International Journal of Food and Nutritional Science

ISSN: 2377-0619
Research Article

OPEN ACCESS
DOI: 10.15436/2377-0619.20.2900

Optimization of the Reduction of Phytates and Trypsin Inhibitors of Soybeans (*Glycine Max L.*): Effect of Soaking and Cooking

Bruno Fotso Saah[1], Stephano Tambo Tene[1], Gires Teboukeu Boungo[1], Julie Mathilde Klang[1*]

[1]Research Unit of Biochemistry, Medicinal plants, Food Sciences and Nutrition, Department of Biochemistry, Faculty of Science, University of Dschang, Cameroon

***Corresponding author:** Julie Mathilde Klang, Research Unit of Biochemistry, Medicinal plants, Food Sciences and Nutrition, Department of Biochemistry, Faculty of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon, Tel: (+237) 6999399317/673787501; E-mail: klangjulie@gmail.com

Abstract

Legumes grain seeds are broadly developed and consumed all over the world. They are considered as an important constituent of human diet, economical source of energy, proteins, carbohydrates, fiber, B-group vitamins and minerals. Among this legumes, soybeans is consider to contain good amounts of proteins, fats, carbohydrates and also certain levels of vitamins and minerals. However, the bioavailability of these nutrients in soybeans is negatively impacted by the presence of anti-nutrients. Treatments like soaking with plantain peel ash and cooking significantly reduce these anti-nutrients. The objective of this work is to optimize the reduction of phytates and trypsin inhibitors in soybean meal. To do this, the response surface methodology with the Box-Behnken design was used with three factors: soaking time (6 h-24 h), plantain peel ash concentration (0.5-5%) and cooking time (5-15 min). Soybean meal obtained was characterized to determine amounts of phytates and trypsin inhibitors. The results showed that the interactions of soaking time and cooking time, soaking time and plantain peel ash concentration significantly affect ($p<0.05$) the amount of phytates. On the other hand, the quadratic effects of cooking time, plantain peel ash concentration and the linear effects of soaking time and plantain peel ash concentration significantly impact ($p<0.05$) the amount of trypsin inhibitors. 24 hours of soaking, 5% of plantain peel ash and 15 minutes of cooking allowed an optimal reduction in the amounts of phytates and trypsin inhibitors, giving a reduction of 47.06% and 44.68% respectively.

Keywords: Soya; Soaking; plantain peel ash; Cooking; Optimization; Phytates; Trypsin inhibitors

Introduction

Legume seeds are important food sources especially in developing countries due to their relatively low cost, high availability and high nutritional value[1]. Among these legumes, soybean is one of the most popular because, apart from their high availability, high proteins (32%) and fats (17%) content, they exceptionally contain all essential amino acids and good mineral content (calcium, zinc, iron)[2] (Ketnawa and Ogawa, 2019). However, the presence in this oilseed of anti-nutritional factors such as phytates, trypsin inhibitors, phenolic compounds, flatulence oligosaccharides, is the main obstacle to the bioavailability of the proteins and mineral amounts of soybeans[3] (Hortz et al., 2007).

Therefore, the levels of these anti-nutrients can be significantly reduced by methods such as soaking, roasting, cooking and even the use of basic solutions such as plantain peel ash[4] (Yasothai, 2016). Indeed, the work of Ndagire et al. (2015)[5] showed that soaking (24 h), germination (48 h) and steaming (19 min) reduced the phytates content (47%). Uche et al. (2014)[6] demonstrated that cooking (3h 48 min)

Received Date: September 07, 2020
Accepted Date: October 16, 2020
Published Date: October 20, 2020

Citation: Fotso, Saah., et al. Optimization of the Reduction of Phytates and Trypsin Inhibitors of Soybeans (Glycine Max L.): Effect of Soaking And Cooking. (2020) J Food Nutr Sci 7(1): 70-76.

and roasting (1 h at 100°C) peanut seeds reduced the content of phytates (42%) and trypsin inhibitors (37%). Ikese et al. (2017) demonstrated that roasting (30min) increased the nutritional value of soy flour. On the other hand, a long soaking and cooking time results in a significant loss of minerals, nutrients and water-soluble vitamins (Aviles-Gaxiola et al., 2018)[7]. One solution to this problem is the use of basic solutions during soaking. Mallemo et al. (2009)[8] showed that the use of 31% banana peel ash during cooking reduced cooking time (52%) and phytates content. The present work has been initiated with the objective of optimizing the reduction of phytates and trypsin inhibitors in soybeans.

Material and Methods

Material

The plant material used for this work consisted of soybeans (*TGX-1850-10E* variety) obtained at IRAD in Foumbot. The sample, once collected, was sent to the laboratory for the various assays and analyses.

Methods

Production of plantain peel ash solution: The plantain peels collected in a household in Bafoussam were dried in the oven Venticell (MM-group) at 50°C for 72 hours. These peels were then incinerated (oxidizing atmosphere) in an oven at 400°C for 15 minutes and the powder obtained was macerated with distilled water and the filtrate obtained constituted the plantain peel ash solution.

Production of optimized soybean flour: The response surface methodology (RSM) was used to optimize the three factors (soaking time, plantain peel ash concentration and cooking time) chosen to obtain a soybeans flour with a low amounts of phytates and trypsin inhibitors. Box-Behnken design was used for this purpose. The Box-Behnken plan requires 15 tests, which are in most cases satisfactory. It has symmetrical structures in which each factor takes three levels. It is the combination of two-level factorial designs, with balanced incomplete blocks according to a particular arrangement with several tests at the centre of the experimental domain.

Matrix of coded and real variables: After determining the levels of the different factors (through literature rewie and preliminary test), which varied between 6-24 hours for the soaking time, 0.5-5% for the Plantain peel ash concentration and 5-15 minutes for the Cooking time, we determined the number of experiments to be performed and made calculations that allowed us to obtain the real variables from the coded variables according to the three-factor Box-Behnken design (Table 1).

Mathematical model proposal: The proposed model simply had the property of representing the experimental response studied well in the experimental area of interest and thus obtained an estimate of the value of the studied responses of acceptable quality. The polynomial models, because of their simplicity, are the ones that have been chosen. On the performance of the experiments, the flow velocity is taken as the response (Y); I the constant; a, b and c the linear coefficients; d, e, and f the square coefficients; g, h and i the interaction coefficients and x_1, x_2, x_3, x_1x_2, x_1x_3, x_2x_3, are the levels of the independent variables. We therefore assumed a second degree model, for two variables. The model is as shown below:

$$Y = I + ax_1 + bx_2 + cx_3 + dx_1^2 + ex_2^2 + fx_3^2 + gx_1x_2 + hx_1x_3 + ix_2x_3$$

Validation of models

In order to predict responses in the area defined for the study, it was important to validate the empirical models obtained. To do this, the determination coefficient (R^2), the Absolute Mean Deviation Analysis (AMDA) which provides information on the

Table 1: Presentation of the experimental matrix

Tests	Coded variables			Real variables			Responses values	
	X_1	X_2	X_3	Soaking time (H)	Plantain peel ash concentration (%)	Cooking time (min)	Phytates content	Trypsin inhibitors content
1	0	0	0	15	2.75	10	101.76 ± 0.15	14.08 ± 0.67
2	-1	0	-1	6	2.75	5	94.58 ± 3.05	9.95 ± 1.88
3	1	-1	0	24	0.5	10	122.05 ± 6.10	14.02 ± 0.88
4	0	0	0	15	2.75	10	104.04 ± 3.96	14.04 ± 0.51
5	0	1	-1	15	5	5	112.89 ± 3.05	5.78 ± 1.27
6	0	1	1	15	5	15	118.69 ± 1.52	7.89 ± 0.96
7	-1	-1	0	6	0.5	10	118.96 ± 1.25	9.22 ± 0.42
8	1	1	0	24	5	10	97.94 ± 0.30	10.96 ± 0.30
9	1	0	-1	24	2.75	5	114.42 ± 1.52	10.82 ± 0.09
10	1	0	1	24	2.75	15	79.33 ± 6.10	10.75 ± 1.62
11	-1	0	1	6	2.75	15	125.102 ± 3.05	7.62 ± 1.95
12	0	0	0	15	2.75	10	102.82 ± 2.13	14.00 ± 0.60
13	0	-1	-1	15	0.5	5	108.625 ± 1.83	9.326 ± 0.65
14	0	-1	1	15	0.5	15	115.948 ± 3.05	8.631 ± 2.38
15	-1	1	0	6	5	10	134.256 ± 6.10	7.157 ± 1.79

average handling error and the Bias factor (Bf) were determined (Baranyi et al., 1999). For the model to be validated, the value of the determination coefficient (R^2) must be greater than 75%; Absolute Mean Deviation Analysis must be equal to 0 and Bias factor (Bf) must be between 0.75 and 1.25.

Soybean flour production: Once the seeds collected were sorted, washed, soaked a different time with variable plantains peel ash concentrations and pre-cooked according to the conditions given by the Box Behnken design; they were drained and dried in the oven for 12 hours at 50°C, then crushed using an electric blender and sieved sieved (300 µm) and stored in polyethylene bags before being used for analysis.

Determination of responses

Phytates contents of soybean meal: 2g of sample will be introduced in a 250 ml Erlen, then 100 ml of 2% HCL will be introduced and the whole will be left to stand for 3 hours. The solution will then be filtered with whatman paper N°3. 50 ml of filtrate will be introduced in a 250 ml Erlen with 107 ml of distilled water. 10ml of 0.3% ammonium thiocyanate has been added as an indicator[9] (Reddy et al., 1999). The whole will be titrated with a standard solution of fecl3 which contains 0.00195 g of iron per ml. The yellow colouring indicates the turn that persists for 5 min. The amount of phytate will be determined by the following formula :

Phytates (mg/100 g) = (X*1.19*100)/0.00195

X = burette descent volume

Trypsin inhibitor contents of soybean meal: Trypsin inhibitors are determined in soya beans by measuring the hydrolysis of a peptic substrate: BAPNA (benzyl-DL-arginine ρ-nitroanilide) (Clifford, Megen, Twaalfhoven & Hitchcock, 1980). In a 100 ml beaker containing 0.5 g of sample, 50 ml of NaOH (10 mM) will be introduced and the pH will be adjusted to pH 9.6 with NaOH or HCl (1 M). The mixture will be stirred at room temperature for 3 hours and stored at 4°C overnight. This mixture will be centrifuged the next day and the supernatant obtained will be used to estimate the trypsin inhibitor content of the samples (AOAC, 1990). The results obtained allow calculation of the activity of the remaining trypsin and are expressed in mg inhibited trypsin / g sample. Thus, the amount of pure inhibited trypsin per unit g of sample can be calculated:

QTI = [(2.632*D*Ar)/m]. With

D= dilution factor of the extracts;

Ar= (DOb - Doa) - (DOd - Doc);

m= mass of the samples.

Statistical analysis

The analysis of variance (ANOVA) was used to determine the influence of each factor and the degree of significance of each of these effects. The significance of each factor was determined by the Fisher test. The regression equations were also subjected to the Fisher test to determine the coefficient of determination R2. The calculations were carried out using MINITAB 18.0 software. The accepted confidence level was P<0.05. Graphical representations of the iso-response curves of the postulated models were made using SIGMA PLOT 12.0 software. GraphPad Prism 5.0 software and the Student Newman-keuls test allowed us to verify the existence of significant differences between the predicted and experimental responses.

Results and Discussion

Effects of treatments on the phytates and trypsin inhibitors contents of soybean meal

The purpose of this part was to define the soaking time, cooking time and plantain peel ash concentration that will make it possible to obtain soybean flour with the lowest phytates and trypsin inhibitors contents. From Table 1 it is noted that the lowest values were obtained under the following conditions: 24 hours of soaking, 2.75% of plantain peel ash and 15min of cooking for phytates content (79.33 ± 6.10 mg/100g) while 15 hours of soaking, 5% of plantain peel ash and 5min of cooking are required for the trypsin inhibitor content (5.78 ± 1.27 mg/100 g).The central tests showed significantly non-different values for the two responses, thus confirming the quality of the tests.

Proposal of mathematical models and contributions of different factors

The contents of phytates and trypsin inhibitors (Y) can be predicted respectively by the following regression equations

$$Y_{Phytates}=56.2+3.28X_1-6.65X_2+7.29X_3+0.02X_1X_1+2.57X_2X_2-0.07X_3X_3-0.48X_1X_2-0.36X_1X_3-0.03X_2X_3$$

$$Y_{Trypsin\ inhibitors} = -3.87+0.42X_1+2.07X_2+2.28X_3-0.01X_1X_1-0.55X_2X_2-0.13X_3X_3-0.01X_1X_2+0.01X_1X_3+0.06X_2X_3$$

It can be seen from these mathematical equations that the amount of phytates (Y) increases with soaking time (X_1), its quadratic effect (X_1X_1), cooking time (X_3) and the quadratic effect of the plantain peel ash concentration (X_2X_2), on the other hand, the plantain peel ash concentration (X_2), The quadratic effect of cooking time (X_3X_3), the interactions of soaking time - plantain peel ash concentration (X_1X_2), soaking time - cooking time (X_1X_3) and concentration of plantain peel ash - cooking time (X_2X_3) decrease the amount of phytates in soya meal. The amount of trypsin inhibitors increases with soaking time (X_1), plantain peel ash concentration (X_2), cooking time (X_3), soaking-to-cooking time interactions (X_1X_3) and plantain peel ash concentration (X_2X_3) but decreases with the quadratic effects of soaking time (X_1X_1), the concentration of plantain peel ash (X_2X_2), the cooking time (X_3X_3), and the interaction of soaking time and plantain peel ash concentration (X_1X_2). These equations also show that the linear effects of the three factors and the quadratic effect of the ash concentration contribute most to the reduction of phytates and trypsin inhibitors in soybeans.

Analysis of variance and validation of models

Table 2 shows the effect of the different factors on the quantities of phytates and trypsin inhibitors in soybeans. It appears that the interactions between soaking time and cooking time, soak time and plantain peel ash concentration as well as the quadratic effect of the plantain peel ash concentration and the linear effect of the soak time significantly affect ($p<0.05$) the amount of phytates. However, it is rather the quadratic effects of cooking time, plantain peel ash concentration, the linear effects of soaking time and plantain peel ash concentration that significantly impact ($p<0.05$) the amount of trypsin inhibitors in soybeans. The table also shows that the determination coefficients (R^2) for each of the two proposed models gave values of 97% for the amount of phytates and 95.65% for the amount of trypsin inhibitors. Similarly, the values of the absolute mean deviation analysis (AADM) obtained are 0.01 (phytates) and 0.03 (inhibitors). Those of the bias factor was 1.00for the two responses, which confirms the validation of the models.

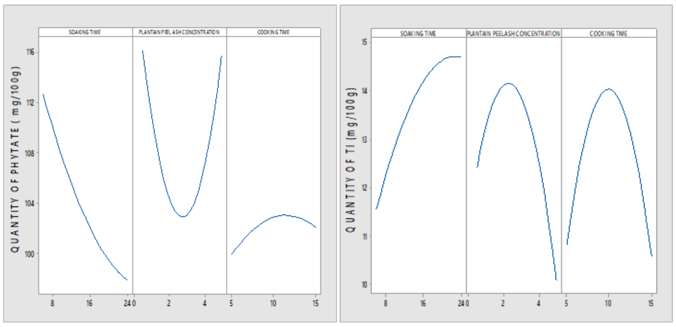
Table 2: ANOVA P-values of soybean (varietyTGX-1850-10E), coefficient of multiple determinations (R2), absolute average deviation (AAD) and bias factor (BF) for optimized reduction of phytates and trypsin inhibitors following Box-Behnken design

	Glycine max		
	P		
X_1 : SOAKING TIME	0,003*		
X_2: PLANTAIN PEEL ASH	0.88		
X_3: COOKING TIME	0.484		
X_1X_1	0.305		
X_2X_2	0.002*		
X_3X_3	0,405		
X_1X_2	0.004*		
X_1X_3	0.000*		
X_2X_3	0.856		
Model validation		Phytates	Trypsin Inhibitors
R^2		97%	95.65%
AAD		0.01	0.03
BF		1	1

**P < 0.05 values indicate that the factors contribute significantly to the response. X_1: linear effect of soaking time; X_2: linear effect of the concentration of plantain peel ash; X_3: linear effect of cooking time;*
X_1X_1: quadratic effect of soaking time; X_2X_2: quadratic effect of the concentration of plantain peel ash; X_3X_3: quadratic effect of cooking time; X_1X_2: interaction between soaking time and the concentration of plantain peel ash; X_1X_3: interaction between soaking time and cooking time; X_2X_3: interaction between the concentration of plantain peel ash and cooking time.

Effect of the factors on the amounts of phytates and trypsin inhibitors

Figure 1 shows the main effects of the different factors on the amounts of phytates and trypsin inhibitors ($p<0.05$). The amount of phytates decreases with the increasing of soaking time and the increasing of plantain peel ash concentration up to a peak value (2.75%) where it begins to increase due to hydrolysis and activation of phytase.Nevertheless, the amount of phytates increases with the increasing of cooking time up to a peak (5 min) where it begins to decrease. Phytates is a labile compound who is destruct by temperature. On the other hand, there is an increase in the quantity of trypsin inhibitors with the increase of soaking time, plantain peel ash concentration and cooking time until it reaches peaks of 15 hours (soaking), 2.75% (plantain peel ash) and 10 minutes (cooking) where it begins to decrease. Releasing of trypsin inhibitor during soaking and activation of that element by minerals content on ashes can explain this variation.

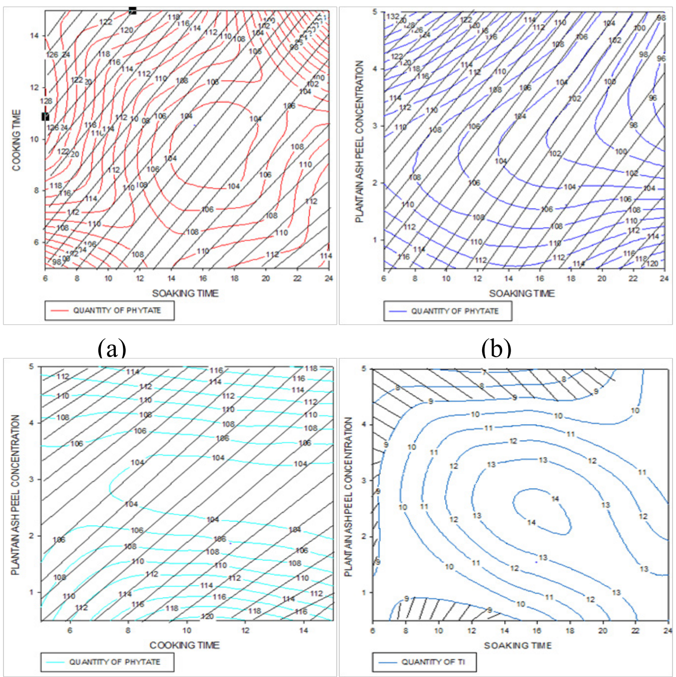


(a) (b)

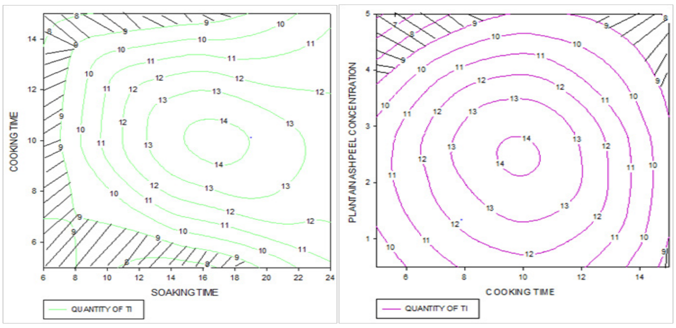
Figure 1: Curves of the principal effects of soaking time (hrs), plantain peel ash concentration (%) and cooking time (mn) on the amount of phytates (a) and trypsin inhibitors (b).

Iso-response curves

Figure 2 shows the iso-response curves of phytates and trypsin inhibitors as a function of soaking time, plantain peel ash concentration and cooking time. The hatched areas of interest are those of phytates less than 250 mg/100 g of soybean and 9 mg/100 g for trypsin inhibitors. This area of interest defines the experimental area in which the application of soaking time, plantain peel ash concentration and related cooking time will result in the production of soybean flours with low phytates and trypsin inhibitors. The combination of the various factors shows that the phytate content can be reduced below the recommended value at any given interval.



(a) (b) (c) (d)

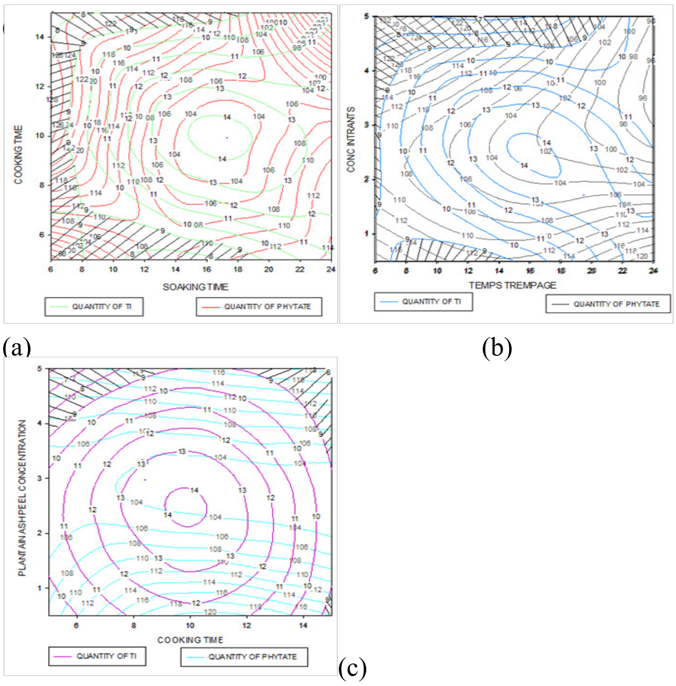


(e) (f)

Figure 2: Iso-réponse curves showing the interest zone shade with the interactions soaking time-plantain peel ash concentration for phytate (a) and trypsin inhibitors (d), soaking time-cooking time for phytate (b) and trypsin inhibitors (e) and plantain peel ash concentration-cooking time for phytate (c) and trypsine inhibitors (f).

TI: T*rypsin Inhibitors*

Figure 3 shows the iso-response curves of the trade-offs to find the necessary treatment conditions to simultaneously have the lowest levels of phytates and trypsin inhibitors. As can be seen from figure 3a below, the firing time range and the soaking interval of 6-14 hours can be used to achieve the desired values. The same is true for graph 3c showing the combination of cooking time and ash concentration where concentrations of more than 4% ash are required to reduce phytates and trypsin inhibitors below 250 mg/100 g and 9 mg/100 g respectively.



(a) (b)

(c)

Figure 3: Iso-réponses curves of compromise showing the interest zone shade with the interaction soaking time-cooking time (a), soaking time- plantain peel ash concentration (b) and plantain peel ash concentration-cooking tme (c) for the amount of phytate and trypsin inhibitors.

Essay of validation of the optimal condition of soaking, plantain peel ash and cooking for and optimal reduction of phytates and trypsin inhibitors in soybeans flour.

The MINITAB 18.0 software has made it possible to define the best treatment conditions to optimally reduce the content of phytates and trypsin inhibitors. These combinations and the values predicted by MINITAB 18.0 software for both responses are shown in Table 3. It appears that soaking for 24 hours, using 5% plantain peel ashand cooking for 15 minutes results in soy flour with a low content of phytates and trypsin inhibitors, which is in the recommended range for infant flours (less than 250 mg/100 g flour for phytates and less than 9 mg/100 g flour for inhibitors). To validate these results, manipulations under the optimal conditions predicted by the software were performed in the laboratory and experimental values were obtained (Table 3). There is no difference between the experimental and predicted optimal values, which allows the optimal conditions defined by the software to be validated. To obtain soya bean flour with a phytates content of less than 250 mg/100 g and trypsin inhibitors of less than 9 mg/100 g and suitable for children aged 6-36 months, the soya beans should be soaked for 24 hours with 5% plantain peel ash and then cooked for 15 minutes. The desirability representing the reproducibility coefficient of the model is 0.86, which shows that the validated model is robust.

Table 3: optimal conditions, predicted values defined by the program and experimental values obtained after manipulations.

	Amount of phytates	Amount of trypsin inhibitors
Soaking time (hour)	24	24
Plantain peel ash concentration (%)	5	5
Cooking time (min)	15	15
Predicted optimal values (mg/100g)	83.23^{a}	8.30^{a}
Experimental optimal values (mg/100g)	82.38^{a}	8.21^{a}
Desirability	0.80	

Discussion

The nutritional quality of infant flour is a key factor for the growth and survival of infants and young children. However, a low nutritional value of flour will lead to nutritional disorders in children[10] (Gupta et al., 2015). The anti-nutrient content is one of the most important factors that negatively impacts this nutritional quality. The amount of trypsin inhibitors decreases considerably when the soaking time (about 15 hours), plantain peel ash concentration (2.75%) and cooking time (5 minutes) exceed the activity threshold of these enzymatic proteins; this may be due to the fact that trypsin inhibitors are enzymes and can only have effective activities under specific conditions[6] (Uche et al., 2014). The combined application of the treatments reduced the content of this anti-nutrient by up to 44.68%. This reduction can be attributed to the inactivation of trypsin inhibitors due to the heat during cooking and the PH of the soaking medium but also to the soaking time which defines the time required for the PH of the medium to have a negative effect on these enzymes. It can also be attributed to deamination reactions that break covalent bonds under the influence of heat during cooking[11] (Osman et al.,2007). These observations are in agreement with those of Rasane et al. (2015b)[12] who demonstrated that the activities of anti-nutrient factors such as trypsin inhibitors, hemagglutinins, tannins and phytates were reduced by 7.59% ,32.6%, 33.3% and 20.7% after de-skinning and dipping. They also agree with those of Ndagire et al. (2015)[5] who showed that protein digestibility increased significantly with the reduction of phytates and phenolic compounds under the combined effect of soaking time (24 h), germination time (48 h) and cooking time (10 mn). The work of Yu-wei et al. (2012)[13] also demonstrated that cooking significantly reduces the content of trypsin inhibitors. The amount of soy flour phytates decreases with increasing soaking time and plantain peel ash concentration, resulting in a reduction of up to 49.01%. These observations are similar to those obtained by Nwosu et al. (2015)[14] who obtained a 59% and 76% phytate reduction in soybean and groundnut respectively after 120 hours of soaking. This reduction may be explained by the activity of phytases, which is an enzyme found in cereal and legume germ and known to have the ability to hydrolyze phytates, increases with soaking and the addition of basic solutions (Herlache, 2007)[15], this reduction is also attributed to leaching with soaking time (Afify et al., 2011)[16] facilitated by the weakening of the seed cell wall by the action of plantain peel ash (Mallemo et al., 2009)[8]. This leaching of phytate into the soaking water may be due to the influence of a concentration of gradient (difference in chemical potential), which governs the rate of diffusion (Reena et al., 2018)[17]. Soaking has been demonstrated by Luo et al. (2014)[18] as allowing the activation of endogenous phytases and the diffusion of anti-nutrients thus facilitating the reduction of these compounds. Similarly, a considerable reduction in phytates by soaking has been demonstrated by Vijayakumari et al. (2007)[19] in Bauhinia purpurea seeds. In this study, it was found that roasting does reduce the content of phytates and trypsin inhibitors, but this reduction is less than the cumulative effect of soaking, concentration of plantain peel ash and cooking on these same anti-nutrients. These results are similar to those of Duhan et al. (2002)[20] who demonstrated that the combined effect of soaking and de-skinning was more pronounced than the unique effect of soaking on phytates and trypsin inhibitors in legumes[21-23]; this may be explained by the fact that these treatments do not have the same mode of action on these anti-nutrients. So the pooling of these treatments makes it possible to optimize this reduction[24-26].

Conclusion

This work was initiated with the objective of optimizing the reduction of phytates and trypsin inhibitors in soybean flour. The analyses carried out show that soaking time, plantain peel ash concentration and cooking time have a negative impact on the content of phytates and trypsin inhibitors. The study of optimal conditions showed that 24 hours of soaking, 5% of plantain peel ash and 15 minutes of cooking made it possible to optimally reduce the content of phytates and trypsin inhibitors, giving respective values of 82.38 mg/100g (47.06% reduction) and 8.21mg/100 g (44.68% reduction) which are below the WHO prescribed values for infant flours (<250 mg/100 g for phytates and <9 mg/100 g for inhibitors) so this flour can be used in the production of weaning foods to combat protein-energy malnutrition in the 0-5 age group which is a major public health problem in developing countries.

Originality of work: The authors declare that this work is original and has not be submitted anywhere.

Additional information: No additional information is available for this paper.

Funding: No funding has received by authors.

Ethical approval: This article does not contain any studies with humans or animal's participants performed by any of the authors. Authors' contribution: Conceptualization, sample collecting, preparation and designing of research work (BSF, JMK, STT); execution of laboratory experiments and data collection (BSF,STT, GTB); Analysis of data and Interpretation (all the authors); supervision of the work (JMK); preparation of manuscript (all the authors).

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