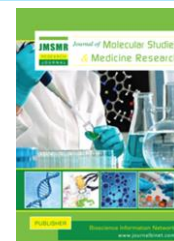


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Micronutrient evaluation of fortified soymilk from sprouted whole soybean for complementary feeding using response surface methodology

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ABSTRACT

Soymilk from TGX 923-2E soybean variety sprouted for 72hrs after 12 hours tap steeping was fortified with ferric ammonium citrate (Fe), calcium (Ca) and vitamin C (VC) according to Box-Wilson (1951) experimental design matrix. A central composite rotatable response surface design (CCRRSD) for $K = 3$ was employed to examine the linear, quadratic and cross product effects of independent process variable concentrations at 5 levels, Fe (1, 2, 3, 4 and 5mg/100ml), Ca (50, 100, 150, 200, and 250mg/100ml) and VC (8, 16, 24, 32 and 40mg/100ml) on the nutrient density of fortified soymilk stored at ambient temperature for 12 weeks. A total of 21 experimental runs were generated and the response surface data for each run were statistically regressed and analyzed for variance using Minitab computer software (version 11.21). Three-dimensional response surface figures were plotted with Matlab software (version R 2007b) to visualize the effects of fortificants variables on the responses. Results obtained showed that all the fortificants were significantly ($p \leq 0.05$) linearly related to their respective responses. None of the response micronutrient met the respective RDI per serving except samples with variable combinations 2, 100, 32; 2, 200, 32; 4, 200, 32 and 3, 150, 40mg/100ml for ferric ammonium citrate, calcium carbonate and vitamin C respectively.

Key words: Complementary feeding, micronutrient, sprouted soybean, fortified soymilk and response surface methodology

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I. Introduction

Extensive nutritional studies have confirmed that beyond the critical infancy period of 4 to 6 months, to up to two years, breast milk alone cannot meet the nutritional and energy needs of infants and young children. Several studies have affirmed that this period is associated with tremendous physical growth, notably double increase in length and triple weight gain, as well as physiological, immunological and mental development (Yeung, 2011). Magnitude of variation in breast milk nutrients among lactating mothers (Lonnerdal, 1985) and the poor sources of iron and ascorbates all demand mandatory introduction of adequate and appropriate nutritious foods with respect to calorie, vitamins and minerals. A complementary food is therefore introduced to improve or meet both the

energy and nutrient demand since the child will no longer gain weight despite appropriate breast feeding, and will be feeling hungry always despite frequent breast feeding (Rarback, 2011).

Complementary foods are nutritional companion (Iwe, 2010; UNICEF, 2010) comprising non-breast milk or specially prepared foods or modified family meals (WHO, 2000) introduced to the diet of breast feeding infants and young children during their transition to adult diet (Iwe, 2010; UNICEF, 2010). They may be solid, semi-solid (Agostoni et al., 2008; Iwe, 2010) or liquid (Agostoni et al., 2008; UNICEF, 2010) foods introduced as from 4 to 6 months to up to two years (UNICEF, 2010). Complementary foods are generally introduced as readily consumed and digested by the young children to provide nutrients that will meet their extra needs most especially during their vulnerable period (UNICEF, 2010; WHO, 2002). Good complementary foods must be rich in energy, protein and micronutrients as well as, clean, safe and easy to prepare (WHO, 2000). Micronutrients are essential vitamins and minerals needed in very small amounts and must be supplied by a variety of foods in the diet (Agbon, 2009) to stimulate cellular growth and metabolism (Kennedy et al., 2003). Complementary feeding is the process of introducing complementary foods within the complementary period (6 months to 2yrs of age) to infants' diet (Iwe, 2010; UNICEF, 2010).

Extensive reports have shown that staples are modified (WHO, 2002; Iwe, 2010) for complementary feeding as they provide energy and proteins but poor sources of minerals (iron, zinc, and calcium) due to the presence of phytates that interfere their absorption. This therefore implies that staple foods must be eaten with other foods, remove phytates or fortified for a child to get enough nutrients. Soymilk, an off white aqueous extract of whole soybeans, has been reported as an economic protein source and good breast milk substitute from vegetable origin (Osuji & Ubbaonu, 2004; Fallon & Enig, 2007). However, soymilk is deficient in vitamins and mineral (lower than RDI) which demands fortification (STS, 1987). Germination/ sprouting of whole soybean and fortification of soymilk have long been adopted to improve the nutritional content of soymilk for school children (STS, 1987; Rafferty et al., 2007). Sprouted soybeans provide readily consumed and digested soymilk thus making it a potential complementary food (WHO, 2002). Food fortification, deliberate addition of one or more essential micronutrients to food (Clarke, 1995), with shortfall essential nutrients has proved to be the optimal strategy in dealing with widespread nutrient deficiencies. Intervention studies have given a good account of successful fortification of modern diets. Soymilk has been successfully fortified with vitamin B₁₂, fat (Omega fatty acids and fish oil), ascorbate and calcium to prevent development of their deficiency symptoms in infants and young children (Fallon & Enig, 2007). The aim of this study is to optimize the essential micronutrient content of fortified soymilk for complementary feeding using response surface analysis.

II. Materials and Methods

Soybean variety, TGX 923-2E, used was procured from the National Cereal Research Institute (NCRI) outstation, Amakama Olokoro in Abia state Nigeria.

Preparation of soymilk: Cleaned and sorted whole soybeans were sprouted at room temperature for 72 hours after 12hrs tap water soaking (Mostafa et al., 1987). The sprouted beans were boiled in 0.5%NaHCO₃ solution for 20min, drained (Omosaiye et al., 1978) and hand-dehulled. The hulls and the shoots were removed by water floatation leaving soybean cotyledons which were milled into slurry with hot water (93°C) in a ratio of 2.7 parts hot water to one part cotyledons (v/w) using QLink (Japan) variable speed kitchen blender (STS, 1987; Nsofor & Maduakor, 1992). Soy extract obtained by screening the slurry through a double layered muslin cloth was evaluated for oil content (Soxhlet extraction) and marked up to 3.5% with soybean oil (IMF, 2004) in line with soymilk definition (Iwe, 2003).

Fortification of soymilk and experimental analyses: Marked up soymilk bulk was fortified according to the experimental design matrix (Table 02). Each run was separately bottled and labeled. All samples were sterilized at 121°C for 5 min, analyzed before storing for 12 weeks at ambient temperature (25 to 32°C) for same analysis fortnightly. Vitamin C was determined according to

Rahman Khan et al., (2006), calcium and dietary iron by AAS-ashing method of Tee et al. (1989 a & b) using Buck Model 200A AAS, oil content by soxhlet method, crude fiber and ash according to AOAC method and carbohydrate by difference (Iwe & Ngoddy, 1998). The energy level of the samples was determined by calculation using energy values of food components (Mullan, 2006).

Experimental design and statistical analyses: Central composite design (CCD) and Box-Wilson (1951) experimental design matrix (Table 02) were employed to optimize the concentrations of the fortificants in each experimental run. Three variable factors (k=3) each with five level coded combinations (Table 01) were used to generate 15 experimental runs which when replicated six times at center point only for estimation of error points gave 21 experimental runs used for the study (Table 02). The model is summarized into equation (1).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

Where, Y = dependent variables, X_i and X_j = coded independent variables in the model, K = number of independent variables, β_0 = intercept (constant or model regression coefficient), ε = random error term, B_{ii} , β_j and β_{ij} = linear, quadratic and cross-product regression terms respectively. The variables were chosen based on their synergetic effects on growth (Fallon & Enig, 2007), and reasonably spaced to differentiate their influence on the responses.

Table 01. Five levels of coded independent variables and their real values used in the design

Independent process variable (mg/100ml)	Code	K = 3	Variable levels				
			-1.682	-1	0	+1	+1.682
Ferric ammonium citrate	Fe	X_1	1	2	3	4	5
Calcium carbonate	Ca	X_2	50	100	150	200	250
Vitamin C	C	X_3	8	16	24	32	40

Table 02. Box-Wilson (1951) experimental design matrix for coded, real independent process and response variables

Expt. Runs	Coded Independent Processes Variables			Real Independent Process Variables			Responses		
	X_1	X_2	X_3	X_1	X_2	X_3	Y_1	Y_2	Y_3
0	0	0	3	150	24	0.525	3.8	4.31	0
1	-1	-1	-1	2	100	16	2.514	103.617	14.84
2	-1	-1	1	2	100	32	2.516	103.606	30.30
3	-1	1	-1	2	200	16	2.518	203.612	14.93
4	-1	1	1	2	200	32	2.518	203.625	31.13
5	1	-1	-1	4	100	16	4.518	103.609	14.79
6	1	-1	1	4	100	32	4.517	103.637	14.89
7	1	1	-1	4	200	16	4.516	203.606	14.90
8	1	1	1	4	200	32	4.516	203.623	30.01
9	1.682	0	0	5	150	24	5.517	153.600	24.80
10	0	0	0	1	150	24	1.516	153.617	23.03
11	0	1.682	0	3	250	24	3.516	253.675	23.80
12	0	-1.682	0	3	50	24	3.518	53.725	23.50
13	0	0	1.682	3	150	40	3.517	153.611	40.60
14	0	0	-1.682	3	150	8	3.517	153.621	8.20
15	0	0	0	3	150	24	3.516	153.604	23.30
16	0	0	0	3	150	24	3.515	153.603	23.31
17	0	0	0	3	150	24	3.516	153.604	23.30
18	0	0	0	3	150	24	3.515	153.605	23.29
19	0	0	0	3	150	24	3.517	153.603	23.31
20	0	0	0	3	150	24	3.517	153.605	23.29
21	0	0	0	3	150	24	3.516	153.604	23.30

X_1 , X_2 and X_3 each represents concentration (mg/100ml) of dietary iron, calcium and vitamin C fortificant used in the fortification trials. Each row represents a fortification trial run or adjustment levels of the process variable combination at one run. Y_1 , Y_2 , and Y_3 represent dietary iron, calcium and vitamin C responses respectively.

Table 03. Regression coefficients, ANOVA and responses of fortified soymilk

Model terms	Coefficients		
	Y_1	Y_2	Y_3
Constant	3.5148	153.673	23.841
X_1	1.08220*	-0.197	-0.700
X_2	-0.00396	54.106*	0.931
X_3	-0.00386	0.198	7.133*
$X_1 X_2$	-0.00784	0.330	1.296
$X_1 X_3$	-0.00709	0.340	-2.549
$X_2 X_3$	0.02684	-1.337	1.979
X_1^2	0.00215	-0.094	-0.519
X_2^2	0.00197	-0.062	-0.626
X_3^2	0.00197	-0.092	-0.361
R^2	99.3%	99.3%	85.3%
R	0.99	0.99	0.92
ANOVA	0.000*	0.000*	0.106**

X_1 , X_2 , and X_3 are respective coded terms of the independent variables, X_1X_2 , X_1X_3 and X_2X_3 are the interaction coded terms of the independent variables, X_1^2 , X_2^2 and X_3^2 are the quadratic coded terms of the independent variables. Y_1 , Y_2 and Y_3 are their respective dependent responses. *significant at 5% level and ** significant at 1% level.

A regression data analyses were carried out (Table 03) on each run using Minitab software (version 11.21) to generate the estimated regression coefficients, coefficient of determination (R^2), correlation coefficients and ANOVA. Statistical significance was acceptable at 5% probability level ($p \leq 0.05$). The 3-dimensional response plots were performed with Matlab software (R2007b) package to have a mental picture of independent variables effects on response.

III. Results and Discussion

Dietary iron content

Dietary iron concentration (Table 02) increased from 0.525mg/100ml for unfortified sample to a range of 1.516 to 5.517mg/100ml for fortified samples. The 0.525mg/100ml recorded was slightly lower than 0.58mg/100ml, 0.58mg/100ml and 0.56mg/100ml reported by [STS \(1987\)](#), [Soya \(2009\)](#), and [Onweluzo & Nwakalor \(2009\)](#) respectively. Samples with same level of dietary iron fortificant concentration indicated insignificant ($p > 0.05$) variations with storage. None of the samples met the RDI of 10mg/d for infants per serving.

Only ferric ammonium citrate had the strongest linear significant ($P < 0.05$) effect on dietary iron concentration of fortified soymilk with positive coefficient to increase the dietary iron concentration. The linear function which accounted for 99.3% of the total dietary iron concentration implies that ferric ammonium citrate is the major iron source of fortified soymilk. Ferric ammonium citrate is the best iron fortificant for milk ([Clarke, 1995](#)). Iron metabolizes protein for energy with the aid of calcium ([Fallon & Enig, 2007](#)). Exceeding iron RDI over time results in over dose diseases ([ODS, 2007](#)).

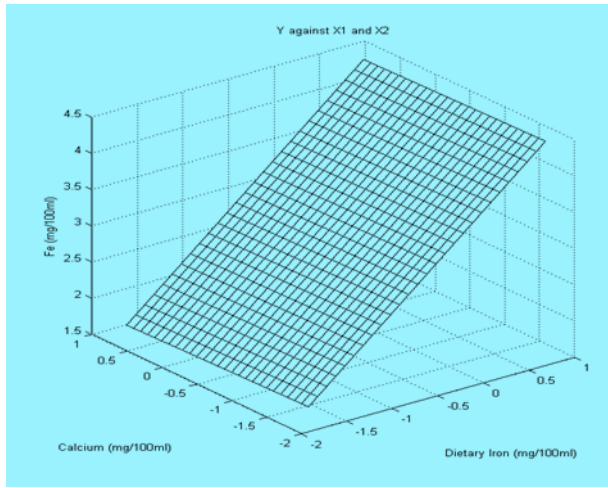


Figure 01. Linear plots of effects of calcium and vitamin C interaction on dietary iron concentration.

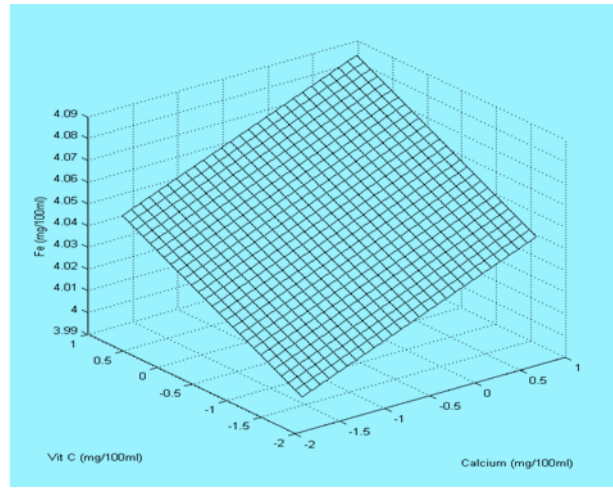


Figure 02. Linear plots of effects of vitamin C and ferric ammonium citrate interaction on soymilk calcium content

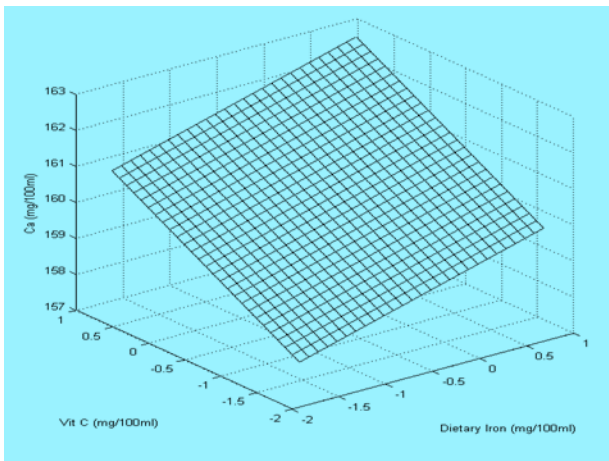


Figure 03. Linear plots of effects of vitamin C and calcium interaction on soymilk calcium content.

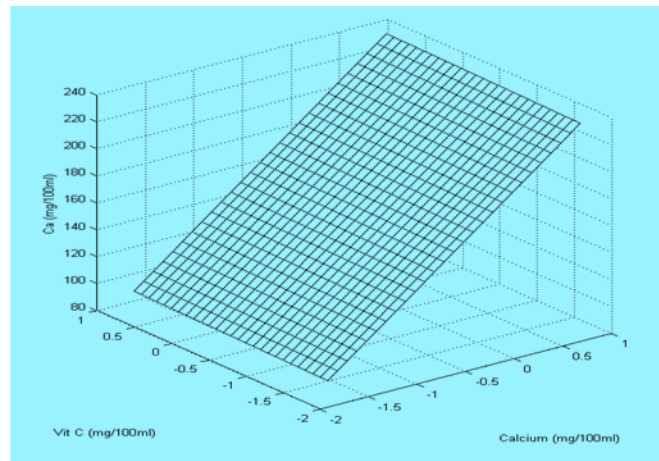


Figure 04. Linear plots of effect of vitamin C and dietary iron interaction on soymilk vitamin C content.

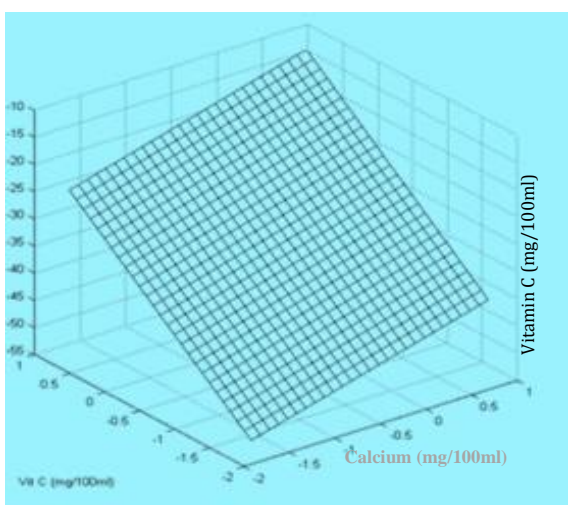


Figure 05. Linear plots of effect of vitamin C and dietary iron interaction on soymilk vitamin C content.

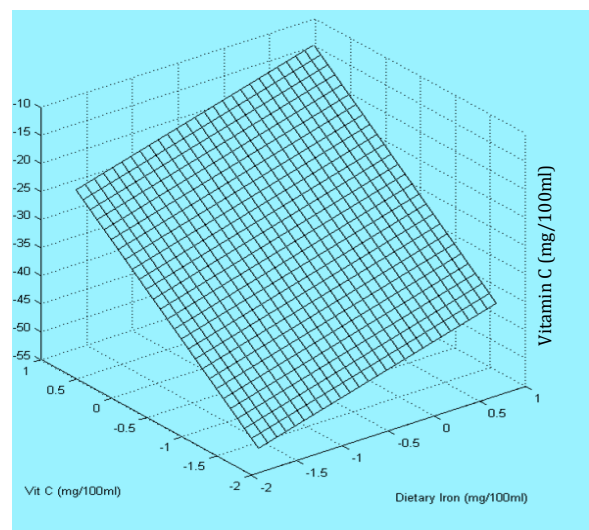


Figure 06. Linear plots of effects of vitamin C and ferric ammonium citrate interaction on dietary iron content.

The fitted model is well adequate to predict iron increase by ferric ammonium citrate with a satisfactory R value of 0.99. Ignoring the non-significant terms, the real prediction equation becomes:

$$Y = 3.515 + 1.082 X_1 \quad (3)$$

Y is the experimental or response value of dietary iron and X_1 is the coded values of ferric ammonium citrate. The yield therefore is a linear function of ferric ammonium citrate content. Linear or first order model is best adjusted in this study. ANOVA (Table 03) indicated that the model had insignificant lack of fit to predict increase in dietary iron by ferric ammonium citrate. Significant influence of ferric ammonium citrate on iron content can be visualized in figures 01 and 02. Slight variations among samples of same fortification levels may be as a result of insignificant ($p > 0.05$) interactive effects between the dietary iron and the soymilk components recorded due to absence of stabilizer (Rafferty et al., 2007) which signified stability (Clarke, 1995). Vitamin C fortificant may have stabilized the dietary iron (Clarke, 1995; DSM, 2011). Minerals are heat resistance (Onwuka, 2005). Experimental run 9 with variable combination 5, 150, 24 for Fe, Ca and vC had the maximum dietary iron content.

Calcium content

Unfortified sample concentration of 3.8 mg/100ml increased to a range of 53.725 to 253.617mg/100ml with fortification (Table 02). Unfortified sample value (3.8mg/100ml) was slightly lower than 4.0mg/100ml reported by Soya (2009). There is insignificant ($p > 0.05$) variations among the samples with same fortification levels with storage. None of the samples met calcium RDI for infants (270 to 600mg/d, BHC, 2009) per serving.

Strong and linear significant ($p \leq 0.05$) effect of calcium carbonate accounted for 99.3% of total soymilk calcium content variations due to variables (Table 02). The yield is therefore reliable, acceptable and can fit into first order polynomial equation well. Calcium carbonate is therefore the major contributor to iron concentration with correlation coefficient of 0.99 which signified a very high correlation between the experimental and predicted values of the variables (Table 03). Therefore, the fitted model is adequate to predict the variation. Dropping the non-significant terms from the regression equation, the real prediction equation becomes:

$$Y = 153.673 + 54.106 X_2 \quad (2)$$

Where Y is the observed value of calcium and X_2 is the coded value of calcium carbonate. Calcium concentration here is dependent on the linear function of calcium carbonate.

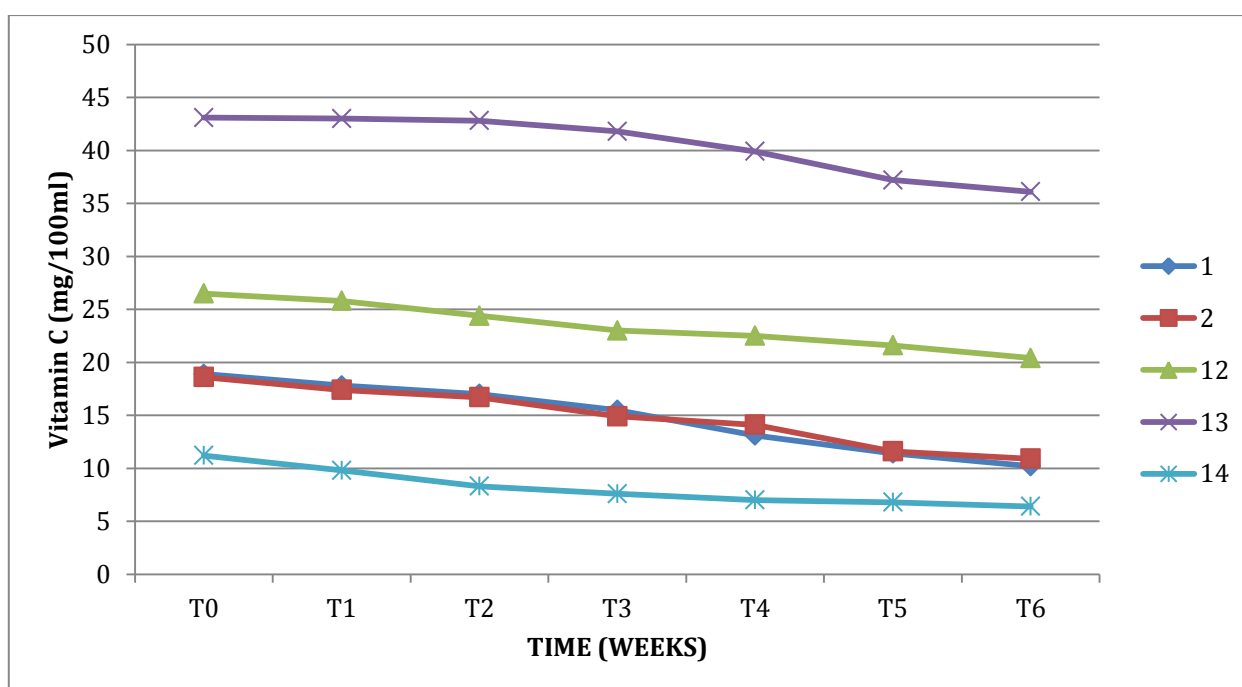
ANOVA (Table 03) indicated strong significant ($P \leq 0.05$) effect of calcium carbonate (X_2) on the calcium concentration of the soymilk. None of the interaction visualized (Figure 03 and 04) between any two of three variables contributed significantly ($p \leq 0.05$) to calcium increase (Table 03) indicating calcium stability and compatibility with other fortificants and soymilk components, hence product stability (Barclay, 2011). Besides, calcium carbonate was not heat labile (Onwuka, 2005). Lack of stabilizer may likely have permitted negligible interactions (Table 03) between calcium and heat denatured soy protein thereby making some of the calcium unavailable for evaluation. Stabilizer works best with calcium fortification by suspending the insoluble calcium and minimize protein-calcium interaction and sedimentation (Rafferty et al., 2007). This may have affected the response results (Barclay, 2011). Iron may have metabolized protein to prevent their interaction with calcium (Fallon & Enig, 2007). With these, fortification levels for calcium in this work were adequate to meet calcium RDI of 210 - 600mg/day (BHC, 2009). Calcium carbonate is an economic source of calcium, effectively absorbed by infants (Bhatia, 2008) for their growth (IMF, 2004), and helps nutrient flow in the cell walls (BHC, 2009).

Experimental runs 3, 4, 7, 8 and 11 contained reasonable percentage of calcium RDI per serving and therefore are good calcium sources (Soya, 2009; SMI, 2009), while samples from runs 9, 10, 13, 14 and 15 followed next. Consumption of 150ml/d for former and 200ml/d for later runs of the fortified samples along breast milk and other calcium containing adjunct foods like fruits, vegetable, and

biscuits will meet the RDI (WHO, 2000; Agostoni et al., 2008). Samples from runs 1, 2, 5 and 6 require consumption of 300ml/d to meet the RDA. Sample of run 12 is a poor source while that of run 11 is an excellent source that requires 120ml/d to meet the RDA. The primary aim of calcium fortification was achieved. Despite these, levels of calcium concentration recorded (Table 03) may likely enhance absorption. Rafferty et al., (2007) had earlier reported that calcium is well absorbed when taken several times in small amounts (300mg/day) throughout the day while excess calcium complexes with protein and may prevent calcium availability.

Vitamin C content

Table 02 and Figure 07 explained the general progressive decrease of vitamin C content with storage as from the first two weeks till the end of the study. Despite the decrease, fortification increased the vitamin C content from 4.31mg/100ml in unfortified to a range of 8.2 (run 14) to 40.60mg/100ml (run 13) in fortified samples. Vitamin C content of unfortified sample (4.31mg/100ml) is slightly higher than 4.2mg/100ml reported by Onweluzo & Nwakalor (2009).



1, 2, 12, 13 and 14 each represents an experimental run of the design

Figure 07. Plots demonstrating rates of vitamin C loss for some selected fortified soymilk samples at two weeks intervals for 12 weeks.

Strong and linear increasing tendency ($P \leq 0.05$) of vitamin C fortificant accounted for 85.3% vitamin C content variation (Table 3). Vitamin C fortificant is therefore the major contributor to vitamin C content of fortified soymilk. This contribution (85.3%) was lower than 99.3% accounted for by both dietary iron and calcium which may be attributed to vitamin C loss (Figure 07 and Table 02) during processing and storage. Correlation coefficient of 0.92 indicated model's goodness of fit to predict 85.3% vitamin C variation due to vitamin C fortificant. Eliminating the non-significant terms from the model equation, real predictive equation becomes:

$$Y = 23.841 + 7.133 X_3 \quad (3)$$

Where Y is the response values of vitamin C and X_3 is the coded values of vitamin C variable. Linear function of vitamin C fortificant was well fitted to predict vitamin C content here.

ANOVA (Table 03) showed that only linear effect of vitamin C fortificant had a significant effect ($p \leq 0.05$) on vitamin C content of soymilk. Interactive effects of the variables on vitamin C are shown in (Figures 05 and 06). Vitamin C enhances calcium and iron absorption (BHC, 2009). Lack of vitamin C

causes anemia (DSM, 2011). Vitamin C loss in this study was inevitable as it was not protected. Retaining vitamin C in food, it must be protected by either appropriate packaging encapsulation or restoration (Clarke, 1995). Lower levels of vitamin C recorded from both fortified and unfortified samples despite fortification and sprouting that increases the vitamin C (Mostafa et al. 1987) may be attributed to the heat treatments during bicarbonate blanch, hot grind, autoclaving and during storage due to its unstable nature, readily oxidized (DSM, 2011). As a nutrient stabilizer, vitamin C may have complexed with the other fortificants and soymilk nutrients thereby not available for evaluation (DSM, 2011). These losses may be responsible for most runs meeting vitamin C RDI levels by consuming more. Vitamin C has to be protected or the fortification levels increased (overage) to accommodate the losses (Hurrell et al. 2004).

Despite the losses, samples of the experimental runs 1, 3, 5, 6, and 7 are good sources that require 210ml/d to meet the RDA. Runs 2, 8, 9, 10, 11, 12, and 15 are very good sources while runs 4 and 13 are excellent (NFH, 2001; Rafferty et al., 2007) of vitamin C. Runs 2, 4 and 8 require 100ml/d, 9, 10, 11, 12 and 15 require 130ml/d, and 13 requires 90ml/d to meet vitamin C RDA. NFH (2001) had earlier reported that nutritionists generally regarded any serving of food that provides 10 to 25% of daily vitamin C need as 'good' source, about 15 to 30mg as 'very good' source and above 30mg as 'excellent' source. If a single serving supplies more than the RDA of vitamin C, it is an 'exceptional' source. As vitamin C is needed in trace amount for proper biological functions (Onwuka, 2005), it implies that the levels of vitamin C obtained in this study were adequate to meet the complementary needs of infants and young children.

IV. Conclusion

Significant nutrient increases due to fortification have projected fortified soymilk in this study as not only protein rich but also nutrient dense liquid food liable to meeting both energy and nutrient needs of infants and young children. Fortified soymilk has the potential of meeting the RDI of nutrients in view and therefore could be used along with breast milk of calorific value of 0.7Kcal alone, or along with adjunct foods like pap or snacks to eradicate protein-energy malnutrition problems prevalent among weaning infants in less developed countries to enhance rapid growth and development. The aim of fortification is therefore met. Statistical optimization using CCD appears to be a valuable tool for the fortification of soymilk to meet the RDI of the deficient micronutrients in view. This will in turn reduce processing cost through identification of maximum fortificant combinations that will achieve the desire RDI of the nutrients in question to avoid overdose diseases associated with exceeding the RDI for a long period.

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