from 40:60 to 60:40, the vitamin D also increases while a decrease kicked in at blends of 70:30 and 80:20 respectively. The analysis of variance (ANOVA) for the cubic model regression on the effect of blending ratio on the yield of vitamin D showed that the model has an F-value of 4393.97 which implies that the model is significant (Table 5). "prob>F" value was less than 0.05 which indicates that the model terms are significant at a confidence level of 95%. In this case, A, B, AB, and AB (A-B) are significant model terms. The "predicted R^2" of 0.9978 was in reasonable agreement with the "adjusted R^2" of 0.9989 which also indicates a good explanation of the variability of the selected model. The lack of fit was found to be insignificant. The Coefficient of Variation, CV % (0.45) gives the precision and reliability of the experiment carried out (a lower value of CV % indicates a better precision and reliability of the experiments carried out). The adequate precision of 148.656 indicates an adequate signal which means that the model can be used to navigate the design space.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	1238.44	3	412.81	4393.97	< 0.0001	significant
[1]Linear Mixture	*136.67*	*1*	*136.67*	*1454.74*	*< 0.0001*	
AB	*1100.48*	*1*	*1100.48*	*11713.5*	*< 0.0001*	
AB(A-B)	*2.78*	*1*	*2.78*	*29.61*	*0.0002*	
Residual	1.03	11	0.094			
Lack of Fit	*0.27*	*1*	*0.27*	*3.57*	*0.088*	*not significant*
Pure Error	*0.76*	*10*	*0.076*			
Cor Total	1239.48	14				
Std. Dev.	0.31		R-Squared	0.9992		
Mean	68.81		Adj R-Squared	0.9989		
C.V. %	0.45		Pred R-Squared	0.9978		
PRESS	2.68		Adeq Precision	148.656		
-2 Log Likelihood	2.44		BIC	10.57		
			AICc	10.62		

Table 5: ANOVA for the cubic model for vitamin D

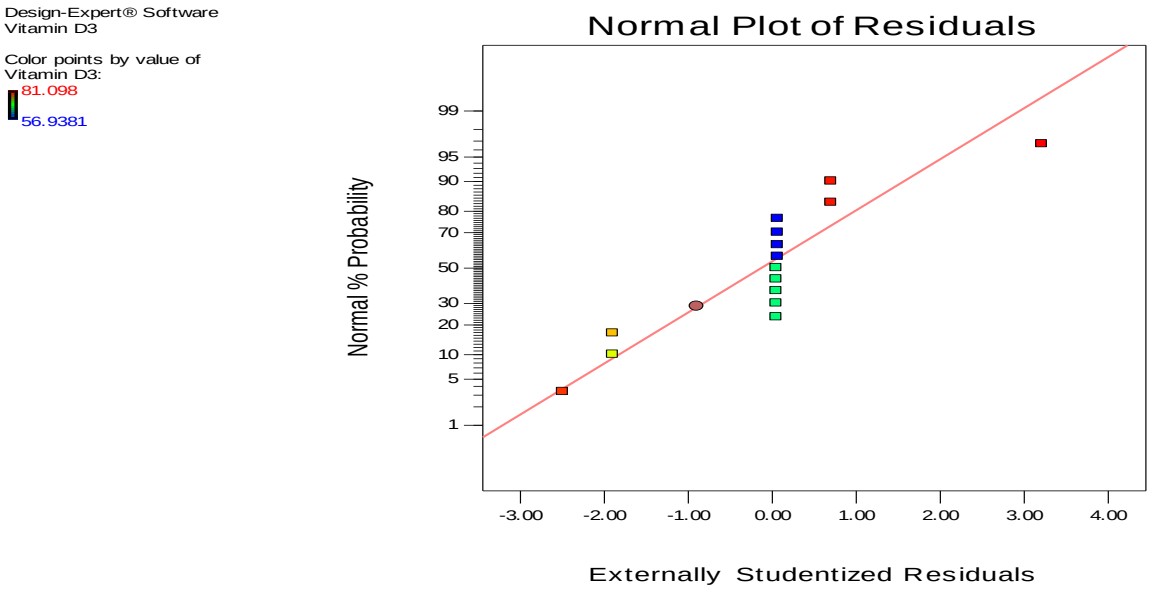

Fig. 3: Normal Plots of Residuals for vitamin D

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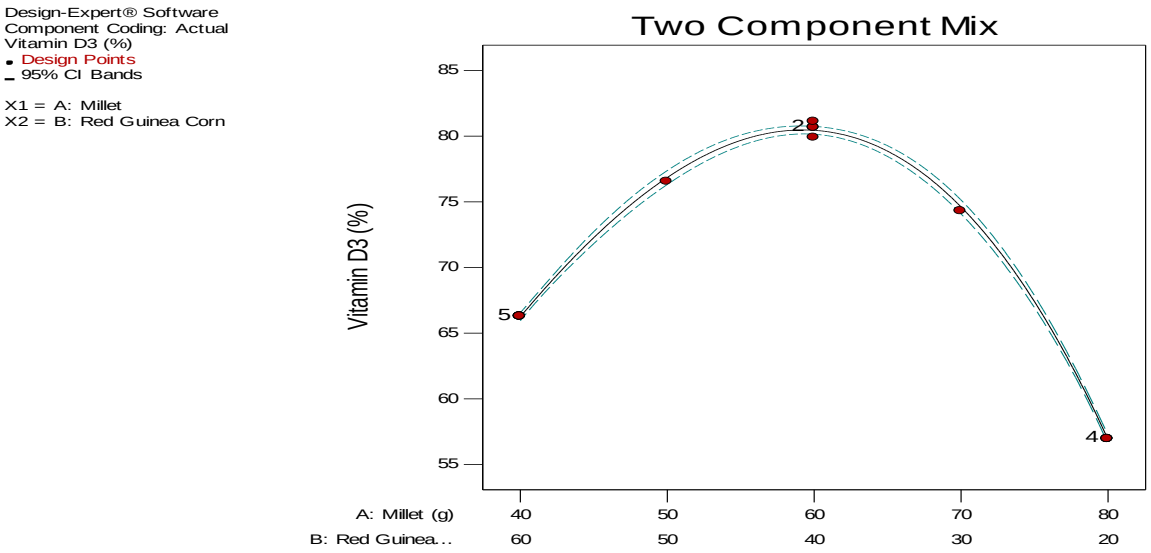

Fig. 4: Two components Mix plots for vitamin D

C. Effect of blending ratio of millet and red guinea corn on Zinc (Zn) yield

From Table 3, Sample C (80:20 blends) was observed to have the lowest yield of Zinc (Zn) (0.434ppm) while sample F (50:50 blends) was the highest (1.386ppm). This means that as the quantity of red guinea corn increases in the mixture, the yield of Zinc (Zn) also increases.

Analysis of variance (ANOVA) for the cubic model regression on the effect of blending ratio on the yield of Zinc (Zn) is presented in Table 4.8. The analysis of variance (ANOVA) shows that the model has an F-value of 193.92 which implies that the model is significant. "prob>F" value was less than 0.05 which indicates that the model terms are significant at a confidence level of 95%. In this case, A, B, AB, and AB(A-B) are significant model terms. The "predicted R^{2}" of 0.9691 as compared to the "adjusted R^{2}" of 0.9764 implies a good explanation of the variability of the selected model. The lack of fit was found to be insignificant which implies that the model reliability of the experiments is a good fit. The Coefficient of Variation, CV % (5.20) gives the precision and reliability of the experiment carried out (a lower value of CV % indicates a better precision and reliability of the experiment carried out). The adequate precision of 39.251 implies an adequate signal which means that the model can be used to navigate the design space.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	1.15	3	0.38	193.92	< 0.0001	significant
[1]Linear Mixture	*0.67*	*1*	*0.67*	*336.65*	*< 0.0001*	
AB	*0.32*	*1*	*0.32*	*161.22*	*< 0.0001*	
AB(A-B)	*0.16*	*1*	*0.16*	*80.18*	*< 0.0001*	
Residual	0.022	11	1.98E-03			
Lack of Fit	*2.44E-04*	*1*	*2.44E-04*	*0.11*	*0.7436*	*not significant*
Pure Error	*0.022*	*10*	*2.16E-03*			
Cor Total	1.17	14				
Std. Dev.	0.045		R-Squared	0.9814		
Mean	0.86		Adj R-Squared	0.9764		
C.V. %	5.2		Pred R-Squared	0.9691		
PRESS	0.036		Adeq Precision	39.251		
-2 Log Likelihood	-55.44		BIC	-47.32		
			AICc	-47.26		

Table 6: ANOVA for the cubic model for Zinc (Zn)

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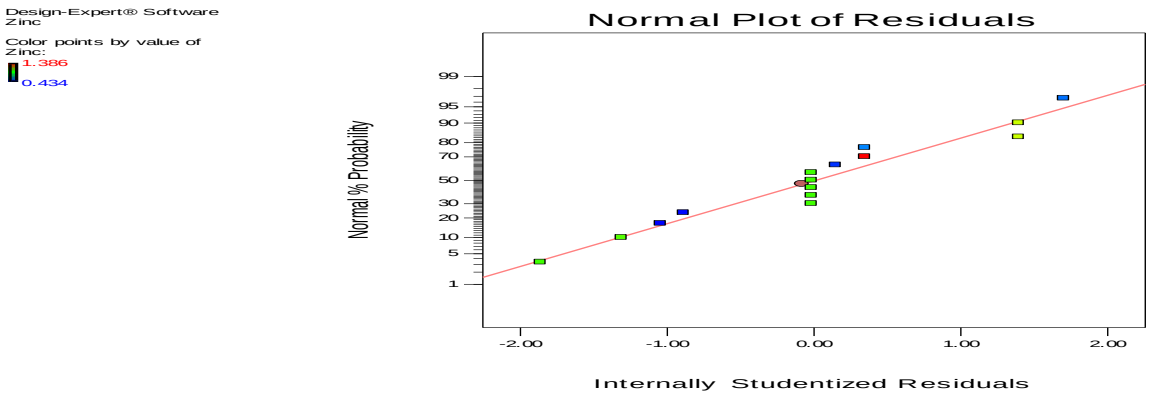

Fig. 5: Normal plots of residual for Zinc

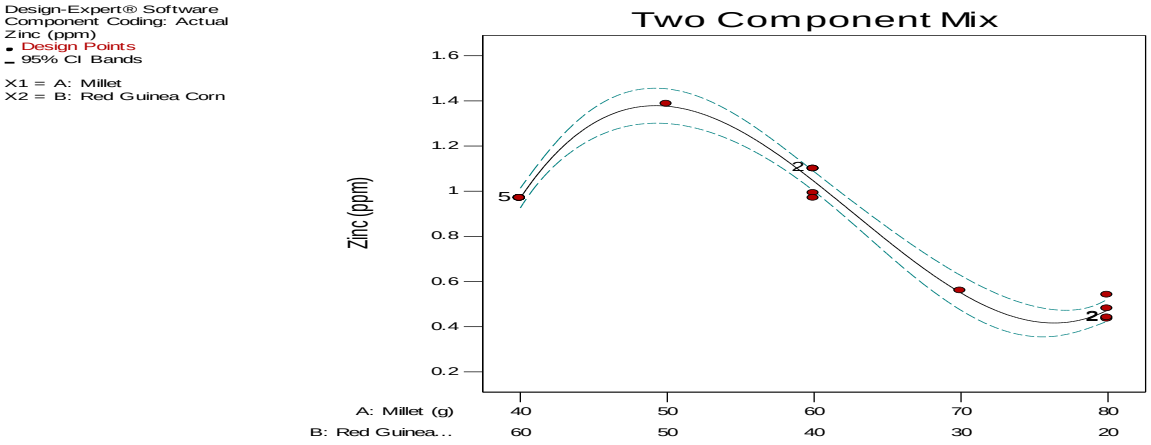

Fig. 6: Two Components Mix for Zinc yield

D. Optimization

Subjecting the analyses to numerical optimization yielded two solutions but the one with 65.1 % was selected as presented in Table 8

Constraints						
Name	**Goal**	**Lower Limit**	**Upper Limit**	**Lower Weight**	**Upper Weight**	**Importance**
A:Millet	is in range	40	80	1	1	3
B:Red Guinea Corn	is in range	20	60	1	1	3
Vitamin K1	maximize	16.98	18.9149	1	1	3
Vitamin D3	maximize	56.9381	81.098	1	1	3
Zinc	maximize	0.434	1.386	1	1	3

Table 7: Constraints for optimization of vitamin K, D, and Zinc minerals

Solutions							
Number	**Millet**	**Red Guinea Corn**	**Vitamin K1**	**Vitamin D3**	**Zinc**	**Desirability**	
1	55.146	44.854	17.633	79.731	1.261	0.651	Selected
2	79.291	20.709	18.82	58.613	0.453	0.11	

Table 8: Solutions for the optimization of vitamin K, D, and Zinc minerals

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V. CONCLUSION

The result from this study has shown that the ash, carbohydrate, and crude protein contents are higher in red guinea corn compared to those obtained from millet. On the other hand, millet has higher moisture, calorific value, crude lipids, and crude fiber contents than red guinea corn. In the same vein, the vitamin K and D were fluctuating in the blend samples while zinc mineral in the blend samples increased as the quantity of red guinea corn was increasing. The analysis of variance (ANOVA) on each of the responses (vitamin K, D, and Zinc mineral) showed that the cubic model was significant at "prob-F" > 0.05 for vitamin K and D as well as Zinc mineral. The statistical study also showed that the F-value, predicted R^2, adjusted R^2, lack of fit, and coefficient of variation (CV) were statistically adequate to check the validity of the model. Optimization of the analysis revealed that a blend in the ratio of 55.146 to 44.854 would be the best blend that maximizes vitamin K, D, and Zinc minerals with a desirability of 65.1 %. Although cereals are not a significant source of vitamins, if frequently consumed can as well provide addition of vitamins to the diet and help to boost our immune system.

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