

# International Journal *of* Food Studies



# International Journal of Food Studies

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# The Impact of Processing Methods on Chemical Composition, Mineral Bioavailability and Functional Properties of Nigerian-Grown Cashew Flour

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## Abstract

In this study, the effect of processing methods on the nutritional quality and functional properties of cashew (*Anacardium occidentale* Linn) kernels were investigated. The kernels were soaked, autoclaved, roasted or germinated at varying time duration; raw kernel served as control. The samples were analysed for chemical, mineral bioavailability and functional properties. Data was subjected to analysis of variance and means were separated by the Duncan multiple range test. The result of chemical composition analyses revealed that raw cashew kernels contained  $3.55 \pm 0.08\%$  moisture,  $21.3 \pm 0.05\%$  protein,  $45.0 \pm 0.15\%$  fat,  $2.53 \pm 0.02\%$  fibre,  $1.59 \pm 0.02\%$  ash,  $26.1 \pm 0.01\%$  carbohydrate,  $521.75$  Kcal/g energy,  $2210.09 \pm 0.02$  mg/kg calcium,  $1712.54 \pm 0.03$  mg/kg magnesium,  $60.04 \pm 0.01$  mg/kg iron and  $36.74 \pm 0.02$  mg/kg zinc. Tannin, phytate and oxalate concentrations in the raw cashew kernel were  $10.14 \pm 0.03$  mg/kg,  $99.30 \pm 0.02$  mg/kg and  $11.03 \pm 0.03$  mg/kg respectively. Increased fat, ash and fibre levels were noted for treated samples compared to raw kernels. Mineral concentrations were increased significantly by various treatments compared to raw kernel; however, germination resulted in the highest increase of mineral content. A reduction trend was observed in phytate, oxalate and tannin concentrations in the treated samples with respect to increased processing time. Consequently, various treatments influenced the bioavailability of mineral elements. Treated samples exhibited significant differences in loose and packed bulk densities, water and oil absorption capacities when compared to raw kernels. Germination shows potential to generate not only much needed nutrients in cashew for human development, but also improved bioavailability of nutrients and functionality compared to other processing methods. This approach can be used in Community Nutrition and Emergency Feeding Programmes, in developing countries, where the consequence of anti-nutritional factors may worsen the incidence of malnutrition and disease.

**Keywords:** Cashew; Nutrient; Mineral bioavailability; Functionality; Processing effects

## 1 Introduction

The nutritional inadequacies in developing countries that arise from high cost of animal proteins have necessitated the use of other protein food sources such as those from plant origins. Tree nuts such as almonds, cashews, hazel nuts,

macadamias, pecans, pine nuts, pistachios and walnuts are readily available and offer great potential as a ready to eat food for human consumption.

The cashew tree (*Anacardium occidentale* Linn) is a native of Brazil and widely cultivated across the coastal regions of the tropics (Gibbon &

Pain, 1985). The major producing countries of the cashew nut are Tanzania, India, Mozambique, Srilanka, Kenya, Madagascar, Thailand, Malaysia, Indonesia, Nigeria, Senegal, Malawi and Angola. Cashew was introduced into Nigeria in 16<sup>th</sup> century; however, the cultivation has spread to western, eastern and northern states (Hammed, Anikwe, & Adedeji, 2008). It is important to note that cashew nut is underutilized in Nigeria as only about 10% is utilized locally (FAO, 2008).

The cashew fruit is made up of the apple in which the kernel is embedded. The kernel has high nutrient value and consists of 21.2% protein, 22.0% carbohydrate and 47.0% fat; it offers 575 kcal of energy per 100g and various minerals (Sathe, 1994). As with other tropical nuts, the cashew comes with associated issues such as the presence of secondary plant metabolites that are also known as anti-nutritional factors (ANFs). These include tannin, phytate and trypsin inhibitor which bind with minerals such as Fe, Zn and other similar metals, and protein respectively. These metabolites subsequently interfere with nutrient utilization when fed to human (Ngatchic et al., 2013). This activity remains a major drawback to the direct use of cashew as food ingredient. Several processing techniques have been reported by various investigators that may enhance the nutritional quality of food materials and also reduce or destroy the anti-nutrient present in them (Esenwah & Ikenebomeh, 2008; Slupski, 2011). The knowledge of how processing conditions modify bioavailability of nutrients trapped in the food matrix is indeed a critical factor to be considered in the development of food for various age groups in addition to cost of raw ingredients, processing cost and sensory acceptability of such foods (Abebe, Stoecker, Hinds, & Gates, 2006). Similarly, the functional performance of plant-based protein is also important in determining its usefulness in food systems. This study adds to the current knowledge of cashew nutrition and reports the effect of soaking, autoclaving, roasting or germination on the nutritional quality and functional properties of cashew flour.

## 2 Materials and Methods

### 2.1 Materials

Cashew nuts were purchased from a local market in Kogi State, Nigeria. All chemicals used were of analytical grade and were purchased from Sigma Chemical Company, St. Louis, MO, USA.

### 2.2 Preparation of cashew flour

Cashew nuts were sun dried to facilitate shelling. The dried nuts were shelled manually with the use of hammer to obtain the kidney shaped kernels. The kernels were divided into five portions: one portion was kept as a whole kernel (control), second portion was soaked in distilled water, third portion was autoclaved, fourth portion was roasted and the last portion was germinated.

#### Soaking

Cashew kernels (50g) were soaked in tap water in a ratio 1:10 (w/v) at room temperature ( $25 \pm 2$  °C) for 6, 12 and 18h respectively. The soaked kernels were washed twice with water, followed by rinsing with distilled water and then dried in an oven at 60 °C to a constant weight, according to Kaur and C. Kapoor (1990). The dried samples were milled using Philips laboratory blender (HR2811 model), to produce flour and sieved using a 60 mm mesh. The flour obtained was packed in a glass container and stored in a refrigerator, maintained at 8 °C prior to use.

#### Autoclaving

Cashew kernels (50g) were autoclaved for 5, 10 and 15 min respectively at 121 °C, according to Kaur and C. Kapoor (1990). The autoclaved kernels were dried at 60 °C to a constant weight. The dried samples were milled into flour using Philips laboratory blender (HR2811 model), and sieved using a 60 mm mesh. The flour obtained was packed in a glass container and stored in a refrigerator, maintained at 8 °C prior to use.

## Germination

Cashew kernels (500g) were sorted manually for viable kernels. The selected kernels were planted in a basket containing wood sawdust as the medium/substrate for growth. The planted kernels were watered daily. The kernels were allowed to germinate at  $35 \pm 2$  °C for 7 days producing radical protrusion of about 0.5cm. The germinated kernels were washed with distilled water, drained and dried in a hot air oven at 60 °C, for 24 h, according to Ijarotimi, Oluwalana, and Ogunedojutimi (2012). The dried samples were milled into flour using Philips laboratory blender (HR2811 model), and sieved using a 60 mm mesh. The flour obtained was packed in a glass container and stored in a refrigerator, maintained at 8 °C prior to use.

## 2.3 Chemical analyses

### Determination of proximate composition

Standard methods according to the AOAC (2005) were used to determine moisture (AOAC-925.10), fat (AOAC-2003.05) by soxhlet extraction and ash (AOAC-923.03) by combustion. Protein (AOAC-960.52) content ( $N \times 6.25$ ) was determined by micro Kjeldahl method. Carbohydrate content was determined by difference. The energy value was estimated (kcal/g) by multiplying the percentage crude protein, crude lipid and carbohydrate by the recommended factor (2.44, 8.37 and 3.57 respectively) as described by Eknayake, Jansz, and Nair (1999). All analyses were carried out in triplicate.

### Determination of mineral concentration

The method according to the AOAC (2005) was used for the determination of mineral content. Two grams of the pulverized sample was placed in a crucible and ignited in a muffle furnace at 550 °C for 12 h. The resulting ash was dissolved in 10 mL of 10%  $\text{HNO}_3$ , and heated slowly for 20 min. After heating, it was filtered and the filtrate used for the determination of mineral content. The mineral constituents (Ca, Mg,

Fe and Zn) were analyzed separately, using an atomic absorption spectrophotometer (Hitachi Z6100, Tokyo, Japan).

### Determination of anti- nutritional compounds

#### Determination of phytate content

The phytate content of the flours was determined using method described by Oladele, Osundahunsi, Yemisi, and Adebowale (2009). Two (2g) grams of each finely ground flour sample was soaked in 20mL of 0.2N HCl and filtered. After filtration, 0.5mL of the filtrate was mixed with 1mL ferric ammonium sulphate solution in a test tube, boiled for 30min in a water bath, cooled in ice for 15min, and centrifuged at 3000 rpm for 15min. One millilitre of the supernatant was mixed with 1.5mL of 2,2-pyridine solution and the absorbance measured with a spectrophotometer at a wavelength of 519nm. The concentration of phytic acid was obtained by extrapolation from a standard curve using standard phytic acid solution.

#### Determination of oxalate content

The titration method described by Oladele et al. (2009) was used to determine the oxalate content. One gram of the sample was weighed in a 100mL conical flask, 75mL 3N  $\text{H}_2\text{SO}_4$  was added and stirred intermittently with a magnetic stirrer for 1h. The contents were then filtered using Whatman No.1 filter paper. From the filtrate, a 25mL aliquot was taken and titrated, while maintained at a temperature of 80-90 °C, against 0.1N  $\text{KMnO}_4$  solution until a faint pink colour persisted for at least 30 sec.

#### Determination of tannin content

Tannin content was determined by the method described by Mugabo, Afoakwa, Annor, and Rwubatsa (2017). One gram of sample was dispersed in 10mL distilled water and agitated. The dispersion was left to stand for 30 min at room temperature ( $20 \pm 2$  °C), then centrifuged. 2.5mL of the supernatant (extract) was pipetted into a 50mL volumetric flask. Similarly, 2.5



mL of standard tannic acid solution was pipetted into a separate 50mL flask. One millilitre of Folin-Denis reagent was measured into each flask, followed by 2.5mL of saturated  $\text{Na}_2\text{CO}_3$  solution. The mixture was made up to mark in a 50mL volumetric flask and incubated for 90 min at room temperature. The absorbance was measured at 250 nm with a spectrophotometer (Jenway model 6000).

### Determination of functional properties

The loose and packed densities were determined using the method described by Ikpeme, Osuchukwu, and Oshiele (2010). Water and oil absorption properties of the cashew flour were determined following methods of Adebayo, Ogun-sina, and Gbadamosi (2013) with slight modifications. A flour sample (1g) was mixed with 10mL distilled water for water absorption and 10mL of oil for oil absorption in a Philips laboratory blender (HR2811 model) at high speed for 30 sec. Samples were allowed to stand for 30min at room temperature then centrifuged (Uniscopes, England) at 2000rpm for 30 min. The volume of supernatant in a graduated cylinder was noted. Density of water was taken to be 1g/mL and that of oil was determined to be 0.993g/mL. Analysis was performed in triplicate.

### Mineral bioavailability determination

The bioavailability of minerals (calcium and magnesium, iron and zinc) was calculated as reported by Woldegiorgis, Abate, Haki, and Ziegler (2015). The molar ratio between anti-nutrient and mineral was obtained after dividing the mole of anti-nutrient with the mole of mineral. The mole of phytic acid was calculated as the measured value of phytic acid divided by the molecular weight of phytic acid (660.8) whereas, the mole of mineral (Ca or Mg Fe or Zn) was calculated as the measured value of the mineral divided by the individual mineral molecular weight (Fe: 55.8, Zn: 65.4, Ca: 40.0, Mg: 24.3).

## 2.4 Statistical analysis

Determinations were carried out in triplicates and the error reported as standard deviation of the mean. Analysis of Variance (ANOVA) was performed and the least significant differences were calculated with the SPSS software for windows (release 17.00; SPSS Inc., Chicago IL, USA). Significance was accepted at  $p \leq 0.05$  levels.

## 3 Results and Discussions

The compositions of raw and treated cashew flours are presented in Table 1. The moisture content the flour was in the range of  $2.68 \pm 0.10$  to  $4.78 \pm 0.08\%$ . Soaking of cashew kernel for 18 h (sample  $S_3$ ) resulted in highest value ( $4.78 \pm 0.08\%$ ), while lowest moisture content (2.68%) was exhibited in the cashew kernel flour roasted for 15min (sample  $R_3$ ). The lowest value recorded in sample  $R_3$  may be attributed to a greater moisture loss by evaporation during roasting. This result is in agreement with the findings of Vickers, Peck, Labuza, and Huang (2014) who reported that roasting processes decreased moisture content of almond kernels. The moisture content of roasted cashew flour was however lower than 5.90% reported by Ogungbenle and Afolayan (2015). The protein content of the control sample ( $21.3 \pm 0.05\%$ ) and germinated sample ( $22.21 \pm 0.54\%$ ) were significantly higher compared to soaked, autoclaved and roasted flours respectively. Roasting resulted in a decrease in protein content over time compared to raw cashew flour. This is by virtue of the Maillard reaction, which is an interaction between the carbonyl group of a reducing sugar and the free amino group from an amino acid or protein. Similarly, soaking (6-18h) and autoclaving (5-15 min) of cashew kernel resulted in lower protein values compared to raw kernel due leaching of soluble proteins into processing water. However, the observed significant increase in protein value of germinated cashew flour could be attributed to the liberation of bound proteins during germination. Earlier studies also reported that during germination, carbohydrates are mobilized to synthesize amino acids for the

growing seedling (Ocheme & Chinma, 2008). There was significance difference ( $p \leq 0.05$ ) in the fat content of the flour samples. The highest value ( $53.0 \pm 0.08\%$ ) was observed in sample S<sub>3</sub> while the lowest value ( $45.0 \pm 0.15\%$ ) was exhibited in the control (sample C). Fat content of cashew kernel flour in this study increased with all processing techniques applied. The higher value noted in soaked flours compared to raw flour is perhaps due to the fact that soaking initiated the cleavage of the protein-lipid or carbohydrate-lipid linkages thereby facilitating the easy extraction of the oil by the extracting solvent (Madigan, Martinko, Parker, et al., 1997). Similarly, the higher lipid content in roasted sample compared to the control could be explained by the fact that oil bodies and the endoplasmic network were largely destroyed, and the volume of extracellular pores enlarged during roasting (Grundy et al., 2015). The fat content presently reported for roasted cashew flour was higher than that reported by Ogungbenle and Afolayan (2015). However, it is important to note that lipid oxidation, a critical factor in limiting shelf life, may be controlled to a large extent by roasting of nuts. Non-enzymatic reactions facilitate the release of antioxidants though the formation of acrylamide has been observed only in almonds and only at excessive roasting temperatures (Perren & Escher, 2013). Furthermore, in terms of its physical behaviour during mastication, roasted nuts were found to be more brittle and crunchy and produced more loose particles post chewing than raw nuts (Vickers et al., 2014).

The ash content of the flour samples ranged from  $1.59 \pm 0.15$  to  $2.66 \pm 0.05\%$ , with the lowest value observed in the control sample. The significant increase in ash content of roasted samples compared to raw sample may be attributed to concentration of the organic compounds in the former during roasting. Soaking and autoclaving of cashew kernels with time also improved the ash content. The increase in ash content observed in soaked kernels could be attributed to leaching of some constituents, notably anti-nutrients into the processing water which in turn improved mineral availability. Similarly, higher ash content is expected especially at above ambient temperature employed

during autoclaving through the swollen and ruptured cell walls which permeate water and soluble constituents. Germination process also improves the ash content of cashew flour. The fibre content of the flour samples ranged from  $2.53 \pm 0.02$  to  $5.76 \pm 0.12\%$ , with the lowest value observed in the control sample. The carbohydrate content of flour samples ranged from  $15.8 \pm 0.16$  to  $26.1 \pm 0.01\%$ . Carbohydrate has the highest composition by percentage in raw kernel flour (control) with a value of  $26.1 \pm 0.01\%$ . The lower range recorded for soaked and autoclaved samples respectively, may be due to leaching of soluble carbohydrate (i.e. sugars) into the processing water. Similarly, the decrease in carbohydrate content of the roasted cashew kernel with time could be due to Maillard reaction, which occurred between amino acids, amines, aldehydes and carbonyl group of reducing sugars at high temperature to produce the roasted cashew flavour. The decreased carbohydrate content in germinated cashew flour (sample G) could be explained by the fact that hydrolysis of carbohydrate to sugar occurs during germination. The energy content of treated cashew samples was significantly different to that of the control sample. Energy value of the germinated flour was significantly ( $p \leq 0.05$ ) lower than that of other flour samples. Generally, long-term consumption of nuts is associated with a decreased risk of weight gain and obesity (Bes-Rastrollo et al., 2009). Flores-Mateo, Rojas-Rueda, Basora, Ros, and Salas-Salvado (2013), in recent studies, observed no association between nut consumption and weight gain as demonstrated in a meta-analysis of clinical trials. Additionally, it is quite important to note that there was spontaneous reduction of energy intake subsequent to nut consumption, relative to a no-load or alternate load condition. That is, the majority, probably 65-75%, of the energy reportedly provided by nuts is offset by lower energy intake at later eating events. Surprisingly, no single component of nuts appears responsible for this effect. The fatty acid profile is often mentioned but it does not appear to be responsible for spontaneous reduction of energy intake, because dietary compensation to walnuts, almonds, and peanut is comparable and they vary markedly in pro-



Table 1: Proximate composition of cashew flours

Sample	Moisture (%)	Protein (%)	Fat (%)	Fiber (%)	Ash (%)	Carbohydrate (%)	Energy (Kcal/g)
A <sub>1</sub>	3.80 <sup>e</sup> ±0.10	19.69 <sup>ef</sup> ±0.03	45.16 <sup>b</sup> ±0.02	3.85 <sup>f</sup> ±0.10	2.44 <sup>e</sup> ±0.04	22.05 <sup>d</sup> ±0.11	515.50 <sup>b</sup>
A <sub>2</sub>	3.94 <sup>f</sup> ±0.10	19.63 <sup>e</sup> ±0.10	47.51 <sup>c</sup> ±0.10	3.76 <sup>d</sup> ±0.10	2.51 <sup>f</sup> ±0.10	22.63 <sup>e</sup> ±0.10	526.34 <sup>d</sup>
A <sub>3</sub>	3.99 <sup>fg</sup> ±0.06	19.42 <sup>d</sup> ±0.03	48.25 <sup>g</sup> ±0.71	3.01 <sup>b</sup> ±0.03	2.56 <sup>g</sup> ±0.06	22.77 <sup>fd</sup> ±0.03	526.98 <sup>f</sup>
S <sub>1</sub>	4.61 <sup>h</sup> ±0.02	21.11 <sup>i</sup> ±0.04	51.06 <sup>i</sup> ±0.11	3.82 <sup>e</sup> ±0.05	1.67 <sup>b</sup> ±0.04	16.76 <sup>c</sup> ±0.99	542.18 <sup>i</sup>
S <sub>2</sub>	4.69 <sup>i</sup> ±0.10	20.57 <sup>g</sup> ±0.10	52.81 <sup>j</sup> ±0.10	3.77 <sup>d</sup> ±0.10	1.76 <sup>c</sup> ±0.10	16.40 <sup>b</sup> ±0.10	550.76 <sup>j</sup>
S <sub>3</sub>	4.78 <sup>j</sup> ±0.08	20.93 <sup>h</sup> ±0.87	53.00 <sup>k</sup> ±0.08	3.63 <sup>c</sup> ±0.05	1.87 <sup>d</sup> ±0.04	15.97 <sup>a</sup> ±0.16	551.05 <sup>k</sup>
R <sub>1</sub>	3.04 <sup>c</sup> ±0.04	19.13 <sup>c</sup> ±0.02	45.29 <sup>c</sup> ±0.04	5.76 <sup>i</sup> ±0.12	2.47 <sup>ef</sup> ±0.03	24.43 <sup>i</sup> ±0.14	530.97 <sup>g</sup>
R <sub>2</sub>	2.79 <sup>b</sup> ±0.10	17.95 <sup>b</sup> ±0.10	47.66 <sup>f</sup> ±0.10	5.52 <sup>h</sup> ±0.10	2.54 <sup>fg</sup> ±0.10	23.54 <sup>h</sup> ±0.10	526.75 <sup>e</sup>
R <sub>3</sub>	3.04 <sup>c</sup> ±0.04	17.73 <sup>a</sup> ±0.05	48.87 <sup>h</sup> ±0.09	5.18 <sup>g</sup> ±0.02	2.66 <sup>h</sup> ±0.05	22.91 <sup>g</sup> ±0.18	533.98 <sup>h</sup>
G	2.79 <sup>b</sup> ±0.10	22.21 <sup>j</sup> ±0.54	45.68 <sup>d</sup> ±0.16	3.80 <sup>e</sup> ±0.02	2.72 <sup>i</sup> ±0.03	22.02 <sup>d</sup> ±0.02	512.00 <sup>a</sup>
C	2.68 <sup>a</sup> ±0.08	21.25 <sup>j</sup> ±0.05	45.03 <sup>c</sup> ±0.15	2.53 <sup>a</sup> ±0.02	1.59 <sup>a</sup> ±0.02	26.14 <sup>j</sup> ±0.01	521.75 <sup>c</sup>

Data are mean values of triplicate determination ± standard deviation. Means within a row with different superscript are significantly different ( $p \leq 0.05$ ).

A<sub>1</sub>-cashew kernels autoclaved for 5min; A<sub>2</sub>-cashew kernels autoclaved for 10min; A<sub>3</sub>-cashew kernels autoclaved for 15min, S<sub>1</sub>-cashew kernels soaked for 6 h; S<sub>2</sub>-cashew kernels soaked for 12 h; S<sub>3</sub>-cashew kernels soaked for 18 h, R<sub>1</sub>-cashew kernels roasted for 5min; R<sub>2</sub>-cashew kernels roasted for 10min; R<sub>3</sub>-cashew kernels roasted for 15min; G-cashew kernels germinated for 7days, C-raw cashew kernels

portions of monounsaturated fatty acids and PUFA (USDA, 2002).

The mineral concentrations of raw and treated cashew flours are presented in Table 2. In parallel, with the observed increase in ash content in treated kernels, there was increase in mineral levels relative to the raw cashew flour. The highest concentration of mineral elements was noted in germinated samples followed by roasting, autoclaving and soaking. The enhancement of mineral concentrations during germination may be due to decomposition of anti-nutrient (notably phytate), thus releasing the bound nutrients. This result agrees with the report of Ihemeje, Ukauwa, and Ekwe (2015) who reported higher mineral concentrations in germinated walnut compared to raw walnut, though there was depletion as the germination time increased. The only factor that could account for higher mineral concentrations in roasted samples is the observed decrease in level of anti-nutrients. Similarly, soaked and autoclaved samples had higher mineral concentrations than the raw sample.

The anti-nutritional factors in the raw and treated flour samples are presented in Table 3. The phytate, oxalate and tannin contents of raw and treated cashew kernel flours were found to be in a range of  $50.27 \pm 0.03$  to  $99.33 \pm 0.03$ mg/kg,

$4.62 \pm 0.03$  to  $11.03 \pm 0.02$ mg/kg and  $4.47 \pm 0.04$  to  $10.14 \pm 0.03$ mg/kg respectively. Generally, flour samples from soaked, germinated, autoclaved and roasted cashew kernels produced significantly lower phytate, oxalate and tannin concentrations that were typical of products that had received some degree of treatment during preparation. However, the anti-nutritional factors were better deactivated by germination, roasting and autoclaving compared to soaking of the kernels. Furthermore, when the impact of different heat treatment (roasting and autoclaving) was compared to germination, germination was found to be more efficient in terms of deactivation of anti-nutrients as shown in Table 3. Water uptake of dry dehiscent nuts causes a rise in general metabolic activity and the formation of a seedling from the embryo during germination. Such growth requires phosphorus that is mobilized from the main storage form i.e. phytic acid and the process is accompanied with increase in phytase activity. The lower phytate and oxalate concentrations observed in roasted and autoclaved samples respectively may be attributed to the fact that they are heat labile. Similarly, observed decrease in anti-nutrients in soaked samples could be attributed to losses due to leaching during soaking and rinsing of the kernels.

Table 2: Mineral concentrations of cashew flours (mg/kg)

Sample	Calcium	Magnesium	Iron	Zinc
A <sub>1</sub>	3312.00 <sup>f</sup> ±0.03	1904.67 <sup>e</sup> ±0.03	69.83 <sup>e</sup> ±0.02	64.97 <sup>c</sup> ±0.04
A <sub>2</sub>	3445.11 <sup>g</sup> ±0.03	1963.23 <sup>g</sup> ±0.03	70.97 <sup>h</sup> ±0.03	68.54 <sup>d</sup> ±0.03
A <sub>3</sub>	3553.02 <sup>i</sup> ±0.04	1997.53 <sup>i</sup> ±0.03	72.05 <sup>i</sup> ±0.03	72.57 <sup>i</sup> ±0.03
S <sub>1</sub>	2231.24 <sup>b</sup> ±0.05	1869.42 <sup>b</sup> ±0.03	67.39 <sup>c</sup> ±0.03	63.09 <sup>b</sup> ±0.02
S <sub>2</sub>	2257.43 <sup>c</sup> ±0.03	1884.31 <sup>c</sup> ±0.03	68.12 <sup>d</sup> ±0.03	68.79 <sup>e</sup> ±0.03
S <sub>3</sub>	2289.67 <sup>d</sup> ±0.03	1892.33 <sup>d</sup> ±0.03	69.98 <sup>f</sup> ±0.15	70.60 <sup>g</sup> ±0.03
R <sub>1</sub>	3240.33 <sup>e</sup> ±0.05	1929.42 <sup>f</sup> ±0.03	65.32 <sup>b</sup> ±0.04	69.76 <sup>f</sup> ±0.02
R <sub>2</sub>	3465.44 <sup>h</sup> ±0.03	1987.48 <sup>h</sup> ±0.03	70.23 <sup>g</sup> ±0.03	72.33 <sup>h</sup> ±0.03
R <sub>3</sub>	3585.27 <sup>j</sup> ±0.05	2060.33 <sup>j</sup> ±0.04	75.12 <sup>j</sup> ±0.05	77.95 <sup>k</sup> ±0.05
G	3664.42 <sup>k</sup> ±0.03	2118.05 <sup>k</sup> ±0.02	79.22 <sup>k</sup> ±0.03	75.96 <sup>j</sup> ±0.03
C	2210.09 <sup>a</sup> ±0.02	1712.54 <sup>a</sup> ±0.03	60.04 <sup>a</sup> ±0.01	36.74 <sup>a</sup> ±0.02

Data are mean values of triplicate determination ± standard deviation.

Means within a row with different superscript are significantly different ( $p \leq 0.05$ ).

Table 3: Anti-nutrients content of cashew flours (mg/kg)

Sample	Phytate	Oxalate	Tannin
A <sub>1</sub>	69.41 <sup>i</sup> ±0.02	7.12 <sup>h</sup> ±0.02	6.48 <sup>f</sup> ±0.06
A <sub>2</sub>	57.12 <sup>e</sup> ±0.02	6.93 <sup>g</sup> ±0.02	6.23 <sup>e</sup> ±0.02
A <sub>3</sub>	53.94 <sup>b</sup> ±0.04	6.60 <sup>f</sup> ±0.04	6.09 <sup>d</sup> ±0.02
S <sub>1</sub>	65.86 <sup>h</sup> ±0.05	9.24 <sup>j</sup> ±0.03	7.14 <sup>i</sup> ±0.02
S <sub>2</sub>	59.26 <sup>f</sup> ±0.02	7.54 <sup>i</sup> ±0.02	6.94 <sup>f</sup> ±0.02
S <sub>3</sub>	55.92 <sup>d</sup> ±0.03	6.16 <sup>c</sup> ±0.05	6.73 <sup>g</sup> ±0.03
R <sub>1</sub>	70.43 <sup>j</sup> ±0.02	6.20 <sup>d</sup> ±0.04	6.48 <sup>f</sup> ±0.03
R <sub>2</sub>	62.54 <sup>g</sup> ±0.02	5.13 <sup>b</sup> ±0.02	6.03 <sup>c</sup> ±0.02
R <sub>3</sub>	50.27 <sup>a</sup> ±0.02	4.62 <sup>a</sup> ±0.03	5.73 <sup>b</sup> ±0.05
G	55.61 <sup>c</sup> ±0.03	6.38 <sup>e</sup> ±0.03	4.47 <sup>a</sup> ±0.04
C	99.33 <sup>k</sup> ±0.03	11.03 <sup>k</sup> ±0.02	10.14 <sup>j</sup> ±0.03

Data are mean values of triplicate determination ± standard deviation.

Means within a row with different superscript are significantly different ( $p \leq 0.05$ ).

The presence of anti-nutrients in human diets affects nutrient absorption in infants and adults. Phytate chelates metal ions such as calcium, magnesium, zinc, copper and iron to form insoluble complexes that are not readily absorbed from the gastrointestinal tract. Its effect on mineral elements, especially in the case of iron is intense as even small amounts of phytic acid inhibit iron absorption in biological systems (Hurrell et al., 1992). Iron occurs in two forms in foods, as haem and as non-haem iron. Haem iron, which is present as haemoglobin and myoglobin, is absorbed directly as the intact iron porphyrin complex. Haem iron is well-absorbed (15-35%) and little influenced by physiological or dietary factors. The absorption of non-haem iron varies widely and is influenced by dietary components and iron status of the individual (Monsen et al., 1978). In essence, different methods to reduce phytic acid during food preparation will have a positive effect on iron absorption. However, it is important to note that ascorbic acid has been shown to increase iron absorption and to at least partly overcome the inhibiting effect of phytic acid (Hallberg, Brune, & Rossander, 1989). The mechanism of this effect is thought to be due to the prevention of the formation of insoluble iron compounds not available for absorption, and due to the reduction of ferric to ferrous iron (Hallberg et al., 1989). Tannin has been implicated in the formation of complexes with protein and minerals thereby limiting their availability. The toxicity effects of the tannin may not be significant since the total acceptable tannic acid daily intake for a man is 560 mg (Gemedé & Fekadu, 2014). Similarly, anti-nutritional activity of oxalates lies in their ability to form complexes with metals like calcium, zinc, magnesium and iron making them unavailable for absorption. However, it is imperative to note that the risk of calcium deficiency due to the consumption of oxalate-rich plants has been reported to be minimal as humans are able to efficiently use very low amounts of calcium in food (Reddy, Balakrishnan, & Salunkhe, 1978). Moreso, the concentrations of oxalate reported in this study are unlikely to pose toxicity problems to man since it is below the toxic levels of 2-5g (Oke, 1969). Generally, the amount of anti-nutrients

in nuts is highly variable; the levels found in a specific food probably depends on growing conditions, harvesting techniques, processing methods, testing methods and even the age of the food being tested (Bello, Salami-Jaji, Sani, Abdulhamid, & Fakai, 2013).

The mineral bioavailability of raw and treated flours is presented in Table 4. Phytic acid has six strongly dissociated protons (pK<sub>n</sub> 1.1-2.9) and six weaker dissociated protons (pA<sub>a</sub> 4.6-12.0) as earlier reported by Cosgrove and Irving (1980). Phytate chelates metal ions to form insoluble complexes. Phytate works in a broad pH-region (2.0-12.0) as reported by De Carli, Rosso, Schnitzler, and Carneiro (2006). Therefore, at pH values that normally occur in foods and under physiological conditions (pH 1 to 10); phytic acid being negatively charged has the potential to bind cations or positively charged divalent and trivalent mineral ions such as Zn<sup>2+</sup>, Fe<sup>2+/3+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup> and Cu<sup>2+</sup> (Fredlund, Isaksson, Rossander-Hulthen, Almgren, & Sandberg, 2006). The pH is also an important factor influencing the solubility of phytate, it being more soluble at lower than at higher pH values (Torre, Rodriguez, & Saura-Calixto, 1991). The molar ratio of phytate to iron, calcium, magnesium and zinc were reduced when treatments (soaking, autoclaving, roasting and germination) were applied. The molar ratio of phytate to zinc and calcium were far below the recommended maximum value 15 and 0.24 in all the flour samples indicating better bioavailability for absorption. Similarly, raw and treated cashew flours had phytate to iron ratio within the recommended value of less than 1 (Walingo, 2009). As with iron, zinc, and calcium, it is assumed that magnesium-phytic acid complexes are formed in the intestine, which are insoluble at a pH > 6 (Champagne, 1988) and thus are not absorbable. However, the stability of the magnesium-phytic acid complex is weaker than phytic acid complexes with iron, copper, and zinc (Vohra, Gray, & Kratzer, 1965). Any significant effect, due to the reduction in the level of anti nutrients in these samples, should be expected in the bioavailability of mineral elements. Therefore, the reduction achieved for the anti-nutrient in treated samples when compared to raw sample could be directly

Table 4: Mineral bioavailability of cashew flours

Sample	Phytate: Fe molar ratio	Phytate: Ca molar ratio	Phytate: Mg molar ratio	Phytate: Zn molar ratio
A <sub>1</sub>	0.08 <sup>ab</sup> ±0.00	n.d	n.d	0.12 <sup>d</sup> ±0.00
A <sub>2</sub>	0.07 <sup>a</sup> ±0.00	n.d	n.d	0.08 <sup>ab</sup> ±0.00
A <sub>3</sub>	0.07 <sup>a</sup> ±0.00	n.d	n.d	0.07 <sup>a</sup> ±0.00
S <sub>1</sub>	0.08 <sup>ab</sup> ±0.00	n.d	n.d	0.10 <sup>c</sup> ±0.00
S <sub>2</sub>	0.07 <sup>a</sup> ±0.00	n.d	n.d	0.08 <sup>ab</sup> ±0.00
S <sub>3</sub>	0.06 <sup>a</sup> ±0.00	n.d	n.d	0.08 <sup>ab</sup> ±0.00
R <sub>1</sub>	0.09 <sup>b</sup> ±0.00	n.d	n.d	0.10 <sup>c</sup> ±0.00
R <sub>2</sub>	0.08 <sup>ab</sup> ±0.00	n.d	n.d	0.09 <sup>b</sup> ±0.00
R <sub>3</sub>	0.06 <sup>a</sup> ±0.00	n.d	n.d	0.06 <sup>a</sup> ±0.00
G	0.06 <sup>a</sup> ±±0.00	n.d	n.d	0.07 <sup>a</sup> ±0.00
C	0.14 <sup>c</sup> ±±0.00	n.d	n.d	0.27 <sup>e</sup> ±0.00

n.d - not detected.

Data are mean values of triplicate determination ± standard deviation.

Means within a row with different superscript are significantly different (p≤0.05).

Table 5: Functional properties of cashew flours

Sample	Bulk density (g/mL)		WAC (mL/100g)	OAC (mL/100g)
	Loose	Packed		
A <sub>1</sub>	0.45 <sup>b</sup> ±0.01	0.63 <sup>d</sup> ±0.01	90.00 <sup>b</sup> ±0.15	166.00 <sup>e</sup> ±0.17
A <sub>2</sub>	0.45 <sup>b</sup> ±0.01	0.60 <sup>c</sup> ±0.01	94.00 <sup>c</sup> ±1.15	182.00 <sup>g</sup> ±1.15
A <sub>3</sub>	0.45 <sup>b</sup> ±0.02	0.58 <sup>b</sup> ±0.01	100.00 <sup>d</sup> ±0.45	200.00 <sup>h</sup> ±0.28
S <sub>1</sub>	0.45 <sup>b</sup> ±0.01	0.55 <sup>a</sup> ±0.03	96.00 <sup>cd</sup> ±0.17	173.00 <sup>f</sup> ±0.14
S <sub>2</sub>	0.44 <sup>b</sup> ±0.01	0.55 <sup>a</sup> ±0.01	102.00 <sup>d</sup> ±1.15	207.00 <sup>i</sup> ±1.15
S <sub>3</sub>	0.44 <sup>b</sup> ±0.03	0.55 <sup>a</sup> ±0.02	110.00 <sup>e</sup> ±0.14	225.00 <sup>j</sup> ±0.21
R <sub>1</sub>	0.49 <sup>c</sup> ±0.01	0.57 <sup>b</sup> ±0.01	90.00 <sup>b</sup> ±0.14	137.00 <sup>b</sup> ±0.15
R <sub>2</sub>	0.49 <sup>c</sup> ±0.01	0.60 <sup>c</sup> ±0.01	107.00 <sup>e</sup> ±1.15	146.00 <sup>c</sup> ±1.15
R <sub>3</sub>	0.53 <sup>d</sup> ±0.02	0.66 <sup>e</sup> ±0.02	110.00 <sup>f</sup> ±0.07	150.00 <sup>d</sup> ±0.14
G	0.49 <sup>c</sup> ±0.01	0.61 <sup>c</sup> ±0.02	95.00 <sup>c</sup> ±0.35	165.00 <sup>e</sup> ±0.21
C	0.40 <sup>a</sup> ±0.01	0.65 <sup>e</sup> ±0.01	87.00 <sup>a</sup> ±0.11	130.00 <sup>a</sup> ±0.12

WAC-Water absorption capacity; OAC- Oil absorption capacity.

Data are mean values of triplicate determination ± standard deviation.

Means within a row with different superscript are significantly different (p≤0.05).

related to this improved availability. In essence, bioavailability of nutrients in processed food products could be enhanced when they contain minimal amount of residual anti nutritional factors. This is of great concern in Community Nutrition and Emergency Feeding Programmes in developing countries where the consequence of anti-nutritional factors may worsen incidence of malnutrition and disease among infants and other vulnerable groups.

The functional properties of raw and treated flours are as shown in Table 5. Determination of functional properties of food ingredient is essential for the development of different food products especially for children. Processing methods had significant effect ( $p \leq 0.05$ ) on the functional parameters under consideration. The loosed bulk density value ranged from  $0.44 \pm 0.01$  to  $0.53 \pm 0.02 \text{ g/mL}$ . Roasting significantly ( $P \leq 0.05$ ) increased the loosed bulk density of cashew flour. Sample  $R_3$  had the maximum value, while the control sample had the least. The increase in loose density of the flour sample as a result of soaking and germination could be attributed to enzymatic activities. The packed bulk densities of the flour samples ranged from  $0.55 \pm 0.01$  to  $0.66 \pm 0.02 \text{ g/mL}$ . The reduction in packed density observed in germinated sample might have been as a result of decrease in weight of the flour owing to the breakdown of complex denser compounds inherent in cashew kernel into simpler ones (Gernah, Ariahu, & Ingbian, 2011). Similarly, Ihemeje et al. (2015), in earlier studies, observed a reduction in bulk density of germinated walnut compared to raw sample. The decrease in packed density of the flour sample, as a result of soaking, could also be attributed to enzymatic activities. The high volume per gram of flour material is important in relation to its packaging. It is desirable to have high bulk density in that it offers greater packaging advantage, as greater quantity may be packed within a constant volume (Adepeju, Gbadamosi, Adeniran, & Omobuwajo, 2011). Moreso, higher bulk density is important factor in convalescent child feeding.

Water absorption capacity (WAC) of flour samples ranged from  $87.0 \pm 0.11$  to  $110.0 \pm 0.14 \text{ mL/100g}$ . Water absorption capacity of food product is an index of the maximum

amount of water the product absorbs and retains and it is important to soften and increase digestibility (Ijarotimi et al., 2012). Differences in WAC of raw and treated flour samples could be, as a result of differences in the content of polar amino acid residues of proteins or charged side chains, which have an affinity for water molecules (Jitngarmkusol, Hongsuwankul, & Tananuwong, 2008). The oil absorption capacities (OAC) of cashew flours were in the range of  $130.0 \pm 0.12$  to  $225.0 \pm 0.21 \text{ mL/100g}$ . The oil absorption capacity values were generally higher than those of the water absorption capacity. Evidence on oil absorption of the samples indicate that there was significant ( $p \leq 0.5$ ) difference in the values obtained in raw and treated samples. Autoclaving and roasting significantly increased the oil absorption compared to the control. The increased oil absorption capacity of heat-processed samples may be due to the denaturation and dissociation of their constituent proteins that occur on heating which unmasks the non-polar residues from the interior of the protein molecule (Odoemelam, 2005). Similarly, germination improved the oil absorption capacity of cashew flour compared to the raw flour. The increase in oil absorption capacity of germinated sample may be attributed to the increased activity of lipolytic enzymes, which produce more free fatty acids during sprouting (Ihemeje et al., 2015).

## 4 Conclusion

The present study has demonstrated that processing of cashew kernel by soaking, roasting, germination or autoclaving had significant adjustments in the nutrient density and functional properties. Various processing methods significantly decreased the levels of phytate, oxalate and tannin with corresponding improvement in mineral bioavailability. Germination shows potential, to generate not only much needed nutrients in cashew for human, but also improved bioavailability of nutrients and functionality compared to other processing methods.

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# Use of Response Surface Methodology (RSM) for Composite Blends of Low Grade Broken Rice Fractions and Full-fat Soybean Flour by a Twin-screw Extrusion Cooking Process

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## Abstract

In this study, seventeen (17) composite blends of broken rice fractions and full-fat soybean, formulated using response surface methodology and central composite design within a range of barrel temperatures (100–140°C), initial feed moisture content (15–25%) and soybean composition (8–24%), were extruded with a twin-screw extruder and the expansion and color indices were optimized. The results indicated a significant ( $p < 0.05$ ) effect of extrusion conditions on the responses. Fitted predictive models had coefficients of 88.9%, 95.7%, 97.3%, 95.4% and 95.2%, respectively, for expansion index, bulk density, lightness, redness and yellowness. The p-value and lack-of-fit tests of the models could well explain the observed variability and therefore could be used to establish production setting for the twin-screw extruder. The optimum extrusion conditions were found to be 130 °C (barrel temperature), 20% (feed moisture level) and 23% feed soybean composition and optimum responses in terms of bulk density, expansion index, lightness, redness and yellowness chroma indices were 0.21 g cm<sup>-3</sup>, 128.9%, 17.1, 3.13 and 24.5, respectively. This indicates that optimum conditions can be established in twin-screw extrusion cooking of broken rice fractions and full-fat soybean composite blends that can result in product of low bulk and maximum expansion with a satisfactory light yellow product color that can be used to produce products that valorize broken rice and reduce qualitative postharvest loss.

**Keywords:** Broken rice; Soybean; Extrusion; Response surface methodology; Optimization

## 1 Introduction

Rice (*Oryza sativa* L.) is an important source of food for a large number of the population in sub-Saharan Africa (SSA), its production and utilization is an important economic program for most governments in the region. Though production does not match consumption demand and there are huge import bills at the detriment of other critical sectors, significant improvement has been

made over the last few years in increasing yield per ha in most countries. But little improvement has been recorded in the postharvest value chain of the production system (Manful, Quaye, & Gayin, 2004; Ndindeng et al., 2015). Recently, research investment for the reduction of postharvest losses has resulted in increased interest in the utilization of rice flour from low grade broken rice fractions for the production of value added products (Chinma, Anuonye, Simon, Ohiare, &

## Nomenclature

QPL	Qualitative postharvest loss	L*	Lightness
SSA	sub-Saharan Africa	a*	Redness
RSM	Response surface methodology	b*	Yellowness
CCRD	Central composite rotatable design	D	Deviations
EC	Extrusion cooking	RD	Relative deviation
FARO	Federal agricultural research oryza	BRT	Barrel temperature
NCRI	National Cereals Research Institute	FMC	Feed moisture content
EI	Expansion index	FSC	Feed soybean composition
BD	Bulk density	DOE	Designed of experiment

Danbaba, 2015; Danbaba, Nkama, & Badau, 2016; Danbaba et al., 2017) mainly due to its high expansion during extrusion, ease of digestion, high calcium content, low glycemic index, low sodium, attractive white colour, low gluten, low allergenicity and low cost as it is primarily produced from low grade broken rice fractions (Danbaba et al., 2016; Kadan, Bryant, & Pepperman, 2003). However, rice is relatively low in protein (6-8 g/100 g db) and its amino acids limited by lysine (Chaiyakul, Jangchud, Jangchud, Wuttijumnong, & Winger, 2009). Interestingly, lysine content of rice is higher (3.5-40/16 g Nitrogen) than that of other common cereals such as corn, sorghum and millet and therefore can easily be improved nutritionally by the addition of high lysine food materials to attain 5.5 g/16 g N considered adequate for humans.

The utilization of soybean to complement cereal protein has been increasing rapidly in most parts of Africa. Soybean is a rich source of lysine; hence rice-soybean foods can provide balanced dietary protein. Uses of locally-grown high protein crops, and shelf stable and affordable recipes have been proposed for addressing protein-energy malnutrition (PEM) in developing countries (Asefa & Melaku, 2017); combining this with processing technologies that result in shelf stable, convenient and consumer accept-

able products has also been advocated (Iwe, Van Zuilichem, Ngoddy, & Ariahu, 2001) (Iwe, 2003). Extrusion cooking technology and blends of cereals and legumes have been used in several parts of Africa to produce products that are nutrient dense, consumer acceptable, with improved physical and functional qualities (Danbaba et al., 2017; Filli, 2011, 2016; Gogoi, Oswalt, & Choudhury, 1996). During extrusion, enzymes are partially or totally inactivated, anti-nutritional factors destroyed and microbial load significantly reduced thereby improving widespread uses of legumes (Abd El-Hady, Mostafa, El-Samahy, & El-Saies, 1998). In other parts of the world, the technology has been employed to produce foods such as breakfast cereals, snack foods, pasta products, extruded bread, modified starches, beverage powders, meat and cheese analogues, textured vegetable proteins and meat products (Abd El-Hady et al., 1998; Moore, Sanei, Hecke, & Bouvier, 1990). The process has high versatility, efficiency, low cost of operation, and high yields with relatively no waste generation.

But to maximize the use of EC in new food product development, it is important to be able to alter process conditions to produce consumer acceptable products, improved physical and functional properties, minimized cost of production by adopting low-cost raw materials, maintained

high quality standards and minimized losses during production (Chaiyakul et al., 2009). This can only be achieved by systematically selecting appropriate production conditions using designed experiments (DOE) (Abd El-Hady et al., 1998; Danbaba et al., 2016; Moore et al., 1990). Using DOE, Eggum, Juliano, Ibabao, and Perez (1986) and Asare, Sefa-Dedeh, Sakyi-Dawson, and Afoakwa (2004) studied the behaviour of low amylose rice flour blended with cowpea during extrusion cooking. Effects of protein content and extrusion parameters on sensory and physical properties of extruded high protein, glutinous rice-based snacks were reported by Chaiyakul et al. (2009) and Asare et al. (2004), while the effect of the addition of cowpea and groundnut on the quality of rice-based extrudates has also been reported (Asare et al., 2004). In relation to rice-soybean extrusion, Coutinho, Batista, Caliari, and Soares Junior (2013) studied the effect of moisture and extrusion temperature on broken rice blended with by-products of soybean processing, Marengo et al. (2016) and Garg and Singh (2010) used partially defatted soybean blended with rice to produce snacks, while Noguchi, Kugimiya, Haque, and Saio (1982) reported on the physical and chemical characteristics of extruded rice flour and soybean protein isolate. Omwamba and Mahungu (2014) developed extruded ready-to-eat snacks from blends of rice, sorghum and soybean. In the current study, effects of moisture, extrusion temperature and amount of full-fat soybean flour on the physical and other functional characteristics of twin screw extruded snacks were evaluated. Also, little or no information is available on the extrusion of full-fat soybean flour blended with broken parboiled rice fractions on physical indices of extruded snacks. Response surface methodology (RSM) and central composite design are mathematical techniques used for optimizing process conditions where multiple independent variables influence the response value. In food processing, the main objective of using this DOE is to locate more precise combinations of the process variables that will have the highest probability of producing food products of desired quality and also to describe interrelations between the independent variables and product quality (Colonna, 1989; Nwabueze, 2007) for sustainable quality as-

surance.

Little work regarding the effects of extrusion temperature, initial feed moisture content and ratio of broken parboiled rice fractions to full-fat soybean blends on the physical and chemical properties of rice-extruded foods has been reported. Therefore, the objective of the current study is to produce extruded foods from blends of low grade broken parboiled rice fractions and full-fat roasted soybean flour, optimize the process parameters of twin-screw extruder parameters and to develop and validate mathematical models for the relationship between the response variables and the process variables using designed experiments. We believe that this will provide new sources of cheap and available raw materials, reduce qualitative rice postharvest loss and provide nutritious convenient foods for mitigating protein-energy-malnutrition in SSA.

## 2 Materials and Methods

Broken fractions of parboiled improved high amylose (28.97%) rice variety (Federal Agricultural Research *Oryza* - FARO 52) and full-fat soybean (TGX-1448-2E) flour were obtained from the National Cereals Research Institute, (NCRI) Badeggi, Niger State, Nigeria. The materials were manually sorted, washed and sun dried ( $32 \pm 2$  °C) for 6 h to  $10.11 \pm 0.08$  and  $9.41 \pm 0.04$  (db) moisture content, respectively, before storing under refrigeration until required for analysis. Seventeen (17) rice-soybean formulations containing between 14 to 28 kg/100 kg soybean were mixed and moisture content adjusted (Danbaba et al., 2016) to between 15 and 25 g/100 g (db) (Table 2) by adding a given quantity of water to each sample and mixed through in a laboratory mixer for 5 min before standing for 3hr prior to extrusion at specified temperatures (Table 2). Preliminary experiments were conducted by extruding 8 samples with moisture and soybean contents ranging between 11.0 and 30% and 4 and 30%, respectively, and extruded at a temperature of 70 to 140 °C. Extruded samples that were not burnt and had expanded appreciably were selected and their processing conditions were used for the main experiments. Extrusion cooking was carried out in a twin-screw

extruder (Model - SLG 65, Jinan Saibaino Technology Development Co. Ltd, China) powered with a 16 HP electric motor, screw speed of 300 rpm and length to diameter ratio of barrel (20:1).

## 2.1 Experimental design and Extrusion Cooking

Response surface methodology (RSM) in a five-level  $(-\alpha, -1, 0, 1, +\alpha)$  central composite rotatable design (CCRD) of three variables ( $X_1$  = barrel temperature,  $X_2$  = feed moisture content,  $X_3$  = feed soybean composition) was used (Table 1) where  $\pm\alpha$  is equal to 1.682 and defines the distance of the axial runs from the design centre (Montgomery, 2001). The experimental runs consisted of 17 trials (8 cube points, 6 star points and 3 centre points). The expansion (EI), bulk density (BD), and colour characteristics ( $L^*$ ,  $a^*$ ,  $b^*$ ) were set as the response variables ( $y_1, y_2, y_3, y_4$ , and  $y_5$ ), respectively. Experimental data were fit to a second order polynomial response surface model, equation 1.

$$Y = b_0 + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n b_{ij} x_i x_j + e_i \quad (1)$$

where  $Y$  is any response variable;  $n$  is the number of independent variables;  $x_i$  and  $x_j$  are coded variables;  $b_a$  is the intercept;  $b_i$ ,  $b_{ii}$  and  $b_{ij}$  are the linear, quadratic and interaction effects, respectively, and  $e_i$  is the error term. The goodness of fit of the models were measured by considering the coefficient of determination ( $R^2$  and Adjusted- $R^2$ ), the adequacy of the quadratic models was confirmed by the analysis of variance (ANOVA) using Fishers test value (F-value) and lack-of-fit test. For statistical analysis, the natural variables values ( $X_i$ ) were transformed to coded forms according to equation 2.

$$x_i = \frac{x_i - x_0}{\delta x} \quad (2)$$

Where  $X_i$  = coded (dimensionless) value of the independent variables,  $X_0$  = the value of  $X_i$  at the centre point and  $\delta_i$  = the step change in  $X_i$  and the results are presented in Table 2. Optimization of process variables was based on the maximum values of all the responses (EI, BD, and  $L^*$ ,

$a^*$ ,  $b^*$ ). Responses were individually assigned to different importance levels varying between 1 and 5 and optimized based on higher desirability for expansion index,  $L^*$ ,  $b^*$  and lower desirability for bulk density and  $a^*$ . The deviations (D) and relative deviations (RD) between the experimental values and those predicted by the models for the responses at the optimal condition were calculated by equations 3 and 4, respectively.

$$\text{Deviation (D)} = Y - \Upsilon \quad (3)$$

$$\text{Relative deviation (RD)} = \frac{D}{\Upsilon} \times 100 \quad (4)$$

where  $Y$  is the experimental data and  $\Upsilon$  is the response predicted by the model.

## 2.2 Moisture content determination

The moisture content of feed materials and extruded samples was determined using the dry oven method with accuracy of about four digits (0.0000 g/ g).

## 2.3 Bulk density (BD) and Expansion Index (EI)

Extruded samples were analysed for EI by using the mean diameter of 10 readings and ER calculated as the ratio of diameter of the extrudates and diameter of extruder die (3mm) (Alvarez-Martinez, Kondury, & Harper, 1988). After measuring the weight per unit length of the extruded samples, the BD was calculated using the relationship:

$$\text{BD} = \frac{4}{\pi d^2 L} \quad (5)$$

where: BD = Bulk density ( $\text{g cm}^{-3}$ ),  $d$  = Diameter of the extrudate (cm),  $L$  = Length per gram of the extrudate ( $\text{cm g}^{-1}$ ).

## 2.4 Color characteristics determination

Colors of the extruded foods were measured with a Monilta Color Reader (CR-10, Minolta Co. Ltd., Tokyo, Japan) using the  $L^*$ ,  $a^*$ ,  $b^*$  system, where the  $L^*$  value is a measure of product



Table 1: Codes, ranges and levels of independent variables in RSM design

Levels of variables	Barrel Temperature (°C, A)	Feed Moisture content (g 100g <sup>-1</sup> sample, B)	Feed Composition (g 100g <sup>-1</sup> sample, C)
- $\alpha$ (-1.68)	86.36	11.6	2.6
Low (-1)	100.0	15.0	8.0
Medium (0)	120.0	20.0	16.0
High (1)	140.0	25.0	24.0
+ $\alpha$ (+1.68)	153.6	28.4	29.5

Level of each variable was established based on preliminary extrusion trials. The distance of the axial points from the center point was  $\pm 1.68$ , and calculated from Equation  $\alpha = (2n)^{1/4}$  where n is the number of variables.

lightness, a chromatic color attribute of absolute black (0) to absolute white (100);  $a^*$  value means mixed red-to-green color of samples ranging from negative on the red side to positive on the green side ( $a^+ = 0 - 60$  for red and  $a^- = 0 - (-60)$  for green); and the  $b^*$  value means mixed blue-to-yellow colour of samples ranging from negative to positive value ( $b^+ = 0 - 60$ ) for yellow and  $b^- = 0 - (-60)$  for blue.

## 2.5 Statistical analysis

Homogeneous variance is a necessary prerequisite for linear regression model development (Filli, 2011). Therefore, to reduce the variability within the response variables, the natural variable values ( $X_i$ ) were transformed to coded forms according to equation 2. For the standardized data, one-way analysis of variance (ANOVA) was carried out to determine significant differences ( $p \leq 0.05$ ) among the treatment combinations using response surface analysis of Minitab 16 software. Fishers Least Significance Difference (LSD) was used to check equality of variances. Results are presented as mean of three replications.

## 3 Results and Discussion

### 3.1 Experimental results of extrusion exercise

Based on the seventeen (17) composite blends of broken rice fractions and full-fat soybean flours, formulations were grouped into 5 based on the

extrusion temperature, 86.0 °C (1), 100 °C (4), 120 °C (7), 140 °C (4) and 154 °C (1) (Table 2). These extrusion temperatures were selected based on preliminary exercise (results not presented). Digital images showing physical characteristics of snacks as they emerged from the die at steady state at each experimental condition are presented in Fig. 1 and internal macro structure in Fig. 2. There were great variations in physical appearance in terms of size, colour and puff levels and surface smoothness, therefore indicating variability due to differences in extrusion conditions. They were mostly in the form of cylindrical puffed strands with different sizes and surface textures. Samples containing lower moisture content (8%) and extruded at temperatures above 100 °C had slightly expanded size and rougher surface than those extruded at higher moisture contents. As the moisture level increased to 24%, strands became thinner and smoother surfaced. These observations are similar to those reported by Nwabueze (2007), that thin-smooth to thin-fine-smooth extrudates were obtained when African bread fruits blended with soybean and yellow maize were extruded at a higher moisture level. These indicate the need to systematically define optimum moisture level for the extrusion of rice-full-fat soybean blends for optimum product quality.

The extrudates also had varying honeycomb cellular structure of different numbers and sizes; with samples 2, 5, 8, 10, 11, 12, 13 and 14 showing more and larger spaces (Fig. 2). Structurally, starch-based extruded foods have been reported to have a honeycomb cellular structure

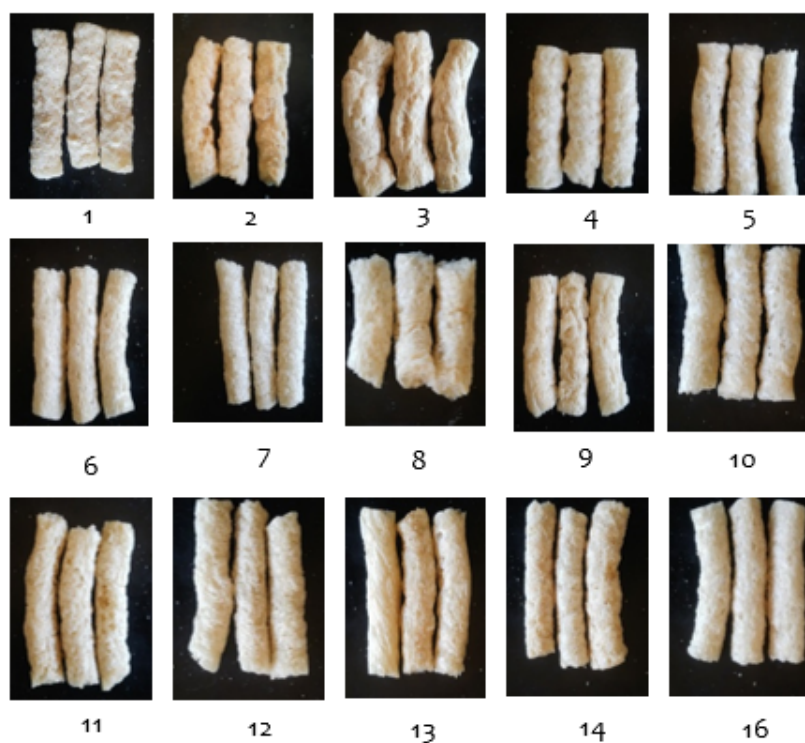


Figure 1: Digital images of expanded rice-full fat soy blends extruded at different conditions

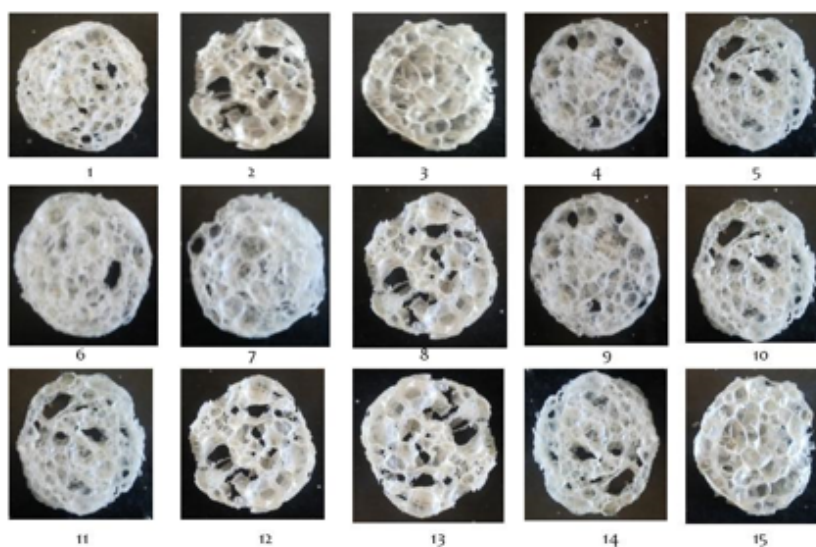


Figure 2: Cross section showing void spaces in rice-full fat soy blends extruded at different conditions

(Faubion & Hosney, 1982; Lai, Guetzlaff, & Hosney, 1989) and the size and number of the cells formed have been reported to be dependent on the feed moisture content and the composition of protein and lipids in feed material, as demonstrated in this study. Similar reports were put forward by Faubion and Hosney (1982), Lai et al. (1989), Lai and Kokini (1991).

In the reports of Agbisit, Alavi, Cheng, Herald, and Trater (2007), Yagci and Gogus (2008), it had been demonstrated that variation of feed moisture content and temperature during extrusion cooking affected the degree of expansion through transformation of the extrusion matrix, leading to differences in void space formation of puffed products. From the observed cross section of the extrudates, it could be inferred that samples 2, 5, 8, 10, 11, 12, 13 and 14 would have low breaking strength, high crispiness and better sensory acceptability.

Varying physical appearance of the extrudates in terms of color (Fig. 1) is an indication of varying response of the different formulations to different extrusion conditions and therefore clearly defines the need to locate appropriate processing conditions to have products of acceptable color. Though change in total reducing sugar variation during the process is not reported, variation in surface color of the different extruded samples may be attributed to browning reactions and pigment degradation that take place during the process (Altan, McCarthy, & Maskan, 2008).

### 3.2 Effects of individual variables and interaction on responses

#### Bulk density

Table 3 shows the mean effects of the barrel temperature, initial feed moisture content and feed soybean composition on the BD of extruded rice full fat soybean. The experimental values for BD range between 0.035 and 0.358 g cm<sup>-3</sup> with a mean value of 0.098 g cm<sup>-3</sup>. The deviation which measures the difference between the observed value and the predicted value, ranged between 0.021 and 0.329 g cm<sup>-3</sup> and the relative deviation was 60.0 and 115.28% indicating wide variation between different treatments in terms of BD. It

may be observed from the results that the BD increased as the feed moisture content decreased from 25 to 15 g/100 g sample in runs 3 and 5, respectively, while relative deviation, which indicates percentage deviation of the observed value from the predicted value, was highest (115.28%) at run 6 and lowest (60.0%) at run 3. Our observations coincide with those reported by Kulkarni and Joshi (1992) and Garg and Singh (2010) that there is a strong correlation between initial feed moisture content and BD of extrudates. As the value of the initial moisture content increased, the value of BD decreased and vice versa. Chang and El-Dash (2003) and Leonel, de Freitas, and Mischán (2009) also reported similar findings for extruded cassava starch. They reported that moisture content of 24-26% as observed in this study, followed by barrel temperature of 120-200 °C, resulted in a significant effect on expansion and therefore bulk density. At low moisture content, there is an increased shear force in the extruder barrel which increases residence time and gelatinization of starch and subsequently increases bulk density, as observed in these results. BD is an important quality parameter in the production of extruded products as it indicates expansion at the die in all directions. In this study, barrel temperature and feed soybean content did not show significant impact on the BD (Table 3) even though several authors (Case, Hamann, & Schwartz, 1992; Hernandez-Nava, Bello-Perez, San Martin-Martinez, Hernandez-Sanchez, & Mora-Escobedo, 2011) reported that BD value decreases when the extrusion temperature is increased due to aggravated starch gelatinization. The lack of agreement between this study and earlier reports may be due to high temperature and possible contribution of soybean oil content to the flow of the matrix in this study. Minimal addition of soybean flour was also observed to affect BD. At 8 g/100 g sample of soybean flour in a blend processed at high moisture content, the highest BD was recorded (Table 3). These observations may be attributed to the behaviour of protein and oil in the formulations, since friction and shear forces during extrusion may cause extensive interlacing between protein molecules and lead to their texturization, and because high protein content promotes denser and more rigid extrudates (Hernandez-Nava et al.,

Table 2: Outline of experimental design with design points, coded and uncoded values of independent variables

Runs	Points	Coded variables			Natural variables		
		A	B	C	BRT	FMC	FSC
Group 1 (100°C extrusion temperature)							
1	Factorial point	-1	-1	-1	100	15	8
3	Factorial point	-1	1	-1	100	25	8
5	Factorial point	-1	-1	1	100	15	24
7	Factorial point	-1	1	1	100	25	24
Group 2 (140°C extrusion temperature)							
2	Factorial point	1	-1	-1	140	15	8
4	Factorial point	1	1	-1	140	25	8
6	Factorial point	1	-1	1	140	15	24
8	Factorial point	1	1	1	140	25	24
Group 3 (120°C extrusion temperature)							
11	Star point	0	-1.68	0	120	11.6	16
12	Star point	0	1.68	0	120	28.4	16
13	Star point	0	0	-1.68	120	20	2.6
14	Star point	0	0	1.68	120	20	29.5
15	Centre point	0	0	0	120	20	16
16	Centre point	0	0	0	120	20	16
17	Centre point	0	0	0	120	20	16
Group 4 (86°C extrusion temperature)							
9	Star point	-1.68	0	0	86.4	20	16
Group 5 (154°C extrusion temperature)							
10	Star point	1.68	0	0	153.6	20	16

BRT = barrel temperature °C, FMC = feed moisture content (%), FSC = feed soybean composition,

A = Barrel temperature, B = Feed moisture content, C = Feed composition. Duplicate runs were carried out at all design points and averages recorded. The experimental runs were randomized.

2011; Jorge et al., 2008).

## Expansion index

Generally, high expansion index is a desirable property in puffed extruded foods as this typically indicates a lighter, crispier product. Expansion index has been shown to be dependent on extrusion conditions as well as blend formulations (Maskus & S., 2015) significant factor affecting expansion ratio (Ding, Ainsworth, Tucker, & Marson, 2005). In this study, the EI increased from 125.7% at 100 °C barrel temperature to 133.80% when the temperature increased to 120 °C with concurrent reduction in initial feed moisture content (Table 3). Hagenimana, Ding, and Fang (2006) reported reduction in EI

as the moisture level increased from 16% to 22% in a twin-screw extrusion of rice flour. In our work similar observations were seen, increased EI was observed as a moisture content reduced from 25 to 20%, indicating a narrow range of moisture is required for optimum product expansion of rice flour (between 16 and 22%). Relative deviation ranging between 99.61 and 100.31% (Table 3) is also an indication of a narrow range in percentage variability between observed and predicted values. High moisture level reduces shear strength and energy input to the material resulting in decreased moisture evaporation at the die exit and therefore negatively affects expansion. In our study the use of soybean flour increased the protein level and therefore reduced the starch level in the blend which may have impeded full starch

Table 3: Mean experimental, predicted and relative deviation results for bulk density and expansion index of extruded broken-rice full-fat soybean formulations

Runs	Bulk density (g cm <sup>-3</sup> )			Expansion index (%)		
	Y	D	RD	Y	D	RD
1	0.089	0.092	103.37	127.8	128.2	100.31
2	0.058	0.041	68.97	131.1	130.7	99.69
3	0.035	0.021	60.00	125.7	125.8	100.08
4	0.156	0.162	103.85	130.9	130.8	99.92
5	0.358	0.329	91.90	130.7	130.8	100.08
6	0.072	0.083	115.28	129.9	129.8	99.92
7	0.075	0.081	108.00	131.7	131.6	99.92
8	0.039	0.028	71.79	132.7	132.9	100.15
9	0.142	0.146	102.82	128.8	128.3	99.61
10	0.059	0.059	100.00	131.5	131.5	100.00
11	0.147	0.151	102.72	128.9	128.9	100.00
12	0.048	0.045	93.75	129.5	129.5	100.00
13	0.058	0.062	106.90	129.8	129.8	100.00
14	0.154	0.148	96.10	133.8	133.8	100.00
15	0.059	0.058	98.31	133.5	133.5	100.00
16	0.058	0.057	98.28	133.7	133.8	100.07
17	0.059	0.056	94.92	133.6	133.8	100.15
Mean	0.098	0.095	95.11	130.8	130.8	100.00
SD	0.076	0.073	14.33	2.22	2.24	0.16
LSD	0.012	-	-	2.12	-	-

Y = experimental results, D = deviation which represent fitted value, RD = Relative deviation, SD = Standard deviation. Equation on which responses are predicted =  $\beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$ . Mean in the same column having differences greater than LSD are significantly different ( $p \leq 0.05$ ) according to Fishers Least Significance Test.

gelatinization with resultant reduction of expansion index from 133.8% to 125.7%, representing about a 6.05% reduction. Yu, Ramaswamy, and Boye (2013) and Devi et al. (2013) in separate studies reported a reduction in expansion ratio with the addition of protein. This result indicates that the EI of extruded products depends on starch gelatinization as an increasing starch-protein ratio leads to formation of a continuous starch matrix that affects its expansion as it exits the die.

### Color characteristics of extruded foods

In extruded starch-based puffed products, color is one of the important quality indicators.

Change in color may indicate extent of browning reactions such as Maillard reaction, caramelization, and degree of cooking and possibly pigment degradation that occurs during heat treatments (Altan et al., 2008; Danbaba et al., 2016). Assessment of the color components of the extruded samples showed that the value of the L\* component varied from 0.31 to 10.31 with a mean value of 5.80, as compared to 87.34 for the raw materials before extrusion, indicating a decrease in luminosity.

L\* value was highest at temperatures between 100 °C and 120 °C, feed moisture levels of 15 to 20% and soybean composition of 8 to 16% (Table 3). At moisture content less than 20% and extrusion temperature greater than 100 °C, there was



darkening of extruded starch mainly due to increased residence time and viscosity, i.e. lesser  $L^*$  value. Our results were in agreement with those of Gutkoski and El-Dash (1999), who reported that with initial moisture content of 17 to 24% and barrel temperature of 90 °C to 150 °C, luminosity decreased linearly with barrel temperature in an extruded oat product. The deviation range of 0.06 to 10.32 and relative deviation of 19.35 to 124.46% is an indication of variation among treatments in terms of this parameter.

The chroma  $a^*$  (redness) values ranged from 0.73 to 5.21, and the deviation and relative deviation were between 0.92, 59.13 and 81.3, 140.91% which indicates slight variability in this color parameter during extrusion cooking considering the observed value can vary from -60 to +60, and low RD indicates slight variability in percentage. The highest value occurred at a temperature range of 120 to 140 °C, feed moisture level of 15 to 20% and feed soybean content of 8 to 16% (runs 2 and 17) (Table 2 and 4) indicating the need to optimize the process variables to locate the most suitable point that will give acceptable quality in terms of this parameter. Responses of the  $b^*$  color index, which represents transition from blue to yellow on the chroma chart, varied according to the treatment (from 13.13 to 28.80) indicating that all samples had a yellow color. The increase of barrel temperature from 120 to 154 °C (runs 13 and 10) (Table 2 and 4) increased  $b^*$  color intensity at 20% moisture content and 2.6% soybean content of the feed materials. High moisture level resulted in brighter products since increase in moisture decreases the residence time, providing less non-enzymatic darkening of extruded products (Badrie & Mellowes, 1991).

### 3.3 Fitting and validating RSM models for optimizing production processes

RSM models to optimize the relationship between extrusion cooking variables and response variables in terms of expansion and color indices are outlined in the regression model equation 4-8. The coefficients with only one factor ( $X_1$ ,  $X_2$  and  $X_3$ ) represent the effect of that single factor, while those with two factors ( $X_1X_2$ ,

$X_1X_3$ ,  $X_2X_3$ ) are the interaction terms and  $X_1^2$ ,  $X_2^2$  and  $X_3^2$  are the square terms. Positive or negative coefficients indicate synergistic or antagonistic contributions to the observed responses. The results of ANOVA performed on the RSM models to assess the significance of the linear, quadratic and interactive effects of the independent variables on the dependent variables are presented in Tables 5.

From equation 6, it is clear that  $X_1$  and  $X_2$  negatively affected bulk density at both linear and quadratic levels, while only  $X_1X_3$  and  $X_2X_3$  reduced this response at the interaction level. All three factors positively impacted expansion at linear levels and negatively affected expansion index at square levels (equation 7). This demonstrated the inverse relationship between bulk density and expansion index of extrudate under linear and square processing conditions. Equation 8 indicated that increasing barrel temperature ( $X_2$ ) resulted in increased lightness at linear and interaction levels.  $X_1$  and  $X_3$  linearly affected redness positively and all the factors positively affected this factor at square levels (equation 9). Temperature only seemed to negatively affect the yellow color index at both linear and square levels, but interactions between  $X_1X_3$  and  $X_2X_3$  negatively impacted yellow color formation (equation 10).

$$\begin{aligned} \text{Bulk density (g cm}^{-3}\text{)} &= 0.110 - 0.027X_1 - 0.027X_2 + 0.029X_3 - 0.002X_1^2 - 0.007X_2^2 + \\ &0.001X_3^2 + 0.053X_1X_2 - 0.053X_1X_3 - 0.043X_2X_3 \\ (R^2 &= 0.957, R_{adj.}^2 = 0.946) \end{aligned} \quad (6)$$

$$\begin{aligned} \text{Expansion index} &= 1.324 + 0.012X_1 + 0.010X_2 + 0.014X_3 - 0.008X_1^2 - 0.004X_2^2 - 0.004X_3^2 + \\ &0.004X_1X_2 - 0.007X_1X_3 + 0.012X_2X_3 \\ (R^2 &= 0.889, R_{adj.}^2 = 0.860) \end{aligned} \quad (7)$$

$$\begin{aligned} \text{Lightness (L}^*\text{)} &= 16.773 - 2.021X_1 + 0.132X_2 - 0.447X_3 - 1.135X_1^2 + 0.173X_2^2 - 0.330X_3^2 + \\ &0.625X_1X_2 + 0.649X_1X_3 - 1.282X_2X_3 \\ (R^2 &= 0.973, R_{adj.}^2 = 0.966) \end{aligned} \quad (8)$$



Table 4: Mean experimental, predicted and relative deviation results for color characteristics of extruded broken-rice full-fat soybean formulations

Runs	Lightness (L*)			Redness (a*)			Yellowness (b*)		
	Y	D	RD	Y	D	RD	Y	D	RD
1	10.31	10.32	100.10	0.9	0.97	107.78	16.01	15.91	99.38
2	3.67	3.55	96.73	5.21	5.13	98.46	23.26	23.41	100.64
3	7.13	6.92	97.05	1.57	1.54	98.09	21.0	20.97	99.86
4	2.23	2.42	108.52	2.3	1.87	81.30	22.1	21.98	99.46
5	5.51	5.45	98.91	0.75	0.92	122.67	23.7	234.97	991.43
6	1.39	1.73	124.46	3.64	3.41	93.68	26.49	26.67	100.68
7	6.73	6.98	103.71	3.2	3.02	94.38	17.17	17.17	100.00
8	5.4	5.52	102.22	2.01	1.68	83.58	13.13	13.38	101.90
9	6.9	6.97	101.01	2.63	2.49	94.68	25.5	25.49	99.96
10	0.31	0.06	19.35	4.35	4.86	111.72	28.8	28.6	99.31
11	7.16	7.12	99.44	2.61	2.53	96.93	23.7	23.48	99.07
12	7.6	7.46	98.16	1.1	1.55	140.91	16.55	16.56	100.06
13	6.42	6.56	102.18	1.02	1.17	114.71	14.78	14.92	100.95
14	5.38	5.06	94.05	0.73	0.95	130.14	14.81	14.47	97.70
15	8.41	8.44	100.36	1.36	1.3	95.59	23.12	23.16	100.17
16	10.31	10.32	100.10	0.9	0.97	107.78	16.01	15.91	99.38
17	3.67	3.55	96.73	5.21	5.13	98.46	23.26	23.41	100.64
Mean	5.80	5.79	96.65	2.32	2.32	104.17	20.55	32.97	152.39
SD	2.84	-	-	1.53	-	-	4.75	-	-
LSD	1.04	-	-	0.21	-	-	2.02	-	-

Y = experimental results, D = deviation which represent fitted value, RD = Relative deviation, SD = Standard deviation.

Equation on which responses are predicted =  $\beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$ . Mean in the same column having differences greater than LSD are significantly different ( $p \leq 0.05$ ) according to Fishers Least Significance Test.

$$\begin{aligned} \text{Redness (a*)} &= 2.521 + 0.431X_1 - 0.162X_2 + \\ &0.051X_3 - 1.135X_1^2 + 0.368X_2^2 + 0.154X_3^2 + \\ &0.001X_1X_2 - 0.493X_1X_3 - 0.364X_2X_3 \\ &(R^2 = 0.954, R_{adj.}^2 = 0.942) \end{aligned} \quad (9)$$

$$\begin{aligned} \text{Yellowness (b*)} &= 22.060 + 0.0936X_1 - 0.170X_2 + \\ &1.770X_3 - 0.720X_1^2 + 0.602X_2^2 - 1.652X_3^2 + \\ &1.265X_1X_2 - 0.493X_1X_3 - 1.945X_2X_3 \\ &(R^2 = 0.952, R_{adj.}^2 = 0.939) \end{aligned} \quad (10)$$

It is clear from equations 4-8 and probability values (Table 5), that the bulk density, expansion indices and color parameters were all significantly ( $p < 0.05$ ) affected by varying barrel temperature, initial feed moisture content and feed

soybean composition as predicted by the models. The  $R^2$  and adjusted  $R^2$  values were  $R^2 = 0.957$ ,  $R_{adj.}^2 = 0.946$  for bulk density,  $R^2 = 0.889$ ,  $R_{adj.}^2 = 0.860$  (expansion index),  $R^2 = 0.886$ ,  $R_{adj.}^2 = 0.870$  (L\*),  $R^2 = 0.954$ ,  $R_{adj.}^2 = 0.942$  (redness) and  $R^2 = 0.952$ ,  $R_{adj.}^2 = 0.939$  (yellowness) (equation 4-8, respectively). The  $R^2$  and adjusted  $R^2$  defines the proportion of variability in the observed responses which are accounted for by the fitted models (Danbaba et al., 2016; Filli, 2011; Singh, Sekhon, & Singh, 2007), and the closer the values are to unity, the better the empirical model fits the experimental data (Lee & Wang, 1997), thus, the results of this study suggest that the models appropriately defined the process behaviour by explaining 94.6%, 86.0%, 87.0%, 94.2% and 93.9% of

Table 5: Analysis of variance (ANOVA) and lack-of-fit p-values, R2 and adjusted R2 and estimates of the coefficients of the second-order response surface

	Expansion index		Bulk density		Lightness (L*)		Redness (a*)		Yellowness (b*)	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
b <sub>0</sub>	1.324	0.000	0.110	0.003	16.773	0.000	2.521	0.022	22.06	0.017
Linear										
b <sub>1</sub>	0.012	0.001	-0.027	0.001	-2.021	0.002	0.431	0.000	0.094	0.002
b <sub>2</sub>	0.010	0.002	-0.027	0.002	0.132	0.000	-0.162	0.000	-2.093	0.000
b <sub>3</sub>	0.014	0.000	0.029	0.000	-0.447	0.000	0.051	0.001	-0.170	0.010
Quadratic										
b <sub>1</sub> <sup>2</sup>	-0.008	0.051	-0.002	0.001	-1.135	0.003	0.368	0.002	1.770	0.000
b <sub>2</sub> <sup>2</sup>	-0.004	0.000	-0.007	0.004	0.173	0.012	0.154	0.003	-0.720	0.000
b <sub>3</sub> <sup>2</sup>	-0.004	0.001	0.001	0.008	-0.330	0.017	0.001	0.021	-0.602	0.016
Interaction										
b <sub>1</sub> b <sub>2</sub>	0.004	0.002	0.053	0.027	0.625	0.001	-0.493	0.002	-1.652	0.000
b <sub>1</sub> b <sub>3</sub>	-0.007	0.001	-0.053	0.001	0.649	0.002	-0.364	0.000	-1.265	0.001
b <sub>2</sub> b <sub>3</sub>	0.012	0.001	-0.043	0.000	1.282	0.000	0.031	0.000	-1.945	0.003
R <sup>2</sup>	0.889	-	0.957	-	0.973	-	0.954	-	0.952	-
R <sup>2</sup> <sub>adjusted</sub>	0.860	-	0.946	-	0.966	-	0.942	-	0.939	-
Lack-of-fit	0.710	-	0.071	-	0.319	-	0.082	-	0.982	-

Model on which the coefficients were calculated:  $y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_1^2X_1^2 + b_2^2X_2^2 + b_3^2X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \epsilon$

the variations observed in bulk density, expansion index, lightness, redness and yellowness, respectively. Additionally, the p-values of lack-of-fit test for the models were not significant ( $p < 0.05$ ), which indicates that the models can be used for the prediction of the observed responses within the experimental domain studied. Yagci and Gogus (2008) were of the opinion that with significant probability values ( $p < 0.001$ ) and non-significant lack-of-fit tests, fitted second-order models could be adequately used as predictor models, regardless of low coefficients of determination. It is clear and appropriate therefore to conclude that the developed models adequately approximate the response variables considered in this study and can be used satisfactorily for the prediction of any value of the responses within the defined experimental range.

### Optimization process variables

In this study, simultaneous numerical optimization of the independent and dependent variables was conducted using a Minitab response optimizer. To meet the intended target requirement placed on each response variable and independent factor in this experiment, a goal was set for each independent and dependent variable. An-

uar, Adnan, Saat, Aziz, and Taha (2013), Gupta, Verma, Jain, and Jain (2014) and Danbaba et al. (2016) reported that for locating optimum conditions using numerical methods, there was a need to set goals, which may either be none, maximum, minimum, target or range for both response and independent variables that could be combined into one desirable function. In this work, the barrel temperature, feed moisture content and feed soybean composition were all set within range (100 to 140 °C; 15 to 25%; 8 to 24%) as defined by the preliminary study (Table 1), while the goal for the response variables of bulk density, expansion index and lightness chroma levels were set at maximum, and a\* and b\* set at minimum (Table 6). Anuar et al. (2013) reported that when goal was set at 5, the response target objective was to meet the objective of getting response at a maximum level.

Results of the numerical optimization therefore indicated that the optimum extrusion variables were 130 °C (barrel temperature), 20% (feed moisture level) and 23% (feed soybean composition) (Table 6), while optimum responses in terms of bulk density, expansion index, lightness, redness and yellowness indices were 0.21g cm<sup>-3</sup>, 128.9%, 17.1, 3.13 and 24.5, respectively. This indicates that the optimum conditions can be established in twin-screw extrusion cooking of low-

Table 6: Constraints and goals applied to locate optimum conditions for processing parameters and responses for rice-full-fat soybean based extruded foods

Variables	Goal	Lower limit	Upper limit	Importance	Optimum
Extrusion variables					
Barrel Temperature (°C)	In range	100	140	3	130
Moisture content (% w.b)	In range	8	24	3	20
Blend composition (%)	In range	15	25	3	23
Response variables					
Bulk density (gcm <sup>-3</sup> )	Maximize	0.035	0.36	5	0.21
Expansion index (%)	Maximize	125.7	133.8	5	128.9
Lightness (L*)	Maximize	10.3	18.3	3	17.1
Redness (a*)	Minimize	0.75	4.35	3	3.13
Yellowness (b*)	Minimize	13.1	28.8	3	24.5

grade fractions of parboiled rice and full-fat soybean composite blends which can result in product of low bulk and maximum expansion with satisfactory light yellow color.

## 4 Conclusions

Based on the RSM models proposed, optimum extrusion cooking conditions for broken rice fractions and full-fat soybean were established at 130 °C, 20 g water per 100g sample and 23 g soybean per 100 g rice with overall desirability conditions of 0.996, while the response variables were all desirable. The optimum formulation expands favorably at the die (128.9%), produces light weight product with desirable light yellow color similar to commercial extruded breakfast or complimentary cereals. Based on this result, more alternative raw materials are at the disposal of food industries in Nigeria and indeed Sub-Saharan African countries that are recommended to produce expanded instant foods which could be blended with legumes to improve nutrition and reduce malnutrition and even produce products for food aid among migrant communities.

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# Nutritional and Antioxidant Potential of Rice Flour Enriched with Kersting's Groundnut (*Kerstingiella geocarpa*) and Lemon Pomace

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## Abstract

This study was designed to enhance the nutritional quality, antioxidant properties and product utilization potentials of locally produced 'Igbemo' rice flour by adding Kersting's groundnut and lemon pomace. Kersting's groundnut is an underutilized legume while lemon pomace is a byproduct of lemon utilization; both meant to enhance the protein quality, antioxidant potential and fibre contents of the composite flour. The dependent variables were minerals composition, amino acid profile, antioxidants and antinutrients properties, in-vitro protein digestibility and in-vitro carbohydrate digestibility. The result showed that blends with higher lemon pomace of 10.00 g had the best calcium, iron, potassium and magnesium contents and antioxidant contents, while blends with highest Kersting's groundnut (20.00 g) had the best zinc content. The anti-nutrients in the blends were generally low and safe for consumption.

**Keywords:** Antioxidants; Composite flour; Kersting's groundnut; Response surface methodology; Rice flour

## 1 Introduction

The increase in production and consumers' acceptability of rice flour in the production of non-gluten baked products is promoting further research into rice flour utilization. In addition to its non-gluten characteristics, rice flour has been found to possess good nutritional qualities. Rice, as a cereal, serves as a basic food source for over half the world population whilst it provides about 80 % of the food intake as ready-to-eat convenience and inexpensive gluten-free snacks (Awolu, Oluwaferanmi, Fafowora, & Osayemi, 2015). Nutritionally, cereals are important sources of carbohydrates, dietary fibre and vitamins (Katina et al., 2005) but they are defi-

cient in lysine. Cereal-based flours are therefore supplemented with legumes as credible source of lysine (Awolu et al., 2015; Awolu, Omoba, Olawoye, & Dairo, 2017). In addition, legumes serve as sources of protein and minerals needed for health growth and development.

Kersting's groundnut (*Kerstingiella geocarpa* Harms) is an underutilized legume; it is rich in essential minerals, protein and amino acids. The crop is indigenous to Africa and a viable alternative to high protein content foods (Bayorbor, Dzomeku, Avornyo, & Opoku-Agyeman, 2010). Kersting's groundnut has not been fully exploited and its nutritional importance has not been fully evaluated.

Lemon (*Citrus limon*) comes after orange and

mandarin in the order of citrus importance. It is rich in vitamin C, minerals, dietary fibre, essential oils, organic acids, carotenoids and flavonoids (Gonzalez-Molina, Dominguez-Perles, Moreno, & Garcia-Viguera, 2010).

This study produced composite flour comprising rice, Kersting's groundnut and lemon pomace. The addition of Kersting's groundnut was meant to enhance its protein content and mineral compositions, whilst lemon pomace enhanced its fibre contents. Kersting's groundnut and lemon pomace has been found to be rich in antioxidants, hence, it was expected that their addition would enhance the antioxidant capacities of the composite flour.

## 2 Materials and Methods

### 2.1 Materials

Igbemo rice was sourced from Igbemo-Ekiti, Ekiti State. Kersting's groundnut (*Kerstingiella geocarpa* Harms.) was sourced from Oyingbo market, Lagos. Lemon fruits were sourced at Oja-Oba, Akure, Ondo State. All reagents were of analytical grade.

### 2.2 Preparation of Flours

#### Preparation of rice flour

About 5 g of Igbemo' rice grains were manually cleaned, dry-milled using hammer mill, sieved through 210 µm particle size sieves and then stored in a sealed plastic container at room temperature for further processing (Awolu et al., 2015).

#### Preparation of Kersting's groundnut flour

Kersting's groundnut seeds (2.0 kg) were par-boiled for 35 min, manually dehulled, oven dried at 65 °C until constant weight was obtained and hammer-milled into fine particles. The flour was subsequently kept inside a sealed plastic container prior to usage (Awolu et al., 2015).

### Preparation of lemon pomace flour

Lemon pomace was produced according to the method described by Kolodziejczyk, Markowski, Kosmala, Król, and Plochanski (2007). Fresh lemon fruits were washed with warm water to remove tough dirt and dust from the fruits. The washed fruit was pulverized with a sharp knife and blended into slurry using a Kenwood blender (BL-237). The juice was extracted from the slurry using a muslin cloth whilst the wet lemon pomace was oven dried (Gen-lab hot air oven, model DHG-9101.1SA) at 60 °C for 18 h. The dried pomace was blended into fine flour using the blender.

### 2.3 Experimental design for the development of flour blends

Optimization of the proximate properties of the composite flour was carried out using the optimal mixture model design of response surface methodology (Design expert 8.0.3.1, trial version). The independent variables were rice flour (70.30 – 85.00%), Kersting's groundnut flour (10.00 – 20.00%) and lemon pomace (5.00 – 10.00%) while the dependent variables were the proximate composition.

### 2.4 Proximate composition determination of the composite flour

The moisture content, crude protein, fat, ash content and crude fibre of the composite flour blends were determined according to the standard methods of AOAC (2005).

### 2.5 Minerals analysis

Mineral analysis was determined according to the AOAC (2005) method. The sample was ashed, and about 15 mL of 6 N HCl was added to it and transferred to a 100 mL volumetric flask. Distilled water was used to make up to the 100 mL mark. Atomic Absorption Spectroscopy (AAS) was used for analysis of all the minerals except potassium and sodium which were analyzed using a flame emission spectrophotometer.

(Model A-6200, Shimadzu, Corporation and Kyoto, Japan). Standards for sodium and potassium were prepared from their chloride salts.

## 2.6 Amino acid profile determination

The sample was hydrolyzed using hydrochloric acid (6 N) for 24 h at 110 °C in a vial under vacuum and N<sub>2</sub> atmosphere, evaporated and dissolved in sodium citrate buffer (pH 2.2) and the hydrolysates were analyzed by using a HPLC combined with a Pickering PCX5200 derivatizer (Pickering Laboratories, Inc., USA) and ion exchange column (3.0 × 250 mm, 8 μm). The amino acids were identified spectrophotometrically by measuring at 570 nm (Benitez, 1989).

## 2.7 Evaluation of Antioxidants Properties

### Determination of ABTS scavenging ability

Aqueous 2, 2'-azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS) scavenging ability solution (7.8 M) with K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (2.45 mM, final conc.) was left in the dark for 16 h and the absorbance adjusted at 734 nm to 0.700 with ethanol. About 0.2 mL of the appropriate dilution of the extract was added to 2.0 mL of ABTS solution and the absorbance was read at 732 nm after 15 min. The Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity was subsequently calculated (Re et al., 1999).

### Determination of DPPH free radical scavenging ability

The stock reagent solution (1 × 10<sup>-3</sup> M) was prepared by dissolving 22 mg of DPPH in 50 mL of methanol and stored at 20 °C. The working solution (6 × 10<sup>-5</sup> M) was prepared by mixing 6 mL of stock solution with 100 mL of methanol to obtain an absorbance value of 0.8 ± 0.02 at 515 nm. Exactly 0.1 mL each of extract solutions of different concentrations were vortexed for 30 s with 3.9 mL of DPPH solution and left to react

for 30 min; the absorbance at 515 nm was then recorded. A control with no added extract was also analyzed. DPPH Scavenging activity was calculated using Eq. (1) (Lee, Mulugu, York, & O'Shea, 2007).

$$DPPH = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \times 100 \quad (1)$$

Where Ab = Absorbance

### Total flavonoids

About 0.5 mL aliquot of 20 g L<sup>-1</sup> AlCl<sub>3</sub> ethanolic solution was added to 0.5 mL of extract solution. The absorbance at 420 nm was measured after 1 h at room temperature. The presence of flavonoids was indicated by a yellow colouration. Extract samples were evaluated at a final concentration of 0.1 mg mL<sup>-1</sup> (Eq. 2) and expressed as quercetin equivalent (QE) based on the calibration curve (Ordonez, Gomez, Vattuone, & Lsla, 2006)

$$C = 0.00255 \times Ab \quad (R^2 = 0.9812) \quad (2)$$

Where Ab is the absorbance and C is the concentration (mg QE g<sup>-1</sup> DW)

## 2.8 Determination of Antinutrients

### Determination of oxalate content

About 1 g of sample was weighed into 100 mL conical flask, 75 mL 3 M H<sub>2</sub>SO<sub>4</sub> was added and stirred for 1 h with a magnetic stirrer. Exactly 25 mL of the filtrate (Whatman filter paper No.1) was taken and titrated hot against 0.05 M KMnO<sub>4</sub> solution until a faint pink colour which persisted for at least 30 sec was formed. The oxalate content was calculated by taking 1 mL of 0.05 M KMnO<sub>4</sub> as equivalent to 2.2 mg oxalate using Eq. (3) (Day & Underwood, 1986)

$$Oxalate(mg/100g) = \frac{Titrevalue \times 2.2 \times DF}{W} \quad (3)$$

Where 2.2 mg = mass equivalent value of 1mL of 0.05 M KMnO<sub>4</sub> solution.

DF = Dilution factor (total volume of sample divided by volume of portion used for titration)

\*W = Sample weight in g.

### Determination of phytic acid content

About 2 g of sample was weighed into 250 mL conical flask; 100 mL of 2 % concentrated HCl was thereafter added, allowed to soak for 3 h and filtered. The filtrate (50 mL) was pipetted into 250 mL beaker, with 107 mL ammonium thiocyanate solution added as an indicator and titrated with standard iron III chloride  $FeCl_3$  solution (containing 0.00195 g iron/mL) until a brownish yellow colour appeared and persisted for five minutes. The phytic acid content was calculated using Eq. (4) (Russell, 1980):

$$PHY = \frac{0.00195 \times V_{FeCl_3} \times DF}{W_{Sample}} \quad (4)$$

PHY = Phyticacid (g/kg)

$V_{FeCl_3}$  = volume of  $FeCl_3$  consumed

$W_{Sample}$  = sample weight

DF = Total volume of extraction solvent added/volume of aliquot taken for the titration.

### Determination of tannin content

About 0.2 g of sample was placed in a test tube, 10 mL of 1 % HCl/methanol was added, the test tube was capped, continuously shaken for 20 min and then centrifuged at 2500 rpm for 5 min. Exactly 1 mL of the supernatant was pipetted into fresh tubes, the absorbance was set at zero and 1 mL blank solution was mixed with 5 mL 4 % HCl/methanol and 5 mL vanillin reagent in a test tube. The sample and blank test tubes were incubated for 20 min at 30 °C. Absorbance was read at 500 nm and concentration of condensed tannins was determined from standard curve. Tannin concentration was expressed in % as follows (Trease & Evans, 1978):

$$Tannic\ content = \frac{(C \times 10)}{200} \times 100 \quad (5)$$

Where:

C = Concentration corresponding to the optical density

10 = Volume of extract (mL)

200 = sample weight (mg)

### Determination of saponin content

About 20 mL of 20 % aqueous ethanol was added to 10 g of the ground sample and agitated with a magnetic stirrer for 12 h at 55 °C. The solution was filtered through Whatman No.1 filter paper and the residue re-extracted with 200 mL 20 % aqueous ethanol. The extract was reduced to 40 mL under vacuum and 20 mL diethyl ether added in a separating funnel and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The pH of the aqueous solution was adjusted to 4.5 by adding NaOH, and the solution shaken with 60 mL n-butanol. The combined butanol extracts were washed twice with 10 mL of 5 % aqueous NaCl and evaporated to dryness in a fume cupboard to give a crude saponin (Hudson & El-Difrawi, 1979).

### Determination of trypsin inhibitor

Tris-buffer (0.05 M, pH 8.2) containing 0.02 M  $CaCl_2$ : 6.05 g tris- (hydroxymethyl) aminomethane and 2.94 g  $CaCl_2 \cdot 2H_2O$  were dissolved in 500 mL of distilled water, the pH was adjusted to 8.2 and the volume made up to 1 L with distilled water. About 2.0 mL of trypsin solution was added to 1.0 g of the extracted sample in a test tube and then placed in a water bath at 37 °C. Exactly 5 mL hydrated Benzoyl-DL-arginine-p-nitroanilide (BAPA) solution was dissolved in dimethyl sulfoxide previously warmed to 37 °C. The reaction was terminated 10 min later by adding 1 mL of 30 % acetic acid. After thorough mixing, the contents of each tube were filtered through Whatman No.1 paper and the absorbance was measured against the blank (Kakade, 1974).

### 2.9 In-vitro protein (IVPD) determination

Composite flour samples (200 mg) were weighed into an Erlenmeyer flask and mixed with 35 mL of porcine pepsin (1.5 g of pepsin in 0.1 M  $KH_2PO_4$ , pH 2.0). Samples were digested for 2 h at 37 °C; digestion was stopped by addition of 2 mL of 2 M NaOH and the samples were centrifuged (4900 xg at 40 °C) for 20 min after

which the supernatant was discarded. The residues were washed and centrifuged twice with 20 mL of buffer (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7.0). Undigested nitrogen was determined using Kjeldahl method. Digestibility was calculated using Eq. (6) (Aboubacar, Axtell, Huang, & Hamaker, 2001).

$$IVPD = \frac{(N_{\text{sample}} - N_{\text{Undigested}})}{N_{\text{sample}}} \times 100 \quad (6)$$

## 2.10 In-vitro carbohydrate digestibility determination (IVCD)

Exactly 4 mL of phosphate buffer (pH 6.6), 1 mL of sodium chloride and 1 mL of  $\alpha$  amylase enzyme was added to 5 mL of the sample at room temperature and mixed thoroughly. Aliquots (0.2 mL) of the mixture were taken at zero and 1.0 h (complete hydrolysis was predetermined) after addition of the enzyme and dispensed into 10 mL Lugol's iodine solution (1:100 dilution). The absorbance was measured at 620 nm and the in- vitro carbohydrate digestibility was calculated using Eq. (7) (Shekib, Eliraqui, & Abobakr, 1988).

$$IVCD = \frac{Abs_{\text{time}=0} - Abs_{\text{time}=1h}}{Abs_{\text{time}=0}} \times 100 \quad (7)$$

where Abs is the Absorbance

## 3 Results and Discussions

### 3.1 Proximate composition and functional properties of the composite flour

The result of the proximate composition is presented in Table 1. The moisture content of the composite flour (6.20 to 6.81 g/100g) was within the acceptable range ( $\leq 10\%$ ) for flours to ensure shelf stability. In addition, the variation in moisture content of the composite flour had a

low R-squared and the adjusted R-squared values of 0.3496 and 0.0244 respectively, which was an indication that it would not support moisture; hence, the composite flour would have a good shelf life.

The ash content of the composite flour ranged between 0.98 and 1.81 g/100g. The ash content increased significantly ( $p \leq 0.05$ ) as the levels of lemon pomace incorporation increased. The R-squared and adjusted R-squared values were 0.7546 and 0.7168 respectively. The crude protein of the flour samples ranged between 7.51 and 12.99 g/100g. The protein content increased significantly ( $p \leq 0.05$ ) as the level of Kersting's groundnut incorporation increased. A similar results have been obtained for composite flour consisting of rice, cassava and Kersting's groundnut flours (Awolu et al., 2015). The ANOVA indicated that the model and model term (linear mixture, AB, AB (A-B)) were significant ( $p \leq 0.05$ ) while the R-squared and the adjusted R-squared values were 0.9975 and 0.9937 respectively. The high adjusted R-squared value for protein content showed that the protein had high positive effect on the composite flour.

The fat content of the flours ranged from 4.00 - 4.44 g/100 g. In comparison, wheat flour had fat content of 1.33 g/100g. The fat content decreased with increased incorporation of rice and lemon pomace flours. The composite flour may be better enhancer of flavour and fat-soluble vitamins than wheat flour. In addition, the fat values of the composite flour should not possess any negative effect in terms of rancidity. The crude fibre values ranged from 2.52 to 3.78 g/100g. The result indicated that the addition of Kersting's groundnut flour and lemon pomace increased the crude fibre content of the composite flour. The ANOVA indicated that the model and model terms (linear mixture, AB, AB(A-B) for crude fibre were significant ( $p \leq 0.05$ ) while the R-squared and adjusted R-squared were 0.9975 and 0.9937 respectively.

The carbohydrates content of the flours ranged between 62.03 - 72.98 g/100g. The model and model terms (linear mixture component, AB, AC, A<sup>2</sup>BC, ABC<sup>2</sup>) were significant ( $p \leq 0.05$ ) while the R-squared and adjusted R-squared were 0.9945 and 0.9882 respectively.

Table 1: Proximate Composition of Composite Flour

Run	Variables (g)			Proximate composition (g/100g)					
	A (g)	B (g)	C (g)	Moisture	Ash	Protein	Fibre	Fat	CHO
1	79.84	10.16	10.00	6.40	1.81	7.51	3.71	4.00	66.57
2	75.00	20.00	5.00	6.20	1.25	12.99	2.89	4.28	67.39
3	83.50	11.50	5.00	6.50	1.05	8.56	2.80	4.00	72.09
4	70.30	20.00	9.70	6.30	1.76	12.57	3.34	4.30	62.03
5	76.98	15.75	7.25	6.46	1.70	9.12	3.21	4.27	67.98
6	79.84	10.16	10.00	6.47	1.30	8.43	3.62	4.00	66.18
7	82.28	10.00	7.71	6.30	1.48	8.41	3.70	4.00	68.40
8	75.00	20.00	5.00	6.81	1.30	12.8	2.90	4.44	66.75
9	85.00	10.00	5.00	6.40	0.98	8.41	2.68	4.01	72.52
10	73.16	19.07	7.76	6.62	1.68	12.72	3.18	4.36	63.67
11	82.28	10.00	7.71	6.80	1.60	8.46	3.72	4.11	67.60
12	85.00	10.00	5.00	6.41	1.06	7.99	2.66	4.00	72.88
13	80.31	14.68	5.00	6.43	1.32	9.55	2.52	4.20	70.98
14	77.44	12.55	10.00	6.41	1.80	9.00	3.78	4.10	64.91
15	73.56	16.43	9.99	6.30	1.74	11.62	3.09	4.19	63.10
16	70.30	20.00	9.70	6.30	1.76	12.63	3.30	4.24	62.07

\*A - Rice Flour; B - Kersting's groundnut; C- Lemon pomace; CHO - Carbohydrate

### 3.2 Minerals composition of optimized composite flour

The result of the mineral composition of the composite flour is presented in Table 2. Iron contents in the composite flour ranged between 2.82 and 3.47 mg/100g. Iron deficiency is the most common nutrient disorder worldwide as it accounts for 50 % of the cases of anaemia (World Health Organization, 2001). Iron contents reported in rice, Kersting's groundnut and lemon pomace flours were 0.82 mg/100g, 10.00 mg/100g and 147.65 mg/100g respectively (Adeyeye & Faleye, 2007; Atukorale, 2002; Janati, Beheshti, Feizy, & Fahim, 2012). The iron content in composite flour increased significantly ( $p \leq 0.05$ ) as the level of lemon pomace increased.

Zinc values ranged from 2.37 to 2.67 mg/100g. Zinc content in the composite flour increased significantly ( $p \leq 0.05$ ) as the levels of Kersting's groundnut increased. The values of zinc obtained in this study was higher than 0.58 – 0.66 mg/100g reported for wheat based composite flour enriched with 'orarudi' (Vahigna

sp) (Onoja et al., 2014).

Magnesium values ranged between 4.54 and 4.64 mg/100g. The magnesium content increased significantly ( $p \leq 0.05$ ) as the lemon pomace increased. Magnesium is a cofactor in about 300 enzyme systems which play regulatory roles in several biochemical reactions in the body such as protein synthesis, muscle and nerve function, blood glucose control and blood pressure regulation (Laurant & Touyz, 2000). It also promotes strong bones strong and keeps heart rhythm steady (Twum et al., 2015).

Potassium ranged between 121.77 -166.33 mg/100g. Potassium content increased significantly ( $p \leq 0.05$ ) with increasing lemon pomace flour content. Potassium plays a vital role in maintaining osmotic balance and pH of the body fluids, regulating muscle and nerve irritability, controlling glucose absorption and enhancing normal retention of protein during growth (National Research Council, 1980).

Calcium values ranged from 55.65 to 65.10 mg/100g. Lemon pomace had been reported to contain about 8452.50 mg/100g calcium content (Janati et al., 2012). The calcium content of



the composite flour increased significantly ( $p \leq 0.05$ ) as lemon pomace increased. Calcium functions primarily in the development of strong bones.

### 3.3 Antioxidant properties of the composite flour

The results of the antioxidant property of composite flour are presented in Table 3. The DPPH free radical scavenging ability ranged from 39.57 to 45.10 %. DPPH of the composite flour increased significantly ( $p \leq 0.05$ ) as lemon pomace increased.

The ABTS scavenging ability of the composite flour ranged between 23.81 and 25.40 mMol/g. The ABTS also increased significantly ( $p \leq 0.05$ ) as lemon pomace increased. The increase in both DPPH scavenging activities and ABTS as a result of the addition of lemon pomace is a justification for its addition, which is to improve antioxidant capacities. In addition, lemon pomace flour had positive effect on the flavonoids content of the composite flour; an increase in level of lemon pomace in the composite flour brought about a corresponding significant ( $p \leq 0.05$ ) increase in the flavonoid contents. Flavonoids are major polyphenolic components of foods and display anti-inflammatory, anti-allergic and anti-cancer activities (Crozier, Clifford, & Ashihara, 2008).

### Antinutritional properties of composite flour

The results of antinutritional properties of the composite flour are presented in Table 4. Phytate content of the composite flour ranged between 2.29 and 2.50 mg/100g. Run 2 with the highest Kersting's groundnut content (20 g/100g) had the overall highest antinutrients contents. However, the antinutrients contents were within recommended level that is safe for human consumption; the recommended toxicity level of phytates for humans is 2 – 5 g/day (Hassan, Umar, & Umar, 2004), while the phytate content in the composite flour was far less than this value.

There was no significant ( $p > 0.05$ ) difference between the tannin contents of the composite flour of the samples. The tannin content obtained in

this study was also very safe for consumption (Ikpeme, Ekpeyoung, & Igile, 2012). This low result indicated that the composite flour would have good protein digestibility as high protein contents interferes with protein digestibility.

The trypsin inhibitor activity of the composite flour ranged between 0.26 and 0.34 mg/100g. The higher the Kersting's groundnut content, the higher the trypsin inhibition activity. As with already mentioned antinutrients, the trypsin inhibition levels were very minimal and safe for human consumption. A trypsin inhibitor activity content ranging from 4.01 to 46.01 mg/100g has been reported for Acha-Soybean composite flour (Ikpeme et al., 2012).

Oxalates ranged from 0.73 to 0.87 mg/g. The level of oxalate obtained is also low and safe for human consumption. The toxicity of oxalates is 2 – 5 g/day (Hassan et al., 2004). The saponin content obtained in this study was also low and safe for human consumption.

### In-vitro carbohydrate digestibility and in-vitro protein digestibility of the composite flour

Research has shown that nutrient composition of foods is not enough to determine nutrient bio-availability (Julian et al., 2007), hence the need for in-vitro (starch and protein) digestibility analyses. The result of in-vitro carbohydrate digestibility is presented in Table 5. The results showed that the sample with highest rice content (run 7) had the overall best carbohydrate digestibility. Rice content dictates the extent of the carbohydrate digestibility; the higher the rice content the higher the carbohydrate digestibility and vice versa. In addition, digestion time rather than digestion temperature enhanced carbohydrate digestibility. Carbohydrate digestibility was higher in samples with the same digestion temperature but higher digestion time; hence digestion at 60 min was higher than digestion at 30 min.

The results of in-vitro protein digestibility are presented in Table 6. Unlike carbohydrate digestibility where the sample with the highest rice (with highest carbohydrate content) had the highest digestibility, protein digestibility had the

Table 2: Minerals Composition of the Composite Flour

Sample	Calcium (mg/100g)	Potassium (mg/100g)	Zinc (mg/100g)	Iron (mg/100g)	Magnesium (mg/100g)
Runs 2	55.65 $\pm$ 1.21 <sup>c</sup>	121.77 $\pm$ 0.59 <sup>c</sup>	2.67 $\pm$ 0.05 <sup>a</sup>	2.82 $\pm$ 0.11 <sup>b</sup>	4.54 $\pm$ 0.12 <sup>c</sup>
Runs 7	61.11 $\pm$ 1.18 <sup>b</sup>	139.00 $\pm$ 0.58 <sup>b</sup>	2.55 $\pm$ 0.10 <sup>a</sup>	3.33 $\pm$ 0.14 <sup>a</sup>	4.58 $\pm$ 0.18 <sup>b</sup>
Runs 14	65.10 $\pm$ 0.96 <sup>a</sup>	166.33 $\pm$ 0.58 <sup>a</sup>	2.37 $\pm$ 0.05 <sup>b</sup>	3.47 $\pm$ 0.08 <sup>a</sup>	4.64 $\pm$ 0.12 <sup>a</sup>

\*values are mean  $\pm$  standard deviation of triplicate samples

\*values on the same column with the same superscript are not significantly different at  $p \leq 0.05$

\*Run 2 = 75 g/100g rice; 20 g/100g Kersting's groundnut, 5 g/100g lemon pomace flours

\*Run 7 = 82.28g/100g rice; 10 g/100g Kersting's groundnut, 7.71 g/100g lemon pomace flours

\*Run 14 = 77.44 g/100g rice; 12.55 g/100g Kersting's groundnut, 10 g/100g lemon pomace flours

Table 3: Antioxidant Properties of the Composite Flour

Sample	DPPH (%)	ABTS (mMol/g)	Flavonoids (mg QE g <sup>-1</sup> )
Runs 2	39.57 $\pm$ 1.35 <sup>c</sup>	23.81 $\pm$ 0.08 <sup>c</sup>	1.21 $\pm$ 0.01 <sup>c</sup>
Runs 7	42.73 $\pm$ 3.05 <sup>ab</sup>	24.40 $\pm$ 0.40 <sup>b</sup>	1.28 $\pm$ 0.01 <sup>a</sup>
Runs 14	45.10 $\pm$ 1.89 <sup>a</sup>	25.40 $\pm$ 0.24 <sup>a</sup>	1.26 $\pm$ 0.01 <sup>b</sup>

\*values are mean  $\pm$  standard deviation of triplicate samples

\*values on the same column with the same superscript are not significantly different at  $p \leq 0.05$

\*Run 2 = 75 g/100g rice; 20 g/100g Kersting's groundnut, 5 g/100g lemon pomace flours

\*Run 7 = 82.28g/100g rice; 10 g/100g Kersting's groundnut, 7.71 g/100g lemon pomace flours

\*Run 14 = 77.44 g/100g rice; 12.55 g/100g Kersting's groundnut, 10 g/100g lemon pomace flours

Table 4: Antinutritional Properties of the Composite Flour

Sample	Oxalate (mg/100g)	Saponin (%)	Tannin (mg/100g)	Phytates (mg/100g)	Trypsin (%)
Runs 2	0.87 $\pm$ 0.01 <sup>a</sup>	3.31 $\pm$ 0.20 <sup>a</sup>	0.03 $\pm$ 0.00 <sup>a</sup>	25.05 $\pm$ 0.02 <sup>a</sup>	0.34 $\pm$ 0.12 <sup>a</sup>
Runs 7	0.73 $\pm$ 0.02 <sup>c</sup>	2.82 $\pm$ 0.04 <sup>b</sup>	0.03 $\pm$ 0.00 <sup>a</sup>	24.03 $\pm$ 0.02 <sup>b</sup>	0.26 $\pm$ 0.02 <sup>c</sup>
Runs 14	0.81 $\pm$ 0.04 <sup>b</sup>	2.44 $\pm$ 0.01 <sup>c</sup>	0.03 $\pm$ 0.00 <sup>a</sup>	22.89 $\pm$ 0.01 <sup>c</sup>	0.27 $\pm$ 0.04 <sup>b</sup>

\*values are mean  $\pm$  standard deviation of triplicate samples \*values on the same column with the same superscript are not significantly different at  $p \leq 0.05$

\*Run 2 = 75 g/100g rice; 20 g/100g Kersting's groundnut, 5 g/100g lemon pomace flours

\*Run 7 = 82.28g/100g rice; 10 g/100g Kersting's groundnut, 7.71 g/100g lemon pomace flours

\*Run 14 = 77.44 g/100g rice; 12.55 g/100g Kersting's groundnut, 10 g/100g lemon pomace flours

Table 5: In-vitro Carbohydrate Digestibility of Composite Flour

Sample	Digested Starch			
	50 °C for 60 min	50°C for 30 min	40°C for 60 min	40°C for 30 min
Run 2	28.67 $\pm$ 0.80 <sup>b</sup>	10.48 $\pm$ 0.05 <sup>c</sup>	25.47 $\pm$ 0.12 <sup>c</sup>	10.05 $\pm$ 0.09 <sup>c</sup>
Run 7	30.00 $\pm$ 0.45 <sup>a</sup>	25.62 $\pm$ 0.03 <sup>a</sup>	28.44 $\pm$ 0.42 <sup>a</sup>	23.36 $\pm$ 0.42 <sup>a</sup>
Run 14	28.54 $\pm$ 0.02 <sup>b</sup>	15.78 $\pm$ 0.07 <sup>b</sup>	27.88 $\pm$ 0.11 <sup>b</sup>	12.45 $\pm$ 0.08 <sup>b</sup>

\*values are mean  $\pm$  standard deviation of triplicate samples \*values on the same column with the same superscript are not significantly different at  $p \leq 0.05$

\*Run 2 = 75 g/100g rice; 20 g/100g Kersting's groundnut, 5 g/100g lemon pomace flours

\*Run 7 = 82.28g/100g rice; 10 g/100g Kersting's groundnut, 7.71 g/100g lemon pomace flours

\*Run 14 = 77.44 g/100g rice; 12.55 g/100g Kersting's groundnut, 10 g/100g lemon pomace flours

Table 6: In-vitro Protein Digestibility Determination (IVPD) of the Composite Flour

Sample	% Digestibility 10min	% Digestibility 15min
Runs 2	69.26 $\pm$ 0.01 <sup>c</sup>	67.27 $\pm$ 0.19 <sup>c</sup>
Runs 7	75.05 $\pm$ 0.23 <sup>b</sup>	73.78 $\pm$ 0.48 <sup>b</sup>
Runs 14	77.59 $\pm$ 0.04 <sup>a</sup>	78.31 $\pm$ 0.02 <sup>a</sup>

\*values are mean  $\pm$  standard deviation of triplicate samples \*values on the same column with the same superscript are not significantly different at  $p \leq 0.05$

\*Run 2 = 75 g/100g rice; 20 g/100g Kersting's groundnut, 5 g/100g lemon pomace flours

\*Run 7 = 82.28g/100g rice; 10 g/100g Kersting's groundnut, 7.71 g/100g lemon pomace flours

\*Run 14 = 77.44 g/100g rice; 12.55 g/100g Kersting's groundnut, 10 g/100g lemon pomace flours

Table 7: Amino Acid Profile of the Composite Flour

Essential amino acid	Concentration: g/100g protein	Non-essential amino acid	Concentration: g/100g protein
Leucine	6.80	Glycine	3.89
Lysine	4.53	Alanin	4.09
Isoleucine	3.92	Serine	3.13
Phenylalanine	4.08	Cystine	1.21
Valine	3.97	Aspartic acid	7.01
Methionine	2.40	Glutamic acid	9.54
Histidine	2.55	Proline	2.84
Threonine	3.19	Hydroxyproline	5.76
Tryptophan	1.86	Citrulline	3.78
		Arginine	2.55

highest digestibility when Kersting's groundnut (main protein component) was only 12.55 % of the composite flour. The effect of higher antinutrients (though at safe levels) in run 2 must have accounted for the lowest protein digestibility. In fact, run 2 with the highest protein content had the lowest protein digestibility. As with carbohydrate digestibility, the higher the digestion time the higher the digestibility.

#### Amino acid profile of the composite flour

The amino acid profile of the composite flour is presented in Table 7. Leucine was the highest (6.80 mg/100g) essential amino acid followed by lysine (4.53 g/100g). Lysine is a major limiting amino acid in cereals and the increase in lysine in the composite flour could be as a result of Kersting's groundnut incorporation. Lysine promotes protein synthesis and thus, it is important for

growth and maintenance of the body (Awolu et al., 2017). Glutamic acid and aspartic acid were the most abundant amino acids in the composite flour with values of 9.54 g/100g and 7.01 g/100g respectively. Glycine, together with other essential amino acids such as alanine, arginine, and phenylalanine forms polypeptides that promote growth and tissue healing (Davies & Reid, 1979).

## 4 Conclusions

The utilization of rice flour in the production of nutritionally rich baked products would be enhanced by the addition of Kersting's groundnut and lemon pomace. While Kersting's groundnut enhanced the protein content and minerals composition; lemon pomace enhanced its fibre content and antioxidant potentials. The addition of Kersting's groundnut at level of 20 g/100g of the composite flour was considered nutritionally safe.

In essence, composite flour consisting rice, Kersting's groundnut and lemon pomace at the blend ratios carried out in this study would be beneficial for consumption in terms of its nutritional composition and antioxidant capacities without negative antinutritional factors.

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## Effect of Chestnut and Acorn Flour on Wheat / Wheat-Barley Flour Properties and Bread Quality

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### Abstract

Additions of barley flour alone or with combination of chestnut and acorn flour (30%; 30+5%; 30+10%) were aimed at increasing the dietary fibre content in wheat bread. In this regard, enhancement by acorn flour elevated the dietary fibre by a greater extent (up to 7.80%) compared to barley or chestnut flours. Increasing the proportion of non-traditional raw materials also influenced flour pasting properties during the amylograph test as well as the farinograph and extensigraph properties of non-fermented dough. In contrast to the wheat flour, analysis of Falling Number and Zeleny values showed a decrease in technological potential of flour composites of approximately 30%. Water absorption increased about 2 percentage points, mainly with enhancement by chestnut flour. All the non-traditional raw materials slowed dough development, whilst dough softening degree differed according to actual composition. Dough viscous and elastic properties worsened as shown by a decrease of in energy absorbed, depending on the type and the addition of the non-traditional products. Changes in flour composition were reflected in amylograph viscosity maximum, which became lower with increasing amounts of chestnut and acorn flour. A significant worsening of the bread specific volume as well as of bread shape (vaulting) corresponded with a partial dilution of the gluten matrix. Compared to the wheat bread, 10% chestnut flour caused bread size to diminish to less than one half of the wheat loaf. Statistically, the principal features were water absorption, dough softening degree and extensigraph energy together with specific bread volume. In terms of wheat flour and bread quality, the influence of barley flour overcame the effects of adding chestnut or acorn flours.

**Keywords:** Wheat-barley composite flour; Chestnut flour; Acorn flour; Bread; Principal component analysis

### 1 Introduction

For hundreds of years, fine wheat flour represented a “white gold”, accessible for the richer minority of society. Owing to this, plebeian nutrition was based on rye or barley together with legumes and pseudocereals. In this regard, edible fruits from trees were rarely used, and served primarily as a feed for domestic animals. Nevertheless, in times of crop failure caused e.g. by flooding or local wars, humans gratefully ate plant

products such as chestnut or acorn to stay alive. Later, the industrial revolution suppressed social discrepancies and wheat flour gradually became the basic raw material for nearly all social classes. Alternative plant raw materials have been forgotten, and only with the rising incidence of lifestyle diseases have we returned to their intentional consumption.

Chestnuts, fruits of tree *Castanea sativa* Mill., are traditionally treated by hydrothermal pro-



cess to transform them into flour. Compared to wheat, chestnut flour contains much more simple sugars and dietary fibre (ca 2% vs. 16% and 2% vs. 26%, respectively), but approximately a half protein content (6% against 10-15%; Giovannelli (2009) and CREA (2015a, 2015b) respectively). In limited amounts, chestnut flour alone or in combination with rice flour may improve volume of biscuits (Hegazy, Kamil, Hussein, & Bareh, 2014) or gluten-free bread (Demirkesen, Mert, Sumnu, & Sahin, 2010).

Acorns are fruits from several species of oak tree, especially holm oak *Quercus rotundifolia* or *Q. ilex*. To produce acorn flour, fruits traditionally undergo drying or roasting, peeling and milling. A further step may be flour debittering, i.e. tannins extraction. The nutrition characteristics of this naturally gluten-free flour are mentioned by Silva et al. (2016): half the protein content (4-5%), ten-times higher fat content (10-14%) and around seven-times higher dietary fibre level (13-17%) compared to wheat flour. Pasting properties of acorn starch differ from that from chestnut - on viscograph curves, no peak and trough points were identified during slurry heating and holding at a higher constant temperature. The viscosity in 40th min of the test was about approximately one-third lower just for acorn flour (900 vs. 1300 Brabender units; Correia and Beirao-da-Costa (2010)). In Sardinia, acorn bread was part of local folklore and its preparation was a ceremony with religious connotations (Pinna, 2013).

Korus, Witczak, Ziobro, and Juszczak (2015) found that only the lowest (20%) replacement of corn and potato starches in gluten-free bread recipe led to a rise in the final product volume. When 50% wheat flour was replaced by acorn meal, composite cookies differed from control in texture, appearance, and flavour slightly; they were still considered as acceptable (Sabrin, 2009).

Barley (*Avena sativa* L.), is the one of the basic cereals, i.e. plants bred for human nutrition for the longest time; in ancient Egypt, emmer wheat and barley served for bread and beer production (Samuel, 1997). In barley flour, protein content is usually around 10%, forming a lower portion than in wheat flour, and but the reverse is true for the polysaccharides (close to

80%). A substantial part of the biopolymers in the latter form  $\beta$ -glucans and arabinoxylans - Belcredi, Ehrenbergerova, Belakova, and Vaculova (2009) analysed 12 different samples, and determined level of both components to be between 2.78-6.08% and 3.66-5.46%, respectively. These polysaccharides may help in prevention of lifestyle diseases - the European Commission approved health claims for barley and oat  $\beta$ -glucans (EFSA, 2011). Within the Czech Republic, barley flour found its fixed place among bakery raw materials, and together with e.g. chia and nopal flour, it extended the range of bread types produced. As reported Gill, Vasanathan, Oraikul, and Rossnagal (2002), quality of wheat-barley bread depends also on barley type (regular, waxy). For 15% substitution of wheat flour by regular barley one, bread loaf volume was larger and crumb softer than for counterpart containing waxy barley flour. Similarly, the molar mass of barley  $\beta$ -glucans predetermines the resulting bread properties. In terms of increasing the specific bread volume and reducing the crumb firmness, addition of  $\beta$ -glucans characterised by molar masses of  $2.03 \times 10^5$  and  $1.00 \times 10^5$  resulted in preference of the loaves made with the higher mass  $\beta$ -glucans (Skendi, Biliaderis, Papageorgiou, & Izidorczyk, 2010). These authors mentioned that 0.6% of such  $\beta$ -glucan type improved rheological properties of wheat dough as shown by prolongation of both farinograph dough development and stability during kneading. With respect to dough machinability,  $\beta$ -glucans supported dough extensigraph elasticity: elasticity-to-extensibility ratio increased almost twice (0.52 for weak bakery wheat control 'Dion', and 0.95 for that composite dough). The rise in dough elasticity alleviates the expansion of pores in dough during fermentation and thereby it increases bread crumb softness (compressibility). The aim of this work was to evaluate basic analytics, viscous and elastic behaviour of wheat or wheat-barley flour composites containing two different amounts of chestnut or acorn flour. Baking potential of the flour composites prepared was evaluated directly in a laboratory baking trial. To distinguish the influence of the non-traditional plant material types and dosage levels, results were statistically analysed by principal components.

## 2 Materials and Methods

### 2.1 Wheat and barley flour

Two samples of white wheat flour (WF1 and WF2), used as a bases for bi- and tri-composite blends, were produced by the Czech commercial mill Delta Prague in years 2015 and 2016. They were characterised by protein contents of 11.2% and 13.2%, Zeleny values of 50 and 39 mL and Falling Number values of 341 and 432 s, respectively. Barley flour (BF) was supplied by the Czech commercial mill Křesín (production year 2015), and was described by protein content, Zeleny value and Falling number as 9.23%, 23 mL and 119 s, respectively. Total dietary fibre (TDF) ratios were similar (3.40, 3.18, and 4.25% for WF1, WF2 and BF, respectively; Table 1). In the cereal premixes WF1BF and WF2BF, barley flour replaced 30% of WF1 or WF2 (mixing ratio 30:70 w/w, respectively).

### 2.2 Non-traditional flour samples

Further non-traditional plant materials, namely chestnut (C) and acorn (A) flour, were bought in local specialised food shops. The former was produced by Sonnentor Kräuterhandelsgesellschaft mbH (Austria) and the latter was from Bioobchod, Czech Republic (agriculture production EU mentioned on a label). Declared protein contents were 4.9% and 4.1%, respectively. Ratio of TDF was determined analytically as 13.52% and 44.49%, respectively. Replacement levels of both wheat flour and wheat-barley premix were chosen as 5 or 10 wt. % (coding e.g. WF1+5C, WF2+10A, WF1BF+5A).

### 2.3 Analytical tests

Analytical testing of wheat controls and prepared flour composites consisted of the technological parameters Falling number (ISO 3039) and Zeleny sedimentation value (ISO 5529), determined in two replications each. In correspondence to EU legislation (Regulation 1169/2011), TDF was calculated from data for pure flour samples (wheat, barley, chestnut and acorn flour; AOAC method 991.43). Due to method repeatability

determined earlier (actual standard deviation 0.22 percent points), it was possible to carry out the measurement with a single repetition.

### 2.4 Rheological behaviour

Viscous behaviour was determined according to ICC method No 126/1, using the Brander Amylograph (Germany) (80 g of solids, 450 mL of distilled water). Rheological characteristics of wheat and composite non-fermented dough were recorded using the Farinograph and the Extensigraph (Brabender Germany; ISO 5530-1:2013 and ISO 5530-2:2013, respectively). Due to preparation of leavened bread, whose proofing takes 50 min, only the Extensigraph data after 60 min of dough resting was considered.

### 2.5 Bread baking trial

Bread preparation and assessment of its characteristics were carried out as described in previous work (Švec & Hrušková, 2004). Leavened dough was prepared using the Farinograph, with the basic formula: 300.0 g wheat flour, 12.0 g yeast, 5.1 g salt, 4.5 g sugar, 3.0 g margarine and sufficient distilled water needed for preparation of dough to a consistency of  $600 \pm 20$  Brabender units (consistency based on experience of the Research Institute of Milling-Baking Industry, Czech Republic). Recipe water additions were determined to be in the range 53.5%-62.5% on basis of flour weight, i.e. from 160.5 mL to 187.5 mL. In the evaluation of flour composites, wheat flour was replaced by wheat-chestnut/acorn blends, wheat-barley premix, or wheat-barley-chestnut/acorn mixture. Commercial French-type yeast "Fala" and the Czech margarine "Perla" (fat content 40%) were used (producers Lesaffre Czech Republic and Unilever Czech Republic, respectively). Dough fermentation and leavening took 50 and 45 min at constant temperature (30 °C, RH 95%); 70 g dough pieces were moulded manually, then placed on a baking plate. Baking for 14 min was performed in a laboratory oven (Bakery Research Institute, Poland) preheated to 240 °C; steamed was created immediately after full baking plate insertion by injection 50 mL of distilled water. After two-hour cooling under lab-

Table 1: Barley, chestnut and acorn flour influence on polysaccharide pasting properties

Flour, flour composite	Analytics		Amylograph test			
	TFD (%)	Falling number (s)	Temperature of maximum (°C)		Viscosity maximum (BU)	
WF1	3.40	341 de	85.0	cd	460	c
WF2	3.18	420 f	88.4	de	940	d
BF	4.25	107 a	<i>n.d.</i>		<i>n.d.</i>	
WF1BF	3.72	304 c	79.0	b	355	c
WF2BF	3.50	168 ab	69.0	a	215	ab
WF1+5C	3.91	323 cd	85.8	cd	415	c
WF1+10C	4.41	317 cd	88.0	de	420	c
WF2+5A	5.23	408 f	96.0	f	>1000	d
WF2+10A	7.29	388 ef	91.0	ef	>1000	d
WF1BF+5C	4.21	286 c	82.0	bc	345	bc
WF1BF+10C	4.70	276 c	82.0	bc	360	c
WF2BF+5A	5.54	178 ab	70.0	a	190	a
WF2BF+10A	7.58	185 b	72.0	a	185	a
<i>Repeatability</i>	<i>0.22</i>	<i>25</i>	<i>0.20</i>		<i>4.20%</i>	
<i>Number of groups (of total)</i>	<i>n.d.</i>	6 / 12 (a - l)	6 / 12	(a - l)	4 / 12	(a - l)
<i>Distinguishing rate</i>	<i>n.d.</i>	50%	50%		33%	

TFD - total dietary fibre content, feature excluded from ANOVA (calculated, i.e. dependent values for flour composites tested); n.d. - not determined.

WF - Wheat flour; WF1BF, WF2BF - wheat-barley flour premix 70:30 wt. %, respectively; BF, A, C - barley, acorn and chestnut flour, respectively.

a-f - means in columns with the same letter were not statistically different ( $p > 0.05$ ).

Number of groups - number of homogenous groups, statistically differing in their averages, identified by different letters (a-f).

Total number of groups - number of tested samples ( $N = 12$ ; corresponding letters a-l).

Distinguishing rate (%) =  $100 \times \text{Number of homogenous groups} / \text{Total number of groups} (=12)$ .

oratory conditions, the specific volume and the shape (height-to-diameter ratio) was determined in triplicate. To measure the volume of buns, the traditional volume displacement method with rapeseeds was used. For crumb firmness (or softness) evaluation, gravitational penetrometer PNR-10 (Petrotest Instruments, Germany) was employed. This apparatus was equipped with a stainless steel hemisphere (25 mm diameter), locked in a metal screw holder (total weight 150 g). Crumb samples were cut out of the centre of the bun halves (height and diameter of crumb cylinders 35 mm and 30 mm), and the penetration depth was determined after a compression time of 5 s (five replications for each sample). In eight-point sensory analysis, three skilled assessors were employed, thus results of the test

have informative character only. On a hedonic scale, summary sensory profile could reach values from 8 to 24 points as the best and the unacceptable bread, respectively (the higher score, the higher extent of negative changes). Quality attributes evaluated were: bun shape (vaulting), crust colour and shine, crust thickness and crispness, crumb elasticity and porosity, aroma and taste, crumb chewiness, and stickiness to palate. For single parameters, bread quality was classified into three categories: 1 - optimal, typical; 2 - acceptable (soft) change, non-typical, flat; 3 - unacceptable, strange (negative, unfavourable change). Within the evaluation, values 1.5 or 2.5 points were also allowed. This part of research followed the tenets of the Declaration of Helsinki promulgated in 1964 and was approved by the

Experimentation Committee of the UCT Prague, which issued informed consent.

## 2.6 Statistical analysis

The scatter of the data was first described by ANOVA (Tukey HSD test,  $p < 0.05$ ) using Statistica 13.0 software (StatSoft Inc., USA). Secondly, correlation analysis was calculated between flour features (analytical parameters, rheological tests) and bread quality attributes. Multivariate analysis by principal components (PCA) was conducted for 14 representative parameters, selected on basis of the correlation matrix as well as the PCA results of the complete dataset. For ANOVA and PCA, the factors considered were composite flour base (wheat, wheat-barley, wheat-barley-chestnut/acorn) and enhancement level of non-traditional plant material (0, 5/10 chestnut/acorn, BF30, BF30+5/10 chestnut/acorn).

## 3 Results and Discussions

### 3.1 Effects of non-traditional plant materials on properties of wheat polysaccharides

#### Analytical tests

According to the TDF contents in the non-traditional materials tested, the amount of dietary fibre increased in composite flours up to twice that in wheat flour alone, mainly by addition of acorn flour (from 3.18% for WF2 to 7.29 and 7.58%, samples WF2+10A and WF2BF+10A; Table 1). When both WF standards were compared, there was a clear difference shown in the Falling number test; it reflected diverse weather conditions during two consecutive harvest years. For the barley flour sample, the value reached about half that level, signifying higher amylase activity and/or a higher rate of damaged starch. Mechanically, physically or enzymatically damaged starch is more accessible to amylases, which contributes to lowering of the value of the Falling number. Between WF1BF and WF2BF premixes, a significant drop was observed in the latter case (Falling numbers 304 and

168 s) compared to values of 420 and 341 s for WF1 and WF2, respectively (Table 1).

#### Pasting behaviour

For WF controls and the 10 flour composites, differences in amylograph viscosity maxima corresponded with level of Falling number ( $r = 0.92$ ,  $p < 0.001$ ; data not shown). Besides amylograph maximum viscosity, partial differentiation of the flour composites was obtained at the temperature corresponding to this maximum. Table 1 shows that there was a lesser influence of chestnut flour and strong one of acorn and barley flour. A negative influence of chestnut flour confirmed results by Hegazy et al. (2014) using the RVA test - the viscosity for wheat-chestnut flour 90:10 (w/w) was clearly lower than for wheat control (2036 vs. 2651 mPa.s; decrease ca 23%). Conversely, as expected, higher TDF content due to additions of acorn flour caused an increase of the maximal viscosity that exceeded the amylograph technical limit of 1000 Brabender units (BU). In combination with BF, high amylase activity in the alternative cereal flour suppressed the thickening effect of the acorn flour. For wheat-barley-chestnut composites, it seemed that chestnut flour partially buffered the effect of BF. Contrary to our results, direct comparison of pasting of the chestnut and the acorn starches conducted Yoo, Lee, Kim, and Shin (2012) by using of the RVA proof, showed lower peak viscosity for the acorn starch than chestnut starch (4874 mPa.s against 5640 mPa.s, respectively). For acorn starch, these authors determined that this was caused by the smaller size of starch granules, slightly higher total amylose content as well as a weaker resistance to swelling (features swelling power and solubility at 80 °C).

### 3.2 Effect of non-traditional plant materials on properties of wheat proteins

#### Analytical tests

Baking quality of proteins was satisfactory for both wheat controls - the demanded minimum in praxis is 35 mL. Additions of all non-traditional

materials induced predictable decrease of sediment volumes during the Zeleny test - the greatest drop was observed for 5 or 10% of chestnut flour. The combination of barley and especially acorn flour reduced the technological quality of composite flour by approximately one half (Table 2). Reason for this difference may have lain in the different granulation of the alternative materials - chestnut flour appearance was obviously smoother (like a wheat flour) compared to the roughly milled acorn one. On the contrary, BF was produced in industrial mill and its granulation was comparable to wheat flour.

### Viscous and elastic behaviour

Two principal properties of wheat proteins are elasticity and extensibility, expressed as their ratio. For bakery products, the extensigraph elasticity should be 2 or 2.5-times higher than the extensibility; optima of the parameters lie in ranges 450 - 600 BU and 140 - 170 mm, respectively. In addition, extensigraph energy taken as area under the curve gives a complex overview of flour technological quality (Příhoda & Hrušková, 2017); its empirical minimum is 100 cm<sup>2</sup> (Kovaříková & Netolická, 2011). With respect to the present work, both wheat flour controls WF1 and WF2 fulfilled the demand for good technological quality (the ratios 2.03 and 2.81, the energies 139 and 115 cm<sup>2</sup>, respectively; Table 2). In our results, baking quality of WF1 could be considered as better than WF2.

Both chestnut and acorn flour added alone (WF1+C, WF2+A) noticeably increased the energy level. The shape of the curves, i.e. dough machinability, were affected by enhancement level - the elasticity-to-extensibility ratios rose by about twice and three-times, respectively (Table 2). It could be supposed that non-gluten chestnut and acorn proteins (albumins) first supported the dough extensibility and secondly the elasticity, too. In combination with barley flour, the influence of these alternative plant materials was obvious - the BF proteins released in the non-fermented dough caused it to be more elastic and with lower tensile strength. Changes in the shape of the recorded curves were reflected in extensigraph energy - flour composites based on the stronger WF1 were negatively influenced

by a larger extent. For the wheat-barley-acorn counterparts, acorn fibre supported dough elasticity, i.e. it showed a certain potential to correct such quality loss. On the other hand, as the trend registered in the plot attests, there was a stepwise decline in dough handling properties - the higher the wheat flour replacement rate, the greater the increase in extensigraph ratio (up to 14.5 and 11.1 for samples WF1B+10C and WF2B+10A, respectively). Gonzaga, Batista, Guiné, and Correia (2015) tested wheat-acorn blends mixed in ratios of 90:10 and 85:15 (w/w), comparing flour from two Spanish wheat varieties Cerealis and Ceres. Acorn flour was prepared on a laboratory scale with grain size of less than 1.0 mm. As we found, these authors confirmed the clear-cut rise in the extensigraph ratio according to acorn flour ratio and dough resting time.

### 3.3 Effect of non-traditional plant materials on wheat dough during kneading

For both wheat dough controls, behaviour during kneading was closer than indicated the Zeleny test - the WF2 sample had a little shorter dough development time but about 50% longer dough stability. Water absorption values of 67.7 and 65.1% as well as dough softening degrees of 50 and 40 BU could be considered as comparable and satisfactory for enhancement by non-traditional plant materials. With increasing portion of chestnut flour, water absorption of the composites tended to decrease, while dough development was somewhat slowed (time was prolonged) and degree of dough softening slightly increased (Table 3). Hegazy et al. (2014) arrived at different results, owing to differences in technological quality of WF1/WF2 and Egyptian wheat flour - 10% replacement by chestnut flour improved dough rheological properties (e.g. water absorption had been increased from 56.5% to 60%, and stability was shortened from 9.0 min to 7.5 min). The authors characterised their base material by protein content about 2 percent points lower (9.80%) and with approximately half dough development time (1.5 min) - the Egyptian sample had weaker baking quality than both our controls.

Table 2: Barley, chestnut and acorn flour influence on technological quality of proteins and extensigraph parameters of wheat flour

Flour, flour composite	Analytics		Extensigraph test			
	Zeleny value (mL)		Ratio* (1)		Energy* (cm <sup>2</sup> )	
WF1	50	g	2.03	a	139.5	cd
WF2	38	ef	2.81	b	115.3	bc
BF	22	a	<i>n.d.</i>		<i>n.d.</i>	
WF1BF	31	cd	6.50	e	113.7	bc
WF2BF	27	abc	4.78	d	64.8	a
WF1+5C	40	f	3.96	c	148.1	cd
WF1+10C	39	f	5.28	d	146.2	cd
WF2+5A	34	de	3.88	c	135.9	cd
WF2+10A	31	cd	6.42	e	156.2	d
WF1BF+5C	29	bc	11.90	g	87.2	ab
WF1BF+10C	26	ab	14.49	h	86.4	ab
WF2BF+5A	25	ab	6.89	e	89.8	ab
WF2BF+10A	23	a	11.09	f	77.0	ab
<hr/>						
<i>Repeatability</i>	<i>1</i>		<i>0.13</i>		<i>8</i>	
<i>Number of groups</i>						
(of total)	7 / 12	(a - l)	8 / 12	(a - l)	4 / 12	(a - l)
Distinguishing rate	58%		67%		33%	

\* - extensigraph parameters determined after 60 min of dough resting

WF - Wheat flour; WF1BF, WF2BF - wheat-barley flour premix 70:30 wt. %, respectively; BF, A, C - barley, acorn and chestnut flour, respectively.

a-h - means in columns the same letter were not statistically different ( $p > 0.05$ ).

Number of groups - number of homogenous groups, statistically differing in their averages, identified by different letters (a-h).

Total number of groups - number of tested samples ( $N = 12$ ; corresponding letters a-l).

Distinguishing rate (%) =  $100 \cdot \text{Number of homogenous groups} / \text{Total number of groups} (=12)$ .

n.d. - not determined.

The farinograph behaviour of the wheat-acorn flour demonstrated a similar course - water amounts rose by about 2 percent points only, dough development time was increased four-times and dough stability fell from 11.0 min to 9.00 min (Table 3). Farinograph test results for wheat-acorn composites differs according wheat flour base (Gonzaga et al., 2015) - in the case of variety Cerealis, 10% acorn flour lowers water absorption (60.0% vs. 55.7%), and but increases it in the case of the Ceres variety (57.5% vs. 59.3%). They also showed that in terms of dough development time, there is a shortening from 4.0 to 2.0 min in the former and no significant change in the latter. Comparing our own amylograph and farinograph results, acorn polysaccharides are likely to have a better ab-

sorption capacity at lower temperatures than they are when heated. This premise confirmed results by Yoo et al. (2012) that, during the cold phase (beginning) of the RVA test, acorn starch demonstrates a higher viscosity than chestnut starch (5594 mPa.s against 4483 mPa.s, respectively). Yoo et al. (2012) proposed that the reasons for this behaviour are to be found in the different lengths of amylose chains and in content of so-called intermediate materials (slightly branched amylopectin with molar mass between amylopectin and amylose). As far as is known, both starch components influence the properties of water suspension in opposite ways - the amylopectin supports starch swelling, while amylose lowers the viscosity.



Table 3: Barley, chestnut and acorn flour influence on farinograph behaviour of wheat flour

Flour, flour composite	Water absorption (%)		Dough development (min)		Dough stability (min)		MTI (BU)	
WF1	67.7	cd	3.75	ab	7.50	b	50	ab
WF2	65.1	a	2.75	a	11.00	d	40	a
WF1BF	70.0	e	10.00	f	17.00	e	30	a
WF2BF	68.0	d	4.50	b	3.50	a	120	e
WF2+5A	66.1	ab	8.50	e	4.50	a	50	ab
WF2+10A	67.5	cd	10.50	f	9.00	c	70	bc
WF1BF+5C	68.2	d	4.00	b	4.00	a	100	de
WF1BF+10C	67.5	cd	4.00	b	4.00	a	100	de
WF1+5C	66.6	bc	8.00	de	9.00	c	50	ab
WF1+10C	65.5	ab	6.50	c	7.50	b	70	bc
WF2BF+5A	68.3	d	7.00	cd	8.00	bc	90	cd
WF2BF+10A	68.5	d	8.25	e	11.00	d	50	ab
<hr/>								
<i>Repeatability</i>	<i>0.2</i>		<i>4</i>		<i>0.20</i>		<i>0.20</i>	
Number of groups (of total)	5 / 12	(a - l)	6 / 12	(a - l)	5 / 12	(a - l)	5 / 12	(a - l)
Distinguishing rate	42%		50%		42%		42%	

MTI - mixing tolerance index (dough softening degree), BU - Brabender unit.

WF - Wheat flour; WF1BF, WF2BF - wheat-barley flour premix 70:30 wt. %, respectively;

BF, A, C - barley, acorn and chestnut flour, respectively.

a-f - means in columns with the same letter are not statistically different ( $p > 0.05$ ).

Number of groups - number of homogenous groups, statistically differing in their averages, identified by different letters (a-f).

Total number of groups - number of tested samples ( $N = 12$ ; corresponding letters a-l).

Distinguishing rate (%) =  $100 \cdot \text{Number of homogenous groups} / \text{Total number of groups} (=12)$ .

### 3.4 Effect of non-traditional plant materials on baking test results

During leavened dough preparation, recipe water additions were evaluated in the ranges 58.0 - 62.5% and 53.5 - 59.5% within the chestnut and acorn sample subgroups, respectively (Table 4). For flour composites based on WF1, the initial water amount of 62.5% decreased as the chestnut content increased (similarly for samples with the WF1BF base), reflecting an increasing portion of sucrose in dough (Moreira, Chenlo, Torres, & Prieto, 2010). Conversely, Kucerova, Sotnikova, and Nedomova (2013) found increasing recipe water amount during wheat bread preparation that had been enhanced by 1% and 3% of bamboo fibre. Bamboo fibre consists approximately from 97% cellulose, characterised by higher swelling capacity. Within the subgroup with bases of WF2 and WF2BF, a decreasing trend in water amount was observed. This was

the reverse pattern compared to the farinograph water absorption. In spite of a significant rise of TDF content (Table 1), bread formula components such as salt, sugar and fat may have restricted the incorporation of water molecules into the coating of protein chains, and so lowered total amounts of water that had to be added.

The quality of the bread samples was differentiated according to absence or presence of BF in dough recipe (Figure 1; triplicates of WF1 samples numbered 1-3, WF1BF ones 7-9; WF2 samples 4-6, WF2BF ones 10-12). Although chestnut and acorn flour were added in small portions, both materials decreased specific bread volume (Demirkesen et al., 2010) as well as modifying bread shape. Chestnut flour contains fibre characterised by rigid structures (Demirkesen et al., 2010), which limits the expansion of pores in the dough mass during fermentation (Collar, Santos, & Rosell, 2007). Dall'Asta et al. (2013) even tested 50% replacement of soft wheat flour by chestnut flour; such product had

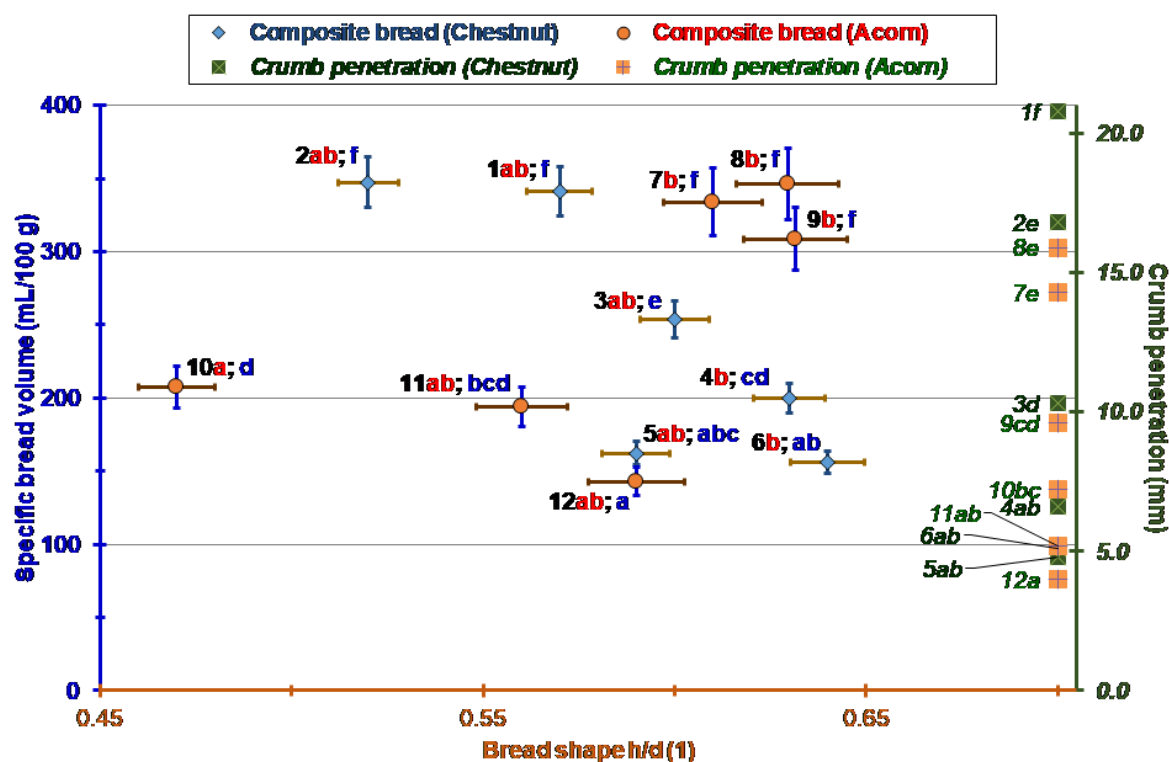


Figure 1: Barley, acorn and chestnut flour influence on wheat bread properties. Samples coding: 1 – WF1; 2 – WF1+5C; 3 – WF1+10C; 4 – WF1BF; 5 – WF1BF+5C; 6 – WF1BF+10C (numbers 7-12 similarly within WF2-acorn subset). Variable coding: h/d – height-to-diameter ratio of bread bun. Data variance analysis: bread shape ‘a-b’ (in red); specific bread volume ‘a-f’ (in blue); crumb penetration ‘a-f’ (in green) – values signed by the same letter are not statistically different ( $p < 0.05$ ).

a clearly lower specific volume ( $1.3 \pm 0.2 \text{ cm}^3/\text{g}$  vs.  $2.8 \pm 0.4 \text{ cm}^3/\text{g}$  for wheat control), homogeneous porosity with correspondingly higher ratio of pores smaller than  $0.049 \text{ mm}^2$ . We also found that there was a strong relationship between specific bread volume and crumb penetration ( $r = 0.93$ ,  $p < 0.01$ ; Table 5), with somewhat harder crumb for bread with acorn flour (Table 4).

### 3.5 Sensory analysis of bread

With the sensory profiling, a value of 8 points meant the lowest score, i.e. optimal bread quality in terms of all 8 quality attributes. Replacing 30% of WF gave a somewhat moister but stickier mouthfeel perhaps due to the presence of  $\beta$ -glucans (+0.5 point) and non-typical flavour

(+0.5 point) - the bread score reached 9 points. For composite bread variants, the most affected parameters were taste and aroma, chewiness and stickiness. For the most enriched bread, the scores rose 14.5 points (Figure 2), where the maximum 24 points represented unacceptable bread, so this bread was of worse sensory quality by about 41%.

Taste of chestnut flour itself is fruity and sweet, due to the higher content of simple sugars (Dall’Asta et al., 2013). Within WF+chestnut samples, free sugars contributed to darkening of the surface of the final products (Man, Pucean, Muste, Muresan, & Frâncu, 2012); further worsening was noticed with the development of an elastic, non-crispy crust and denser sticky crumb. The more compact crumb perhaps limited water

Table 4: Baking test results as affected by barley, chestnut and acorn flour additions

Flour, flour composite	Recipe water addition (%)		Crumb penetration (mm)		Bread sensory score (points)	
WF1	62.5	i	20.8	f	8.0	a
WF2	61.0	h	16.8	e	8.5	a
WF1BF	60.5	gh	10.3	d	9.0	ab
WF2BF	62.0	i	6.6	ab	10.0	b
WF1+5C	60.0	fg	4.8	ab	9.5	ab
WF1+10C	58.0	c	5.1	ab	10.0	b
WF2+5A	59.0	de	14.3	e	8.0	a
WF2+10A	59.5	ef	15.9	e	8.0	a
WF1BF+5C	58.5	cd	9.6	cd	10.5	b
WF1BF+10C	58.4	c	7.2	bc	9.0	ab
WF2BF+5A	56.0	b	5.2	ab	10.5	b
WF2BF+10A	53.5	a	4.0	a	14.5	c
<hr/>						
<i>Repeatability</i>	<i>0.1</i>		<i>0.9</i>		<i>0.5</i>	
Number of groups (of total)	9 / 12	(a - l)	6 / 12	(a - l)	3 / 12	(a - l)
Distinguishing rate	75%		50%		25%	

WF - Wheat flour; WF1BF, WF2BF - wheat-barley flour premix 70:30 wt. %, respectively;

BF, A, C - barley, acorn and chestnut flour, respectively.

a-i - means in columns with the same letter are not statistically different ( $p > 0.05$ ).

Number of groups - number of homogenous groups, statistically differing in their averages, identified by different letters (a-i).

Total number of groups - number of tested samples ( $N = 12$ ; corresponding letters a-l)

Distinguishing rate (%) =  $100 \cdot \text{Number of homogenous groups} / \text{Total number of groups} (=12)$ .

evaporation and together with the higher activity of the amylases (lower Falling number) led to a higher occurrence of sticky dextrins than in wheat control. A partial improvement of overall perception brought about by the addition of the chestnut flour when added at over 10% was due to the increase in sweet taste. In combination with BF, these characteristics were maintained, though mouthfeel stickiness vanished, but typical barley flavour was detected anew. Man et al. (2012) summarised that 10% of that alternative plant raw material led to the best quality product in terms of taste, odour and colour. These authors found significant and positive changes in bread crumb elasticity, although bread porosity was influenced negatively.

In terms of sensory acceptability, bread attributes got worse for acorn flour additions over 10% - taste and aroma became woody with a partial acid aftertaste. For WFBF-acorn samples,

woody flavour was identified for the sample with 10% acorn flour in the recipe. During mastication, acorn flour seemed to be milled roughly, so mouthfeel sensation was also “sandy” with some stickiness. One positive effect of acorn flour was the semi-darkening effect on the bread, mainly for recipes containing 10% or more.

### 3.6 Correlation analysis

The reduced correlation matrix confirmed that bread quality was predetermined by polysaccharides and proteins properties (Table 5). In terms of the analytical and rheological tests, the results of the farinograph proof were less influenced by dough recipe modification - only water absorption influenced specific bread volume negatively ( $r = -0.62$ ,  $p < 0.05$ ; data not shown).

For starch and its constituents, correlation analysis verified a similar relationship between the

Table 5: Baking test results as affected by barley, chestnut and acorn flour additions

Flour component Proof type Feature	Polysaccharide properties				Protein properties				Extensigraph				Baking trial	
	Analytics		Anylograph		Analytics		Farinograph		Extensigraph		Extensigraph		Baking trial	
	Falling number	TDF	Temperature of maximum	Viscosity maximum	Zeleny test	Dough development	MTI	Ratio *	Energy *	Recipe water addition	Specific bread volume	Bread shape h/d	Crumb penetration	
Recipe water addition	0.51	-0.73	0.51	0.23	0.73	-0.10	-0.28	-0.48	0.57	1	0.52	0.03	0.60	
Specific bread volume	0.78	-0.24	0.72	0.72	0.82	0.07	-0.51	-0.83	0.81	0.52	1	-0.01	0.93	
Bread shape h/d	0.54	0.30	0.55	0.50	-0.05	0.25	-0.40	0.31	0.30	0.03	-0.01	1	-0.09	
Crumb penetration	0.66	-0.40	0.63	0.51	0.92	-0.12	-0.51	-0.79	0.71	0.60	0.93	-0.09	1	
Bread sensory score	-0.56	0.76	-0.54	-0.42	-0.68	0.39	0.03	0.62	-0.45	-0.78	-0.68	0.13	-0.69	
(N = 12, p < 0.05; reit = 0.576; p < 0.01; reit = 0.708)														

TDF - Total dietary fibre content; MTI - Mixing tolerance index (degree of dough softening); h/d - height-to-diameter ratio

\* - extensigraph features recorded after 60 min of dough resting.

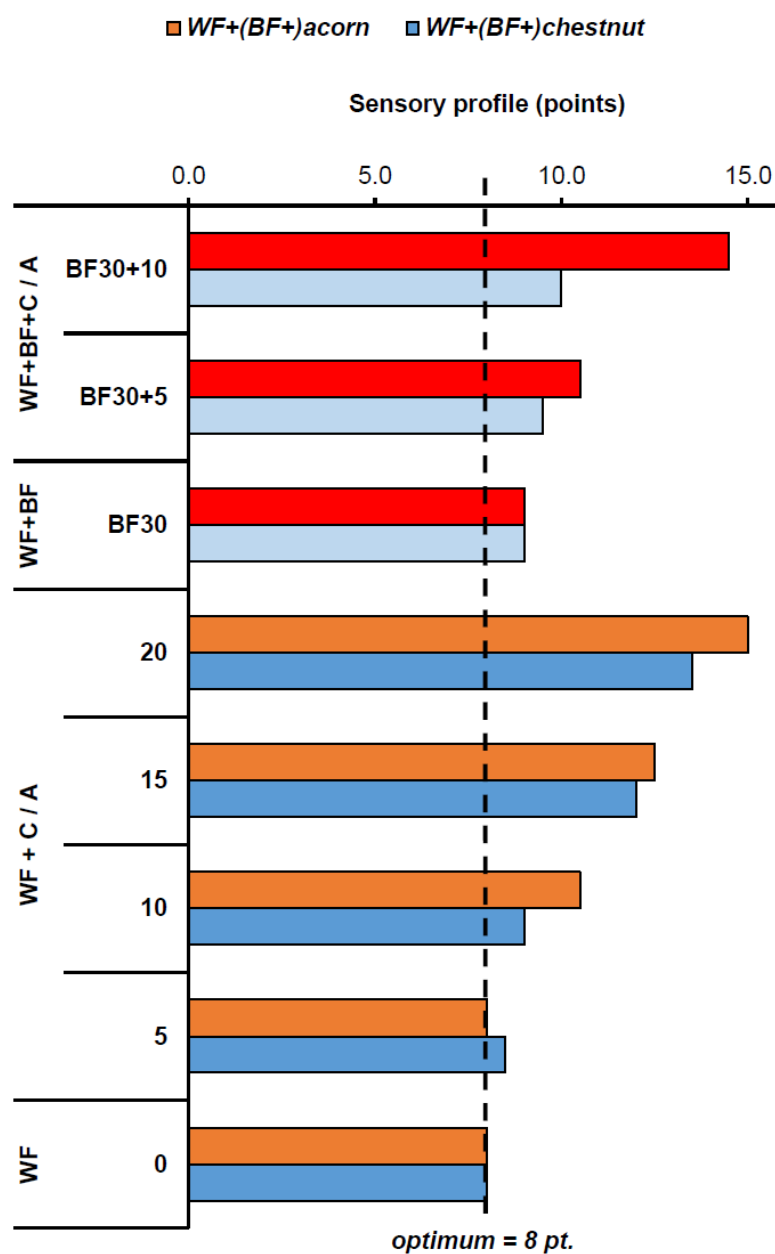


Figure 2: Barley, acorn and chestnut flour influence on sensory profile of wheat bread.

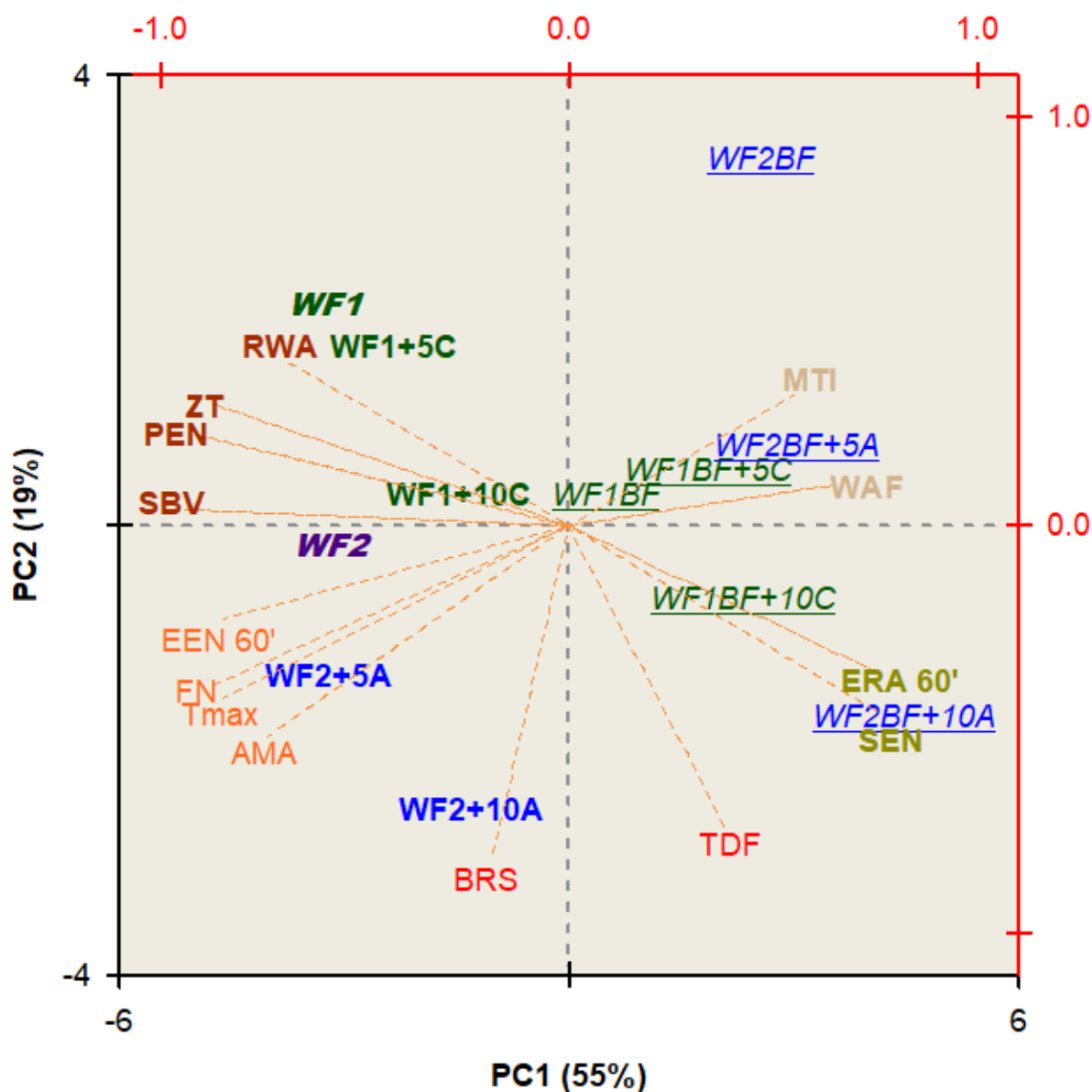


Figure 3: Principal components (PC) biplot of barley, chestnut and acorn flour influence on wheat flour, dough and bread properties and quality. Samples coding: WF1, WF2 – wheat flour (controls), WF1BF, WF2BF – wheat-barley flour premixes 70:30 wt. %; WF1+5C, WF2+10A – flour composites containing 5% or 10% of chestnut and acorn, respectively. Variables coding: TDF – total dietary fibre content; FN – Falling number; Zeleny – Zeleny sedimentation value; WAF – farinograph water absorption; MTI – mixing tolerance index (dough softening degree); ERA 60', EEN 60' – extensigraph ratio and energy after 60 min of dough resting; AMA, Tmax – amylograph maximum and proper temperature; RWA – recipe water addition; SBV – specific bread volume; BRS – bread shape (height-to-diameter ratio); PEN – crumb penetration; SEN – sensory analysis.



screening Falling number method, the detailed amylograph pasting test and specific bread volume and crumb penetration (Pearson's  $r$  between 0.63 and 0.78). In case of dietary fibre polysaccharides, it seems they played more a important role at lower temperatures during dough mixing (in water absorption) and in bread sensory evaluation ( $r = -0.73$  and  $0.76$ , respectively;  $p < 0.05$ ).

Estimation of baking quality of protein (Zeleny value) in flour composites played a significant role in the prediction of bread quality; with exception of bread shape, it was correlated with four further bread attributes (mostly with calculated  $p < 0.01$ ). Likewise for extensigraph elasticity-to-extensibility ratio and energy, it was demonstrated that their principal effect was on bun size development (e.g. correlations to specific bread volume,  $r = 0.82$ ,  $-0.83$  and  $0.81$ , respectively;  $p < 0.01$ , Table 5).

Finally, specific bread volume could also be estimated on the basis of recipe water addition as well as backwards on the basis of crumb penetration ( $r = 0.52$ ;  $r = 0.93$ ,  $p < 0.01$ , respectively). It supported the premise that bread volume is built on 3D crumb structure (2D porosity) and its physico-mechanical properties.

In summary, the tighter the relationships between the recorded features, the higher the extent of their explanation by multivariate statistical exploration.

### 3.7 Principal component analysis

In a biplot of loadings and scores, the first two principal components (PC) explained 74% of data, i.e. 55% was accounted for PC1 and 19% for PC2 (Figure 3). The third PC explained 20% of variance of the features TDF, extensigraph ratio, recipe water addition and bread shape. In addition, PC4 explained both farinograph features, i.e. for the water absorption and the mixing tolerance index (38% of trait variability; data not shown). Fourteen representative variables were split into four main groups, demonstrating relationships to bread quality. Specific bread volume and crumb penetration were positively influenced by protein quality (Zeleny value, extensigraph energy, recipe water addition) and

negatively on polysaccharide pasting behaviour (Falling number and both amylograph features). It could be presumed that the increasing rate of TDF had an impact on dough rheological properties (water absorption, mixing tolerance index, and extensigraph ratio) and bread characteristics including sensory profile.

As mentioned above, the absence or presence of BF in dough or bread recipe was crucial to distinguish between the flour composites. Along the PC1 axis, barley flour lowered baking potential of bi-composite mixtures. The PC2 could be associated with addition level of non-traditional plant materials. In this regard, contradictory effects were identified for 10% of chestnut and 5% of acorn flour - the former had a worsening effect, but the latter an improving effect (i.e. specific bread volumes 253 and 347 mL/100 g, respectively).

In summary, wheat flour composites with 10% of chestnut flour and wheat-barley ones with 5% of chestnut or acorn flour could be recommended for bakery use.

## 4 Conclusion

Enhancement of wheat flour by barley, chestnut and acorn flours contributed to a dietary fibre increase of twice the control level. In flour composites, these non-traditional materials also introduced non-gluten proteins. Such composition changes influenced the technological quality of both polysaccharides and proteins - values of Falling number and Zeleny test were lowered significantly. The effects of chestnut and flour were different, reflecting a wide granulation of the materials. Pasting properties of flour composites were recorded during the amylograph test, and samples could be differentiated according to the temperature of the viscosity maximum. With exception of wheat-acorn samples containing 5% or 10% of the acorn flour, viscosity maxima were determined to be about 10-25% lower in comparison to wheat controls. In this regard, effect of barley flour was stronger than that of chestnut or acorn flour.

Recipe modification also led to changes in dough physico-mechanical properties - non-gluten materials had more influence on elasticity than ex-

tensibility of non-fermented dough. Extensigraph elasticity-to-extensibility ratio rose to approximately double for wheat-chestnut, wheat-acorn and wheat-barley mixtures. For tri-composite blends, this increase reached a still higher level. Modified polysaccharide and protein properties also significantly influenced rheological behaviour during dough kneading. Water absorption was lessened by additions of chestnut flour, corresponding to the higher portion of sucrose in the flour. Conversely, acorn and barley fibre gradually increased the amount of water necessary to reach the required dough consistency by up to 3 percent points. Dilution of the gluten matrix in the dough led to prolongation of dough development time, and to partial loss of dough resistance to overmixing. Such dough modification caused lower specific bread volumes and variation in the shape of the buns in general; the higher the enhancement level, the lower the bread volume. Lower bread sizes also meant denser crumb, as shown by firmness measurement with the penetrometer. Sensory profiles of bread variants differed primarily on basis of the alternative material added. The wheat-barley and wheat-acorn samples were considered to be less acceptable due to characteristic barley aroma and taste and bitter taste plus acid aftertaste of acorn flour. Wheat-chestnut counterparts had a light sweet taste, but a partial stickiness was identified during mastication. Total sensory score of wheat-barley-acorn bread was clearly worse than the wheat-barley-chestnut one, but in both cases, scores could be categorised as “acceptable with some reservation”. As a compromise between fibre content and final properties of a bakery product, wheat flour composites with 10% of chestnut flour and wheat-barley ones with 5% of chestnut or acorn flour could be recommended for practical usage.

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## Stability of Vitamin C in Broccoli at Different Storage Conditions

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### Abstract

In this study, the retention of vitamin C in fresh broccoli stored at different temperatures (i.e. chiller, room, cooking, and roasting or baking; 5-120°C) was investigated. The thermal stability of vitamin C in broccoli was analysed at 5, 20, 45, 60, 70, 80, 110, and 120°C. The vitamin C content was measured by the indophenol titration method. Vitamin C was affected negatively at all stored temperatures. The degradation of vitamin C was modelled by first-order reaction kinetics and the reaction rate constants were observed as  $9.03 \times 10^{-8}$  and  $5.65 \times 10^{-3} \text{ s}^{-1}$  when stored at 5°C and 120°C, respectively. The activation energy was estimated as 74.2 kJ/mol within the temperature range used in this study. The lowest decay of vitamin C was observed during the chilling condition. The data on retention of vitamin C in broccoli could be used to determine their stability, when stored as raw, and when heated at different temperatures.

**Keywords:** Ascorbic acid; First-order reaction; Cooking; Chilling; Activation energy

### 1 Introduction

Vitamins serve as an essential component in metabolism and could be used to protect the human body against diseases, such as cancer, cataracts, and cardiovascular diseases. It is shown from epidemiological studies that a high intake of vegetables and fruits is correlated with a low risk of diseases due to their antioxidants, health functional components and vitamins, such as ascorbic acid (vitamin C), carotenoids, and tocopherols (Bergquist, Gertsson, & Olsson, 2006; Wootton-Beard & Ryan, 2011). Broccoli has gained considerable attention due to its health-promoting ability, which has been attributed to bioactive phytochemicals such as nitrogen-sulfur compounds (glucosinolates and isothio-

cyanates), phenolic compounds (chlorogenic and sinapic acid derivatives and flavonoids) and vitamins (Dominguez-Perles, Carmen Martinez-Ballesta, Carvajal, Garcia-Viguera, & Moreno, 2010; Suresh, Al-Habsi, Guizani, & Rahman, 2017).

Among the vitamins, vitamin C is one of the most important for maintaining human health (Hamad, 2009; Kumar, Ajay Kumar, Raghu, & Manjappa, 2013). Fruits and vegetables contain a high amount of ascorbic acid. Vitamin C in the natural forms of L-ascorbic acid (L-AA) and L-dehydroascorbic acid (L-DA) is found in foods (Ismail, 2013). It is a highly unstable molecule and could decrease during domestic and industrial processing through enzymatic and non-enzymatic reactions (Munyaka, Makule,

Oey, Van Loey, & Hendrickx, 2010). Therefore, processing treatments like peeling, bruising and cutting fruits or vegetables into pieces, and air exposure of the disrupted cells cause oxidation of L-AA in the presence of ascorbic acid oxidase (AAO) and this decreases the retention of ascorbic acid (Munyaka et al., 2010). The degradation can be triggered by many factors such as moisture, temperature, light, pH, metal ions, and oxygen (Munyaka et al., 2010; Spinola, Mendes, Camara, & Castilho, 2012). Enzymatic degradation involves oxidation of L-AA to dehydroascorbic acid (L-DA) and loss of its antiscorbutic activity (i.e. ability to prevent scurvy). The antiscorbutic activity can be lost rapidly and irreversibly by hydrolysis of L-DA to 2,3-diketogulonic acid (2,3-DKG) (Jain & Mulay, 2014). The oxidation is catalyzed by the enzymes, AAO, and ascorbic acid peroxidase (AAP) (Venkatesh & Park, 2014).

The stability of vitamin C depends on many factors, such as the types of fruits and vegetables, growing conditions, level of maturity, storage and processing conditions (Masamba & Mndalira, 2013). For instance, about 8-25% of ascorbic acid is lost when apples are peeled. The best retention of vitamin C is possible with fresh or minimal processing compared with thermal and drying processing methods (Oyetade, 2012; Spinola, Mendes, Camara, & Castilho, 2013). The ripening of fruits causes gradual loss of vitamin; however, this can be slowed down by refrigeration (Oyetade, 2012). Phillips, Council-Troche, McGinty, Rasor, and Teresa Tarrago-Trani (2016) studied the stability of vitamin C in fruit and vegetable homogenates stored at different temperatures (10, -20 and -55°C) and observed that the degradation depended on the storage temperatures, as well as the types of fruits and vegetables. Maximum losses were observed as 23% and 94% after 1 and 7 days of storage, respectively. The vitamin C stability in three types of juices (pineapple, guava and baobab) was influenced by storage temperature, storage time and types of preservatives used. At room temperature, vitamin C in pineapple juice (34.7 mg/100 ml) decreased to 89.9% after 2 months of storage.

Nath, Bagchi, Misra, and Deka (2011) studied the weight loss, ascorbic acid, chlorophyll,  $\beta$ -

carotene, and total antioxidant activity decay in fresh broccoli stored at 15°C and 4°C. The ascorbic acid (i.e. 130 mg/100 g FW) decay of the stored samples at 15°C and 4°C (open atmosphere) were 92.9% and 29.2%, respectively, after 6 days. Favell (1998) studied the stability of vitamin C in peas (three varieties), green beans, broccoli, and spinach at ambient, chill, and frozen conditions. They observed that the decrease of ascorbic acid in peas after post-harvest varied from 26 to 31 mg/100 g, respectively, depending on the variety. The samples stored at 4°C (chilled) showed little change in the first 3 days, but reduced steadily at 2-3% per day. On the other hand, the loss was much faster for the peas stored at ambient temperature (20°C) and the loss was about 10% per day over the first 7 days. The ascorbic acid content of freshly harvested broccoli was reported within the 77.0-93.0 mg/100 g sample. A steady loss was observed for broccoli stored at ambient storage with only 44% ascorbic acid retained after 7 days of storage and 28% after 14 days. However, at chilled temperature, the retention was much better, with no loss after 7 days of storage and 80% retention even after 21 days. Most of the studies determined percent losses during a predetermined storage time. There are relatively few reports available in the literature on the degradation kinetics of vitamin C in fruits and vegetables (Ariahu, Abashi, & Chinma, 2011). Yet the reaction order, rate constant, and activation energy are essential for predicting food quality loss during storage, as well as thermal processing (Nisha, Singhal, & Pandit, 2005).

There are negligible studies in the literature on the loss of vitamin C in broccoli during storage and processing over a wide temperature range. The objective of this study was to determine the stability of vitamin C at different temperatures (i.e. 5-120°C). The experiments were performed in isothermal conditions and the selected temperatures could be used to simulate the conditions of chilling and room temperature storage, cooking, and roasting or baking conditions. The reaction rate was modelled by first-order reaction kinetics.



## 2 Materials and Methods

### 2.1 Sample Collection and Preparation

Fresh broccoli (*Brassica oleracea*, variety: Calabrese) grown in Oman were purchased from a local supermarket. The vegetables were washed in tap water to remove the dirt adhering to them and was spread on tissue paper to absorb the excess surface water. Only florets were cut from the bunch and eight batches of 200 g samples were placed into different aluminium cells and stored at temperatures of 5, 20, 45, 60, 70, 80, 110, and 120°C. Previous reports showed that depending on the type of vegetables and fruits and effects of temperature (low and high), more or less heating time would be needed to measure the vitamin C content (Hal, Bosschaart, Twisk, Verkerk, & Dekker, 2012; El-Ishaq & Obirinakem, 2015; Polinati, Kremer Faller, & Fialho, 2010). Considering this aspect, different time frames were used for different storage temperatures. The heating time and temperature for broccoli samples are shown in Table 1. Samples were taken from each cell at different time intervals and their vitamin C content was measured.

### 2.2 Measurement of Ascorbic Acid in Broccoli

Vitamin C was measured according to the AOAC (1990) using a titration method with 2,6-dichloro-indophenol reagent. Samples of 10.0 g broccoli were weighed at different time intervals from each storage temperature and blended with 100 ml metaphosphoric acid (3%). The solution was then vacuum filtered and transferred to a 100 ml volumetric flask. Ten ml of the diluted sample solution was then titrated against the standardized dye. The dye was standardized by a known concentration of ascorbic acid solution (0.1 mg AA/ml). The end-point was indicated by the appearance of a light pink colour. The result was expressed as mg/100 g fresh broccoli sample and replicated three times.

### 2.3 Reaction Kinetics

The loss or degradation of vitamin C is commonly modelled by the first-order reaction as follows:

$$\ln\left(\frac{C}{C_0}\right) = -k_1 t \quad (1)$$

where,  $C$  is the concentration of vitamin C in a sample at time  $t$  (mg/g sample),  $C_0$  is the initial concentration of vitamin C in the sample (mg/g sample),  $k_1$  is the first-order rate constant ( $s^{-1}$ ) and  $t$  is the storage time (s).

In most of the isothermal experiments, an initial lag period was observed and the above equation was modified with an intercept rather forcing the intercept to zero as Equation 1 (Rahman et al., 2015). The experimental data was fitted with a linear equation with an intercept as follows:

$$\ln\left(\frac{C}{C_0}\right) = -k_1 t + a \quad (2)$$

where,  $a$  is the intercept. The rate constant was determined from the slope of a linear plot  $\ln(C/C_0)$  versus  $t$ . Activation energy of first-order kinetics was estimated using the Arrhenius equation as:

$$\ln(k_1) = -\left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right) + b \quad (3)$$

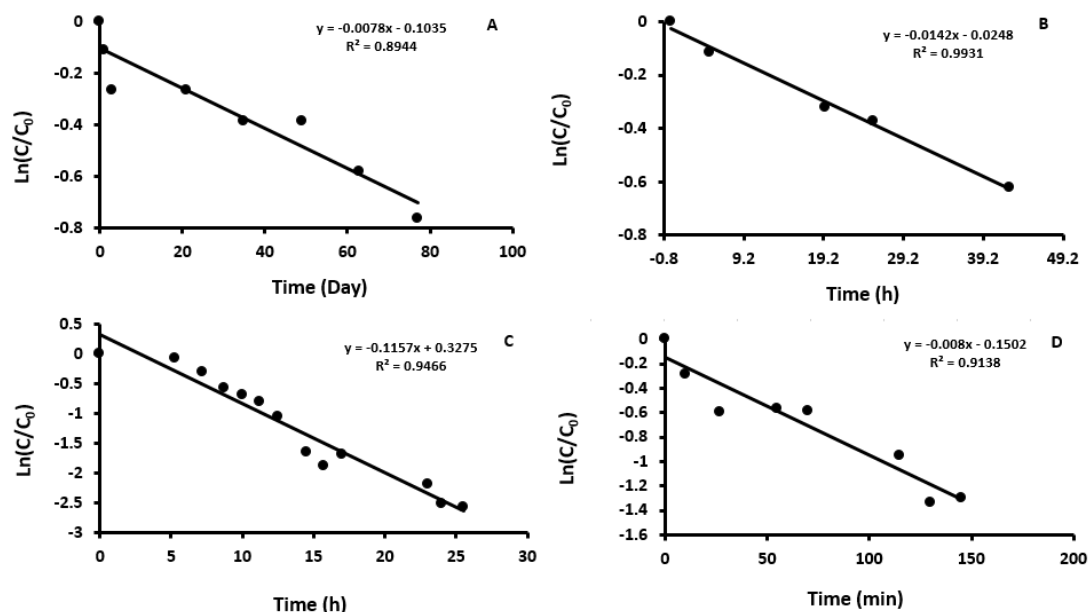
where,  $R$  is the universal gas constant (8.314 J/mol K),  $E_a$  is the activation energy (J/mol),  $T$  is the temperature (K), and  $b$  is the pre-exponent factor. The activation energy was estimated from the slope of the linear plot of  $\ln k_1$  versus  $1/T$ .

### 2.4 Statistical Analysis

Each experiment was replicated three times and the regression analysis of Equations 2 and 3 were performed using Microsoft Excel (MS-Excel, 2016). The regression coefficient was considered as the goodness of the regression equation.

Table 1: Temperature and heating scheme for broccoli

Broccoli								
Temperature (°C)	5 (Days)	20 (h)	45 (h)	60 (min)	70 (min)	80 (min)	110 (min)	120 (min)
Time	0	0	0	0	0	0	0	0
	1	5	5.25	10	5	4	3	2
	3	19.5	7.25	27	15	9	6	5
	21	25.5	8.75	40	25	14	9	8
	35	42.5	10.00	55	40	22	13	11
	49		11.25	70	50	32	19	16
	63		12.50	100	60	42	25	21
	77		14.50	115	70	52	31	26
			15.75	130	80	67	38	31
			17.00	145		82	45	36
			23.00			97		
			24.00					
			25.50					

Figure 1: Plots of  $\ln(C/C_0)$  versus time. A: 5°C, B: 20°C, C: 45°C, D: 60°C

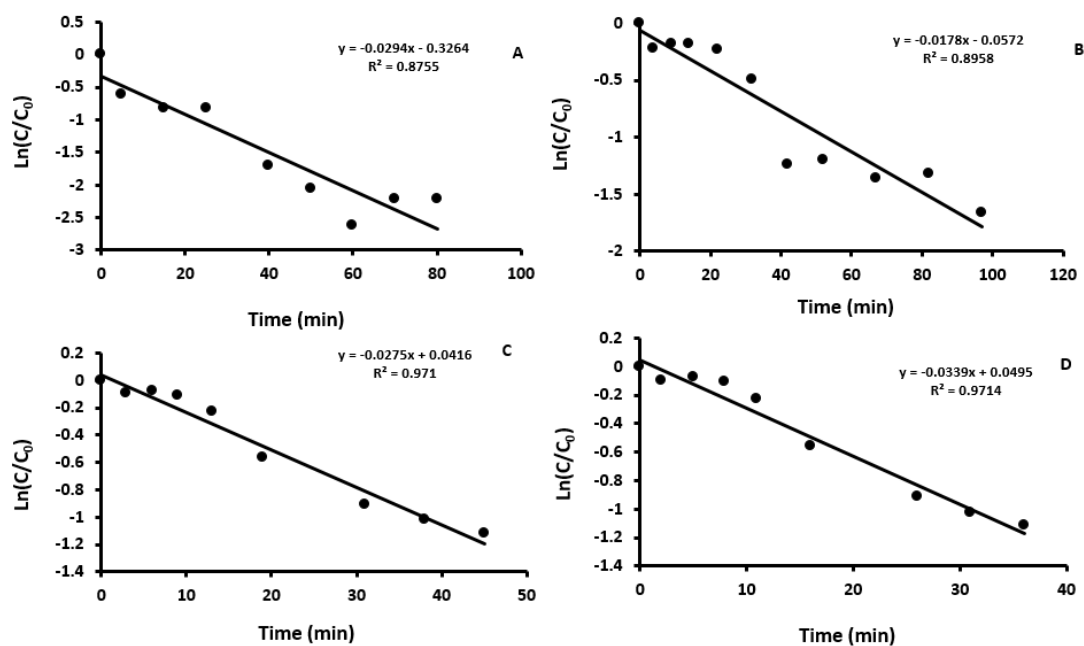


Figure 2: Plots of  $\ln(C/C_0)$  versus time. A: 70°C, B: 80°C, C: 110°C, D: 120°C

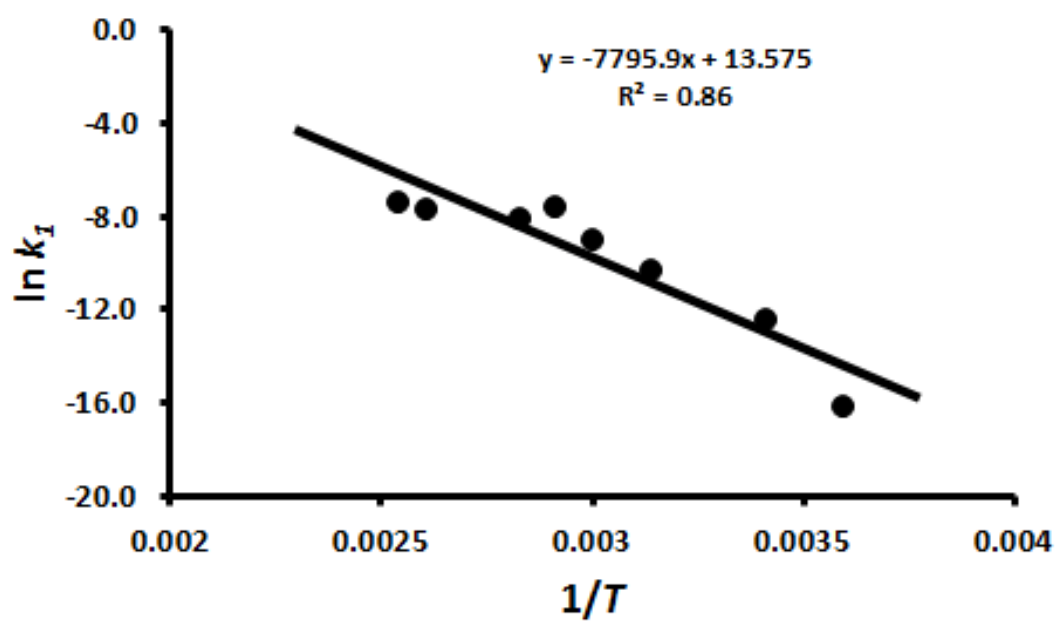


Figure 3: Arrhenius plot  $\ln(k_1)$  versus  $1/T$

### 3 Results and Discussions

The composition of fresh broccoli was determined and presented in our preliminary study (Suresh et al., 2017). Moisture, protein, fat, crude fibre, ash, and carbohydrate content of broccoli was 90.20, 2.08, 0.33, 0.75, 0.74 and 5.90 g/100 g sample, respectively; and pH was determined as 6.8. The vitamin C in the fresh broccoli varied from 53.6 to 64.3 mg/100 g sample. In order to study vitamin C stability with wide variations in temperature, different time frame experiments were used, as also identified earlier by Hal et al. (2012), El-Ishaq and Obirinakem (2015) and Polinati et al. (2010). The heating time frame at different temperatures is shown in Table 1. Different time frames could be used to compare vitamin C stability. For example, the vitamin C reduced to 40.4 mg/100 g sample (i.e. 62.8% loss) after 35 days of storage when stored in chilled conditions at 5°C, whereas vitamin C reduced to 45.0 mg/100 g sample (i.e. 69.9% loss) after 7.2 h when stored at 45°C. Similarly, vitamin C reduced to 38.4 and 36.0 mg/100 g sample (i.e. 59.7% and 56.6% loss, respectively) after 27 and 16 min when stored at 60 and 120°C, respectively. Therefore, time to a specific reduction of vitamin C (for example above, 36-45 mg/100 g sample) could be used to compare data when stored at different temperatures. However, kinetics modelling with rate constants ( $s^{-1}$ ) better standardize the processes when different time frames are used.

The vitamin C (ascorbic acid, AA) losses in broccoli, cauliflower, and cabbage were studied at different stages (delivery, cooked for 30 min and blast chilled, 0-3°C) (Charlton, Patrick, Dowling, & Jensen, 2004). The AA content of broccoli, cauliflower, and cabbage from delivery to blast chilling varied from 2.9-24.0 mg/100 g sample. The dramatic AA losses occurred during cooking (33-81%), with broccoli showing the great loss (81%). During the 4-day chill storage, a steady decline of AA was observed with a loss/day of 4.2%. The loss of AA levels in broccoli during chilled storage was 3.6% per day. The loss/day for cauliflower, and cabbage was 7.6% and 2.3%, respectively.

Goncalves, Abreu, Brandao, and Silva (2011) studied the vitamin C loss in frozen broccoli

(*Brassica oleracea* L. ssp. *Italica*) during storage. The vitamin C content significantly decreased after 121 days of isothermal storage by 80%, 60%, and 29%, respectively at -7, -15, and -25°C. The major loss of vitamin C occurred during the first 55 days of storage. Shobham, Mudhavath, and Sukumaran (2017) studied the effect of microwave and pressure cooking on the stability of vitamin C in vegetables (carrot, potato, spinach, brinjal, cauliflower, green chilli, bitter gourd, and cabbage). The total content of vitamin C in raw vegetables ranged from 3.55 to 91.27 mg/100 g. Microwave and pressure cooking for 2 to 3 min resulted in considerable loss of vitamin C. The percentage loss of vitamin C content in cabbage subjected to microwave and pressure cooking was 9.9% and 49.5%, respectively, whereas it was 20.7% for cauliflower subjected to microwave and 46.2% loss during pressure cooking. A greater loss was observed for potato and spinach. Shams El-Din, Abdel-Kader, Makhoulouf, and Mohamed (2013) studied the effect of cooking methods on natural antioxidants in Brassica vegetables. The result showed that boiling had a greater loss of vitamin C compared to microwave cooking. Boiling for 6 min caused a loss of 64.5% in broccoli, 70.7% loss in white cabbage, and 66.8% in cauliflower.

Figures 1 and 2 show the plot of  $\ln(C/C_0)$  versus time according to first-order reaction kinetics (Equation 2). The high regression coefficient from 0.88 to 0.99 indicates that vitamin C decay can be predicted according to the first-order reaction. Similarly, first-order reaction was used for the degradation of vitamin C in cabbage and lettuce (Awagu, Ekanem, Kolo, & Adamu, 2017), cherry juice (Jirasatid & Noipant, 2015) and orange juice (Calligaris, Manzocco, & Lagazio, 2012). The first-order reaction rate constant was determined from the slope and these increased from  $9.03 \times 10^{-8} s^{-1}$  to  $5.65 \times 10^{-3} s^{-1}$ , when storage temperature varied from 5°C to 120°C. Vitamin losses in fresh capsicum at 5°C and 20°C were observed as  $8.22 \times 10^{-7}$  and  $1.15 \times 10^{-6} s^{-1}$  as compared to the results found in this study of  $9.03 \times 10^{-8}$  (at 5°C) and  $4.03 \times 10^{-6} s^{-1}$  (at 20°C) (Rahman et al., 2015). At refrigerated temperatures, the loss of vitamin C in broccoli was much higher than capsicum, whereas comparable losses were observed at 20°C. In the case of orange juice

stored at 10°C, the rate constant was observed as  $4.63 \times 10^{-7} \text{ s}^{-1}$  (Calligaris et al., 2012). In the case hot water blanching of pumpkin at pH 6.5, the rate constant increased from  $8.10 \times 10^{-4}$  to  $1.37 \times 10^{-3} \text{ s}^{-1}$  when blanching temperature increased from 60°C to 90°C (Ariahu et al., 2011). The reaction rate constant of cherry juice increased from  $3.12 \times 10^{-4}$  to  $1.00 \times 10^{-3} \text{ s}^{-1}$  with increasing temperatures (75, 80, 85, 90 and 95 °C) (Jirasatid & Noipant, 2015). The rate constant observed in this study was similar to earlier reported values. The degradation of ascorbic acid in broccoli could be mainly due to the oxidation of ascorbic acid by oxidizing enzymes, e.g. ascorbic acid oxidase, peroxidase, catalase, and polyphenol oxidase (Mapson, 1970; Venkatesh & Park, 2014). Two types of vitamin C degradation could occur: aerobic and anaerobic degradation. In aerobic degradation, the AA is oxidised to L-dehydro-ascorbic acid (L-DA) followed by hydrolysis and further oxidation, whereas anaerobic degradation has not been clearly studied and reported (Wang, Law, Mujumdar, & Xiao, 2017). During processing, matrix disruption could occur thus facilitating the oxidation of L-AA to L-DA by the enzyme AAO. The L-DA can then be further hydrolysed to 2,3-diketogulonic acid, thus losing its antiscorbutic activity. Other possible chemical reactions associated with changes in flavour, colour, and odour could have occurred with time due to interactions among the components and this resulted in changes in pH (El-Ishaq & Obirinakem, 2015; Munyaka et al., 2010). In this study, low temperature likely caused minimal destruction of structure, thus enzymatic degradation reaction was observed at a slower rate as compared to high temperature. At high temperature, structural damage could enhance enzymatic degradation as well as increase interaction with other released components. Figure 3 shows the Arrhenius plot of  $\ln(k_1)$  versus  $1/T$ , and activation energy was estimated from the slope as 74.2 kJ/mol. Jirasatid and Noipant (2015) observed a similar activation energy of 69.4 kJ/mol for cherry within the temperature range 75-95°C. The higher activation energy indicated that temperature could affect degradation at a faster rate. The activation energy was observed as 58.0, 39.0, and 29.0 kJ/mol, respectively, for guava, mango, and marula pulps

within the temperature range of 80-150°C (Hal et al., 2012). The high activation energy of vitamin C in broccoli implied that the deterioration was more sensitive to temperature as compared to the vitamin C degradation in guava, mango, and marula pulps. The activation energy of ascorbic acid loss during pumpkin blanching (60-90°C) varied from 16.9 to 41.2 kJ/mol, while pH increased from 5.0 to 6.5, respectively (Ariahu et al., 2011). The pH of the fresh broccoli was measured as 6.8 (Suresh et al., 2017). Therefore, the activation energy of broccoli could be expected to be higher than the reported values for pumpkin. In the case of heat treatment (80-90°C) of apple, the activation energy was observed as 88.9 kJ/mol. The activation energy varied from 10.7 to 99.2 depending on the type of fruits and vegetables (Courtois, Vedrenne, & George, 2009). Therefore, the activation energy of vitamin C loss depended on the types of fruits and vegetables, as well as their physicochemical properties and pH.

## 4 Conclusion

The loss of vitamin C increased with storage time and temperature (5-120°C). Vitamin C degradation in broccoli followed a first-order reaction and the temperature dependence of rate constants was described using the Arrhenius model. The activation energy was estimated as 74.2 kJ/mol. Data on the retention of vitamin C could be used when broccoli is stored as raw and when it is cooked at different temperatures. The rate constants at different temperatures could be used in simulation and optimization of thermal processes.

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## Physico-Chemical Composition and Antimicrobial Protein Content of Early Lactation Donkey Milk

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### Abstract

The influence of early lactation on chemical composition and the concentration of antimicrobial proteins of donkey's milk produced in Cyprus were investigated. Milk samples from 10 female donkeys in their first season of lactation were collected at 7, 15 and 30d postpartum. The average contents of donkey milk gross composition were 1.40% protein, 0.16% fat and 8.74% total solids. Results showed that lactation had a significant negative effect on protein concentration, while total solid concentration showed an increased followed by a decrease. Composition of antimicrobial proteins also showed a significant decreased during lactation period except from lactoferrin which showed an increase. On the other hand, throughout the lactation, pH and fat were constant.

**Keywords:** Donkey milk; Antimicrobial proteins; Immunoglobulin-A; Lactation

### 1 Introduction

Milks from non traditional animal species (i.e., donkey, camel, and buffalo) are gaining interest due to the fact that they are considered suitable to supplement the needs of special population groups such as infants and elderly. Donkey milk has been studied less compared to that of ruminant milks, but in recent years interest in donkey's milk production and commercialization have significantly increased. This increase in interest is due to its unique nutritional and physicochemical characteristics as well as its functional properties such as antimicrobial, immunomodulatory, anti-inflammatory, and anti-hypertensive properties (Aspri, Economou, & Papademas, 2017; Brumini et al., 2013; Jirillo & Magrone, 2014; Mao et al., 2009; Zhang, Zhao, Jiang, Dong, & Ren, 2008).

The nutritional composition of donkey milk is very similar to that of human milk and it has been reported to be an adequate replacement for children with cow milk protein allergy, mainly due to its tolerability, nutritional contents and good taste (Aspri et al., 2017; Monti et al., 2012). Donkey milk is characterized by low fat and protein content and high lactose content. It is also characterized by low casein content and a particularly high whey protein concentration, rich in lysozyme which is around 6000 times more than lysozyme content of bovine milk (Guo et al., 2007; Salimei et al., 2004).

Previous studies have shown that the strong antimicrobial activity of donkey milk arise from its high concentrations of antimicrobial agents such as lysozyme and lactoferrin (Tidona et al., 2011; Vincenzetti et al., 2008). Lysozyme is the main protein in donkey's milk that plays an impor-

tant role of fighting infections in breastfeeding infants, while lactoferrin is the second antimicrobial protein in donkey milk (Uniacke-Lowe, Huppertz, & Fox, 2010). In addition, these two antimicrobial proteins work synergistically with other proteins such as lactoperoxidase and immunoglobulins (Polidori & Vincenzetti, 2010). The high content of these antimicrobial proteins are considered to be the reason for the low microbial counts in donkey milk (Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005; Salimei et al., 2004; Sarić et al., 2012; Vincenzetti et al., 2008). As some donkey milk producers are marketing "early lactation milk" the aim of this research was to study the influence of early lactation stage on some physicochemical parameters of donkey milk (TS, protein, fat and pH) and also on the concentration of antimicrobial proteins of donkey milk.

## 2 Materials and Methods

### 2.1 Animals and sampling procedure

This study was carried out at "Golden donkey's farm" located in Skarinou, Cyprus. The farm had three breeds of donkeys (Asia, Cyprus and Israel) and their crosses. Raw milk samples were obtained by hand milked of 10 female donkeys, after parturition on 1<sup>st</sup>, 15<sup>th</sup> and 30<sup>th</sup> day of lactation, always at the same time of the day. The animals were maintained within the same conditions, and receiving the same feeding level and composition. Their diet was composed of hay, wheat and silage. The milk samples of an average volume of 100mL were collected in standard containers, and transported same day to the laboratory, and kept at 4°C for further analysis.

### 2.2 Physicochemical analysis

#### Determination of pH

The pH of the donkey milk samples was determined by potentiometry. The pH meter glass probe was first calibrated using standard buffers at pH 4 and 7 before being used to measure the

sample pH. Sample pH was measured by submerging the tip of the probe into the sample for ~1-2 min until a stable reading was registered on the pH meter scale. Measurements were done in triplicates and average values were reported.

#### Protein content

The protein content of milk samples was determined using the Bradford Protein assay (Bradford, 1976). Bovine serum albumin (BSA) was prepared for standard curve using the following concentrations, 50 µg/ml to 300 µg/ml. After incubated for 5 minutes at room temperature, absorbance measurements were determined spectrophotometrically (Infinite PRO 200, Tecan, Switzerland), at 595 nm. The Bradford reagent was used as a blank. Measurements were done in triplicates and average values were reported.

#### Fat content

Milk fat content was determined by a butyrometric method according to Gerber (IDF ISO 488, 2008). Milk samples (11 ml) were mixed with sulfuric acid (10 ml) and 1 ml of isoamyl alcohol in butyrometer and closed with rubber cork. The mixture was mixed and placed in a water bath at 65°C. The sample was centrifuged in a Gerber centrifuge for 5 min at 1000 rpm and the butyrometer was placed back into the water bath for another 5 min. The fat content is expressed as a percentage on the butyrometer. Measurements were done in triplicates and average values were reported.

#### Total Solids Content

Total solids were determined by the oven method in accordance with ISO 6731 (IDF standard 21, 2010a). 2g of fresh donkey milk samples were transferred to a pre-weighed round flat bottom aluminum dish. Then, the dishes were transferred to a hot air oven at 102 °C for 2 hours. Then the dish was transferred to a desiccator for 30 min to cool down and weighted. Total solids content was calculated by the following formula:

$$\text{Total solids (\%)} = \frac{m_2 - m_0}{m_1 - m_0} \times 100 \quad (1)$$

where  $m_0$  is the mass (g) of the dish,  $m_1$  is the mass (g) of the dish and the test portion and  $m_2$  is the mass (g) of the dish and the dried test portion. Measurements were done in triplicates and average values were reported.

## 2.3 Antimicrobial proteins and IgA

### Lysozyme content of donkey milk

Lysozyme was quantified according to a sensitive fluorescence-based method using EnzChek® kit (Life Technologies, Carlsbad, CA, USA). The assay is based on the assessment of the lytic activity of lysozyme in the cell walls of *Micrococcus lysodeikticus* which have been labeled with the fluorescent dye fluorescein. The test was performed according to the manufacturer's instructions and the absorbance was determined spectrophotometrically (Infinite PRO 200, Tecan, Switzerland), at 497nm and 518 nm. All samples were analyzed in triplicate.

### Lactoferrin content of donkey milk

Quantitative determination of LF in the skim milk samples was performed using a commercial ELISA kit, the Bovine Lactoferrin ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX). The procedures were performed according to manufacturer's instructions. A standard curve was prepared using the supplied lactoferrin standard diluted to concentrations of 31.2 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml using the sample diluent buffer (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 8.0). The final absorbance of the samples and the standards was measured at 450 nm, using an ELISA plate-reader (Infinite F200; Tecan, Mannedorf, Switzerland), and the concentration of lactoferrin calculated by extrapolating from a standard curve. All samples were analysed in triplicate.

### Immunoglobulin A content of donkey milk

Total IgA in milk was determined by using Horse IgA ELISA Quantitation Kit (Bethyl Labora-

tories Inc., Montgomery, TX, USA), according to the manufacturers' instructions. A standard curve was prepared using the supplied lactoferrin standard diluted to concentrations of 1000 nm/ml, 500 nm/ml, 250 nm/ml, 125 nm/ml, 62.5 nm/ml, 31.25 nm/ml, 15.6 nm/ml, 0 nm/ml using the sample diluent buffer. The final absorbance of the samples and the standards was measured at 450 nm, using an ELISA plate-reader (Infinite F200; Tecan, Mannedorf, Switzerland), and the concentration of IgA calculated by extrapolating from a standard curve. All samples were analyzed in triplicate.

## 2.4 Statistical Analysis

All experiments were carried out in triplicates. The data are expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The data were subjected to one-way analysis of variance (ANOVA) to determine the differences of samples. Significant differences were compared by Tuckey test on the level of  $P < 0.05$ .

## 3 Results and Discussions

### 3.1 Physicochemical analysis

The changes in the physico-chemical parameters and composition (protein, fat, pH, TS) of donkey milk during the early lactation period (30 days) are presented in Table 1.

The pH value of donkey milk, ranging from 7.03 to 7.06, did not vary significantly throughout the lactation period (30 days), which was consistent with the findings of Salimei et al. (2004). According to the literature the pH of donkey milk after milking is  $7.19 \pm 0.10$  (Curadi, Giampietro, Lucenti, & M., 2001). This suggests that the pH value was not influenced by breed or stage of lactation (Chiavari et al., 2005; Guo et al., 2007; Malacarne, Martuzzi, Summer, & Mariani, 2002; Polidori, Beghelli, Mariani, & Vincenzetti, 2009). The average pH value ( $7.03 \pm 0.02$ ) of donkey milk was higher than that of cow milk which is in the range of 6.60 to 6.80. According to Salimei et al. (2004), this may be due to the lower casein (CN)

Table 1: Chemical composition of donkey milk at different stages during the lactation period

	Day 7			Day 15			Day 30		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
<b>Protein Content (g/100ml)</b>	1.55 ± 0.41 <sup>b</sup>	1.02	2.19	1.75 ± 0.38 <sup>c</sup>	1.30	2.64	0.90 ± 0.21 <sup>a</sup>	1.02	1.94
<b>Fat (%)</b>	0.15 ± 0.08 <sup>b</sup>	0.05	0.30	0.18 ± 0.22 <sup>c</sup>	0.05	0.80	0.14 ± 0.07 <sup>a</sup>	0.05	0.30
<b>TS (%)</b>	8.73 ± 0.57 <sup>a</sup>	8.13	10.02	9.10 ± 0.40 <sup>b</sup>	8.42	9.69	8.40 ± 0.40 <sup>a</sup>	8.29	9.22
<b>pH</b>	7.03 ± 0.13 <sup>a</sup>	6.83	7.27	7.01 ± 0.11 <sup>a</sup>	6.81	7.19	7.06 ± 0.07 <sup>a</sup>	6.98	7.21

Values are means ± standard deviation; values within the same row followed by different superscript letters significantly differ ( $p < 0.05$ ).

TS = Total Solids

and phosphate contents in donkey milk than in cow milk (Salimei et al., 2004). The phosphate content of cow milk is 1000 mg/L, while the phosphate content of donkey milk is very low, 638, 42 mg/L (Fantuz et al., 2012).

The total protein content of donkey milk (approximately  $1.4 \pm 0.29\%$ ) was similar to that of human milk and much lower than that of cows', goats' or sheep's milk (Guo et al., 2007). The observed average milk protein content (1.4%) was lower (1.72 %, 1.89 %) than reported by Salimei et al. (2004) and Giosue, Alabiso, Russo, Alicata, and Torrisi (2008). The protein content of donkey milk at 15 days after lactation is statistically significant higher compared to the first day and the 30<sup>th</sup> day of lactation ( $1.75 \pm 0.38$ ,  $1.55 \pm 0.41$  and  $0.90 \pm 0.21$  respectively,  $p < 0.05$ ). The results are consistent with the findings of Salimei et al. (2004), which have reported a falling trend in the protein level of donkey milk during lactation. This may be due to the differential expression of milk protein synthesis genes during the lactation period. The levels of early lactation protein mRNA increased after parturition, they reach the maximum after a few weeks and then they start decreasing (Demmer, Ross, Ginger, Pottie, & Grigor, 1998).

In contrast to the protein content, the fat content of donkey milk does not show a statistically significant difference between the different sampling days. However, other studies showed a significant variation of donkey milk fat content throughout the lactation period (Cosentino, Paolino, Freschi, & Calluso, 2012; Martemucci & D'Alessandro, 2012; Salimei et al., 2004). These differences indicate that fat content could be affected by breed,

breeding area and forage, milking technique, and interval between milkings, as also reported by Fox (2003). The average fat content of donkey milk was  $0.16 \pm 0.02\%$ , which is consistent with that reported in the literature for donkey's milk (Ivankovic et al., 2009; Salimei et al., 2004).

The total solids content of donkey milk was significantly higher on the 15<sup>th</sup> day of lactation compared to the 1<sup>st</sup> and 30<sup>th</sup> of lactation ( $9.14 \pm 0.38$ ,  $8.73 \pm 0.56$  and  $8.35 \pm 0.39$  respectively,  $p < 0.05$ ). The observed average dry matter content ( $8.74 \pm 0.25\%$ ) was consistent with the data for dry matter content (8.84 %) reported by Salimei et al. (2004) and are not affected by breed, lactation stage or milking conditions. The mean dry matter observed in current donkey milk study was of  $8.73 \pm 0.11\%$  which is consistent with the values reported in the literature for donkey milk (Chiavari et al., 2005; Guo et al., 2007; Salimei et al., 2004). The total solids content in the donkey milk according to Polidori et al. (2009) can be up to 8.80 g / 100g, which is lower than the solid residue in cow's milk (12.5 - 13.00 g / 100 g), sheep (17.5 - 19.5 g / 100 g) and human milk (11.70 - 12.90 g / 100 g).

### 3.2 Antimicrobial Proteins

The amount of lysozyme, lactoferrin and IgA concentrations (mg/ml) at different stages of donkey lactation are presented in Table 2.



Table 2: Lysozyme, lactoferrin and IgA concentrations in donkey milk at different stages of lactation

	Day 7	Day 15	Day 30
<b>Lysozyme (U/ml)</b>	225.08 ± 7.05 <sup>b</sup>	224.11 ± 6.00 <sup>a</sup>	201.94 ± 10.96 <sup>a</sup>
<b>Lactoferrin (μg/ml)</b>	111.03 ± 41.04 <sup>b</sup>	Nd	135.08 ± 27.68 <sup>c</sup>
<b>IgA (ng/ml)</b>	2674.40 ± 133.72 <sup>c</sup>	2576.47 ± 143.04 <sup>a</sup>	2267.32 ± 145.12 <sup>a</sup>

Values are means ± standard deviation; values within the same row followed by different superscript letters significantly differ ( $p < 0.05$ ).

Nd=Not determined

## Lysozyme

The EnzChek fluorescence-based assay measures lysozyme activity on *Micrococcus lysodeikticus* cells, which are labeled to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching and the increase in fluorescence that is proportional to lysozyme activity. The mean lysozyme activity value was  $217.04 \pm 13.09$  U/ml. Our results were in accordance with other studies carried out by Gubić et al. (2014); Guo et al. (2007); Pilla, Dapra, Zecconi, and Piccinini (2010); Sarić et al. (2012); Sarić et al. (2014); Vincenzetti et al. (2011). The lysozyme content at 30<sup>th</sup> day was significantly lower compared to the lysozyme content at 1<sup>st</sup> and 15<sup>th</sup> day of lactation ( $201.94 \pm 10.96$ ,  $225.08 \pm 7.05$  and  $224.11 \pm 5.99$  respectively,  $p < 0.05$ ).

The high lysozyme content of donkey milk plays an important role in the prevention or reduction of intestine infections in infants and is also responsible for the low bacteria count of donkey milk reported in literature (Salimei et al., 2004). Moreover, the high lysozyme content does not affect the growth or the acidification activity of probiotic strains, making donkey milk a good base for the production of probiotic fermented milk beverages (Chiavari et al., 2005; Coppola et al., 2002). Finally, the high lysozyme activity could explain the low incidences of mastitis in donkeys (Conte et al., 2006). Donkey milk lysozyme belongs to C-type calcium-binding lysozyme and is able to bind calcium ions; this binding leads to more stable complex with an enhanced antimicrobial activity (Wilhelm et al., 2009).

## Lactoferrin

Results of lactoferrin content in donkey milk showed that lactoferrin content at 30<sup>th</sup> day of lactation was significantly higher compared to the 1<sup>st</sup> day of lactation ( $135.08 \pm 27.68$  and  $111.03 \pm 41.04$  respectively,  $p < 0.05$ ), which is in agreement with literature. A study carried out by Gubić et al. (2015) showed that the concentration of lactoferrin is increased over the lactation period. Moreover, according to Adlerova, Bartoskova, and Faldyna (2008), there is an important correlation between lactation and lactoferrin levels. In general, the content of lactoferrin in donkey milk is higher than in ruminant milk, but much lower than in mare and in human milk (Kanyshkova, Buneva, & Nevinsky, 2001).

Lactoferrin is an iron-binding protein that displays many biological functions such as antioxidant, antiviral, anti-inflammatory, immunomodulatory and anti-carcinogenic activity (Kuwata et al., 1998; Ward, Paz, & Conneely, 2005). Furthermore, it controls the proper composition of the intestinal microflora by suppressing the growth of pathogenic bacteria while promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Madhusudan, Ramachandra, Udaykumar, Nagraj, & Jagjivan, 2017). Different studies suggest that lactoferrin and lysozyme work synergistically to inhibit the growth of pathogenic bacteria and contribute to donkey milk's strong overall antibacterial activity against both Gram-positive and Gram-negative bacteria (Sarić et al., 2012; Tidona et al., 2011). In fact, Lf can bind different components in the outer membrane, thereby opening "pores" to enhance susceptibility of Gram-negative bacteria to the lysozyme by increas-

ing in membrane permeability (Ellison & Giehl, 1991; Leitch & Willcox, 1999).

### Immunoglobulin-A (IgA)

The concentration of IgA of donkey milk was significantly lower at the 30<sup>th</sup> day of lactation compared to the 1<sup>st</sup> and 15<sup>th</sup> day of lactation. ( $2267.32 \pm 159.91$ ,  $2674.47 \pm 178.14$  and  $2576.47 \pm 167.34$  respectively,  $p < 0.05$ ). The average value of IgA was 2.50 g/L.

Immunoglobulins (Igs) are a defence family of globular proteins with antimicrobial and other protective bioactivities. They play an important role in transferring immunity to the newborn by establishing an optimal microfloral population in the gut of the newborn inactivate bacteria by binding to specific sites on the bacterial surface, while its own immune system is developing (Gapper, Copestake, Otter, & Indyk, 2007).

## 4 Conclusion

In conclusion, lactation stage affected the gross composition of donkey milk but had no significant effect on pH and fat content. It can be concluded that the chemical composition of milk in terms of fat, protein, pH, total solid residue, lysozyme, lactoferrin and IgA showed significant differences throughout the different stages of lactation except for fat and pH. However, further research is needed to establish how donkey's nutrition affects the quality of milk; as it is a type of milk of particular interest with unique composition.

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## Study of the Self-Stabilization Ability of Tzatziki (a Traditional Greek Ready-to-Eat Deli Salad)

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### Abstract

Traditional Greek yogurt-based salad Tzatziki is one of the most popular ready-to-eat deli salads in Greece. The objective of this study was to estimate the microbial stability of Tzatziki, with and without chemical preservatives, using a rapid method. Determination of the microbial count was carried out using the bioluminescence method (ATP) and traditional microbiological analysis, plate-counting method (CFU) in various batches of the final product of Tzatziki. The results showed that the Tzatziki salad without preservatives initially gave higher relative light units (RLU) values (79,532) than the same salad with preservatives (43,198) because the potassium sorbate and the sodium benzoate, used in the recipe, appeared to suspend the action of microorganisms. After incubation in two different substrates, MacConkey and Sabouraud, the Tzatziki salad without preservatives gave higher RLU values (9,488 and 16,176, respectively) than the salad with preservatives (12,780 and 12,005, respectively). In the two selective substrates, differences appeared between the two methods of microbial count (RLU and CFU). While RLU values were roughly at the same level, the CFU values presented significant differences ( $p < 0.05$ ). It was also shown that there was a strong correlation ( $R^2 = 0.93-0.95$ ) between bacterial counts estimated by traditional CFU and ATP methods. As expected, the dominant microbial population in Tzatziki was *Lactobacillus* spp., originated from yogurt. Coliforms and yeasts were not able to survive in this environment. Generally, according to the results, Greek traditional Tzatziki salad was a microbial stable product and the bioluminescence method could be a rapid method to determine its microbial state.

**Keywords:** Bioluminescence; Microbial stability; Tzatziki; Greek deli salad

### 1 Introduction

Tzatziki, one of the most popular Greek ready to eat deli salads, is widely consumed in Greece, the East Mediterranean region and Balkan

countries, as well as in other parts of the world, including the rest of Europe and USA. Tzatziki is made of strained yogurt (usually from sheep or goat milk) mixed with fresh chopped cucumber, garlic, salt, olive oil, vinegar and dill.



Tzatziki is a low pH food, due to the presence of yogurt and the added vinegar, and it has a low salt percentage, 2-3%. This dressing has shown to be resistant to microbial spoilage by pathogen microorganisms (Skandamis, Davies, McClure, Koutsoumanis, & Tassou, 2002). Tzatziki is generally recognized for its health properties and for more than a hundred years, people accompany meals rich in fat with this salad.

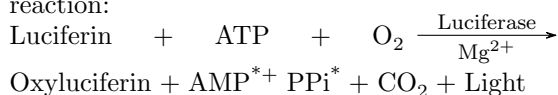
As far as yogurt is concerned, there is a tremendous scientific work related to its microbial stability. The same goes for garlic and garlic extract, with an emphasis on the effect of garlic on microorganisms. Garlic is considered a germicide (Harris, Cottrell, Plummer, & Lloyd, 2001). Although, there is little documentation about cucumber's activity on microorganisms, it has been reported that the volatile oils of cucumber (*Cucumis sativus*) show antibacterial activity, as well as a minimum inhibitory concentration (MIC), 0.09-0.50 mg mL<sup>-1</sup>, against both gram-positive and gram-negative bacteria, and anti-fungal activity against human pathogen fungi (*Candida albicans*, *C. tropicalis* and *C. glabrata*) (MIC = 1.34-2.90 mg mL<sup>-1</sup>) (Sotiroudis, Melliou, Sotiroudis, & Chinou, 2010). (*E,Z*)-2,6-nonadienal and (*E*)-2-nonenal have been found in cucumber and have shown strong activities to several human and foodborne pathogen bacteria such as *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium* (Cho, Buescher, Johnson, & Janes, 2004; Croft, Juttner, & Slusarenko, 1993).

Conventional approaches to the determination of microbial stability rely on fermentation tests and cell counting methods. Among the relatively rapid methods for detection of microorganisms in food, adenosine triphosphate (ATP) bioluminescence is very suitable for on-line monitoring of bacterial contamination in food and beverages. This method does not require a culturing step or large equipment to fulfil the measurement, and it is fast and sensitive (Bottari, Santarelli, & Neviani, 2015). Bioluminescence is proposed to be the technique giving results in the shortest time (Hawronskyj & Holah, 1997). The ATP bioluminescence sensing assay relies on the fact that ATP is a major biological energy source existing in various microbes and, therefore,

reflects the existence of living microbes (Luo et al., 2009).

The Celsis Lumac IMC kit, which was used in this research, was designed to detect the presence or absence of microorganisms in industrial samples and/or screen for microbial level contamination. Results are obtained within minutes when more than 10<sup>4</sup> microorganisms per mL are present in the sample tested. Below this limit or when samples have high quenching levels, either a filtration step or an incubation step is required for the microbial enrichment of the sample.

The assay procedure is based on the ATP bioluminescence technique, which employs the light-producing system of the firefly to detect microorganisms. The test procedure consists of the selective extraction of non-microbial (somatic) ATP by a specific somatic cell extractant (NRS). The extractant makes the cell membrane permeable for molecules like ATP, which leak out. Microbial cells are not affected because they have cell walls. Extracted non-microbial ATP is hydrolysed by an ATPase (Somase). Microbial ATP is then measured in a Celsis Lumac Biocounter, after the addition of NRB and Lumit-PM. The amount of light produced correlates with the amount of ATP in the sample, shown as Relative Light Units (RLU). The light measurement is based on the following reaction:



To our knowledge, Tzatziki has not been subjected to any study of its microbial flora except for some recent works on its stability against pathogen microorganisms (Tsiraki & Savvaidis, 2014).

The aim of the current study is to develop a rapid and low cost ATP bioluminescence-sensing approach to determine the microbial stability of industrial Tzatziki samples.



## 2 Materials and Methods

### 2.1 Material

The reagents (Lumit-PM, Lumit-Buffer, NRS, NRB, Somase) for the determination of the microbial population with the bioluminescence method were obtained from Lumac B.V. (Landgraaf, Netherlands). The reagents used for the determination of Total microbial count, *Lactobacillus* spp., yeast and coliforms (Total Plate Count agar, MRS agar, Malt Extract agar and Violet Red Bile agar) were obtained from Merck Ltd (Darmstadt, Germany). The substrates used for the determination of *Lactobacillus* spp., yeasts and coliforms (Ringer solution, Sabouraud broth and MacConkey broth) were obtained from Merck Ltd (Darmstadt, Germany).

### 2.2 Tzatziki samples

Tzatziki salad was made using a simple home-made recipe with ingredients purchased from the local market. Strained yogurt, cucumbers (peeled and diced), olive oil, cloves of garlic (peeled), vinegar, salt, and chopped fresh dill (at 60, 20, 9, 4.5, 3, 2.5 and 1% of the total weight of the product, respectively) were mixed in a food processor until well-combined. The mixture was divided in two equal batches. In the first batch, sodium benzoate 0.05% and potassium sorbate 0.05% were added as preservatives. No preservatives were added to the second batch. Each batch was then divided into 100 g sub-batches, which were then packed in sealed plastic containers. All the containers were stored at 5 °C.

### 2.3 Determination of the microbial population using firefly luciferase for ATP measurement

The method used for the inoculation of salad is described in Fig. 1. The ATP assay was carried out as follows: 200  $\mu$ L of every sample was aseptically transferred to a cuvette; 100  $\mu$ L of Somase/NRS reagent was added to each sample and the samples were left for a 45 min period for incubation. Then, 100  $\mu$ L from each mixture

were taken and placed into cuvettes. The cuvettes were inserted into the Celsis Lumac Biocounter 1800 (Landgraaf, Netherlands) and the assay started. The instrument automatically added 100  $\mu$ L of NRB solution and the samples were left for 10 sec in order for the extraction to take place. Finally, 100  $\mu$ L of Lumit-PM reagent was added to every sample.

### 2.4 Determination of Colony Forming Units (CFU)

The determination of CFU was carried out according to ISO 4833-1 (International Standard Organization, 2003a) standard methodology for the Total Plate Counts, ISO 21527-1 (International Standard Organization, 2008) for the enumeration of yeasts and moulds, ISO 5541-1 (International Standard Organization, 1986) for the enumeration of coliforms and ISO 9232 (International Standard Organization, 2003b) for the identification of yogurt *Lactobacilli*. Subsamples of 25 g of each Tzatziki sample were diluted in 225 mL of Ringer solution, homogenized with a stomacher (Bioscience International, 11607 Magruder Lane, Rockville MD 20852-4365, USA), and then, incubated for 1 h at 25 °C. Suitable 1:10 dilutions were prepared. The counting of Total Mesophilic Flora and *Lactobacillus* spp. was performed by pouring aliquots of the decimal dilutions in Total Plate Count agar and MRS agar, respectively. For yeast counting, 25 g of each sample was homogenized in 225 mL of Sabouraud broth and incubated at 35 °C for 24 h. Decimal dilutions of Sabouraud broth were prepared and poured in plates for total viable yeast counting, using Malt Extract agar. For coliform counting, 25 g of each sample was homogenized in 225 mL of MacConkey broth and incubated at 30 °C for 24 h. Decimal dilutions of MacConkey broth were also prepared and poured in plates for coliform counting, using Violet Red Bile agar. The incubation of Violet Red Bile agar samples was carried out at 30 °C for 48 h and of Malt Extract agar at 25 °C for 5 days. After the incubation, the results were expressed in colony forming units (CFU) g<sup>-1</sup> based on the average count of a triplicate determination.

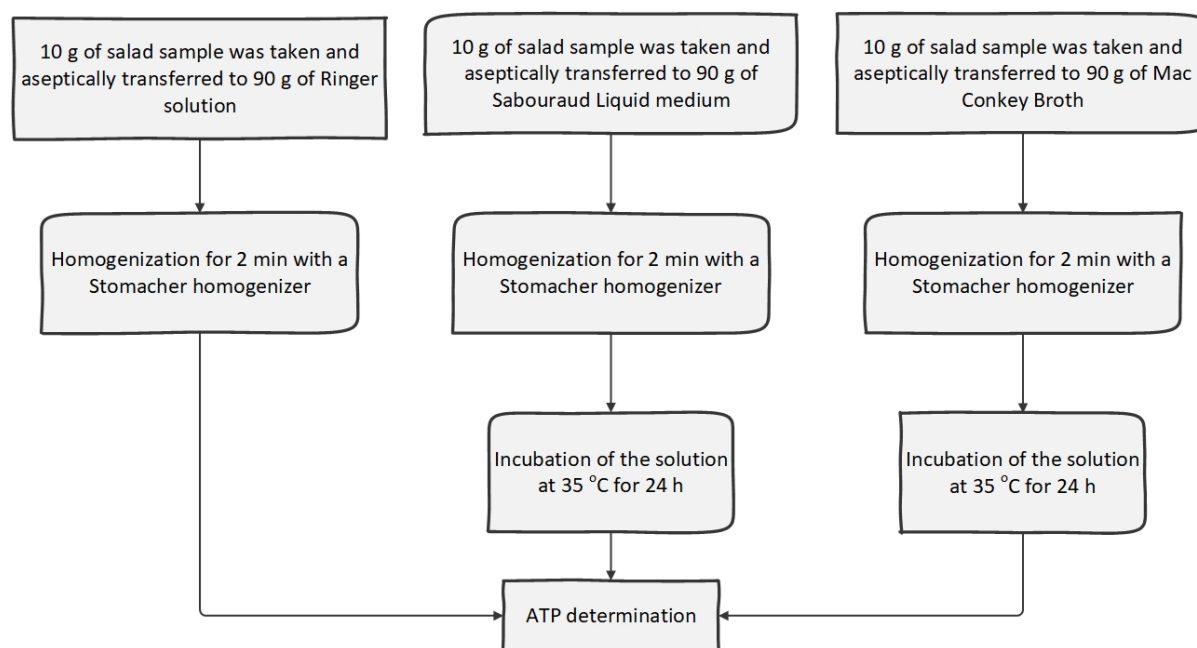


Figure 1: Method for the inoculation of salad

## 2.5 Statistical analysis

Results represent the average of three simultaneous assays. Statistical significance of the differences between mean values was assessed by ANOVA;  $p < 0.05$  was considered as statistically significant.

## 3 Results and Discussion

The microbiological analyses were carried out on various batches of Tzatziki using, each time, three samples from each batch for each measurement in order to evaluate the reproducibility of the method. There was no significant difference between the different batches. The curves presented a similar behavior in all cases (batches). As expected, the Tzatziki salad without preservatives initially gave RLU values higher (79,532) than the same salad with preservatives (43,198). The potassium sorbate and the sodium benzoate, used in the recipe, appeared to suspend the action of microorganisms. In both cases, the RLU values decreased day after day, and 10 days later tended to zero (Fig. 2).

The same procedure took place after incubation for the two selective mediums, in order to have information about spoilage microorganisms. Incubation was carried out because the initial microbial count of yeasts and coliforms was lower than  $10^4$  microorganisms per mL of sample. As expected, the Tzatziki salad without preservatives after incubation in two different substrates (Fig. 3), MacConkey and Sabouraud, gave RLU values higher (9,488 and 16,176) than the salad with preservatives (12,780 and 12,005), respectively. The microbial count in Sabouraud medium, which is selective for the growth of yeasts, compared to MacConkey (selective for coliforms) was higher because the cells of yeasts are bigger than those of bacteria and contain more ATP per cell and hence produce higher RLU values (Deligaris, 1981). The amount of ATP in microbial cells may differ, depending on their physiological state and types of cells present. For example, injured or starved microbial cells may contain approximately 10-30% of ATP present in healthy cells and yeast cells contain approximately 100 times more ATP than bacterial cells (Mendonça, Juneja, & Daraba, 2014). Gener-

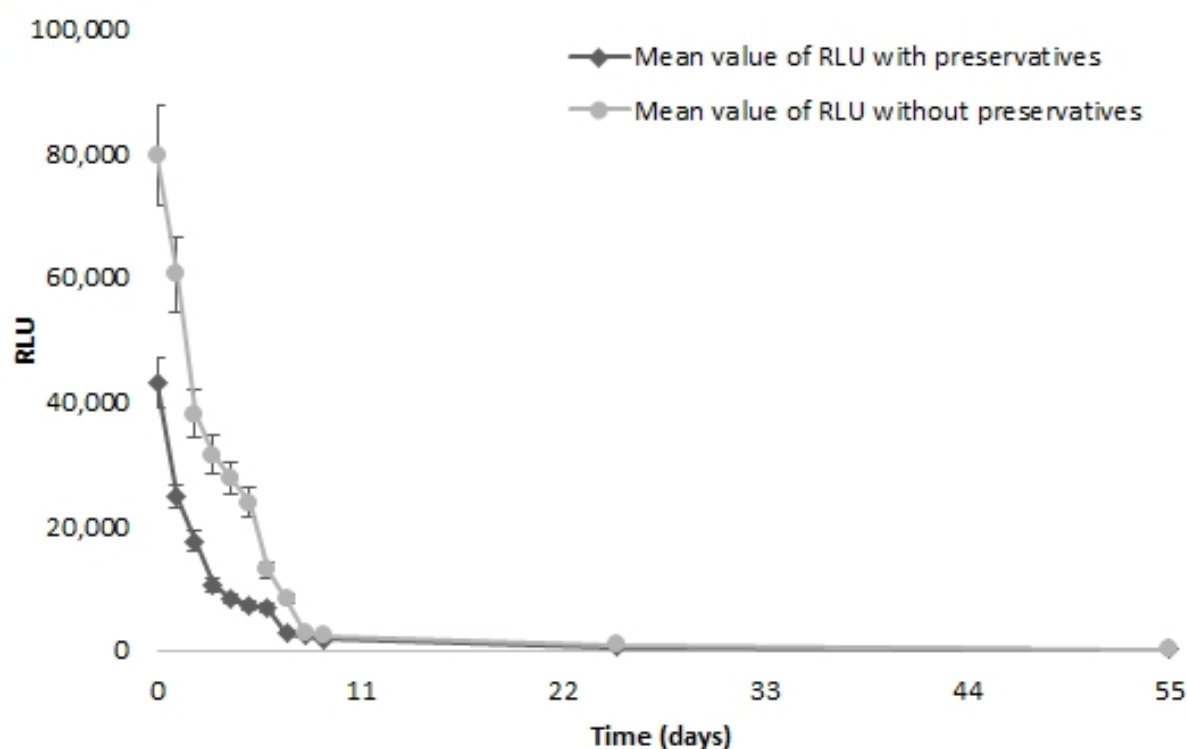


Figure 2: RLU values of Tzatziki, with and without preservatives (Ringer solution)

ally, coliforms, yeasts and fungi that existed in the system were given small possibility to grow due to the higher number of Lactobacilli present that function competitively against them. Thus, (Fig. 2) the decrease in population is progressive while the population of coliforms (Fig. 3a) and yeasts (Fig. 3b) decreases abruptly. The correlation between CFU and RLU measurements of Tzatziki, with and without preservatives, appear in Fig. 4.

In the two selective substrates, differences appeared between the two methods of microbial count (RLU and CFU) (Fig. 5). While RLU values were roughly at the same level, the CFU values presented significant differences ( $p < 0.05$ ). Particularly, the CFU values in the Sabouraud broth were initially lower than those in the MacConkey broth. This is explained by the size of the cells of yeasts that are bigger than those of the bacteria and contain more ATP per cell (Mendonça et al., 2014).

The correlation between RLU and CFU  $\text{g}^{-1}$  of salad in Ringer Solution, MacConkey and Sabouraud was almost linear in all cases.

In yogurt, generally because of the low pH value and the high number of Lactobacilli, the growth of other bacteria (proteolytical, coliforms) or yeasts is not being allowed. However, yogurt is sensitive to alterations from fungi, especially in the areas that are in contact with air. The microbial flora of Tzatziki is mainly composed of Lactobacilli, originated from yogurt (Tsiraki & Savvaïdis, 2014), and the results showed that Tzatziki salad presented an extreme stability from the 2<sup>nd</sup> day of its preparation until the last day (15<sup>th</sup>) of experiments. Lactobacilli and other microorganisms were decreased and this was possibly due to the combination of ingredients (garlic, cucumber). Allicin and its derivative products (diallyl disulfide, diallyl trisulfide) which are found in garlic essential oils, have shown good antimicrobial activities

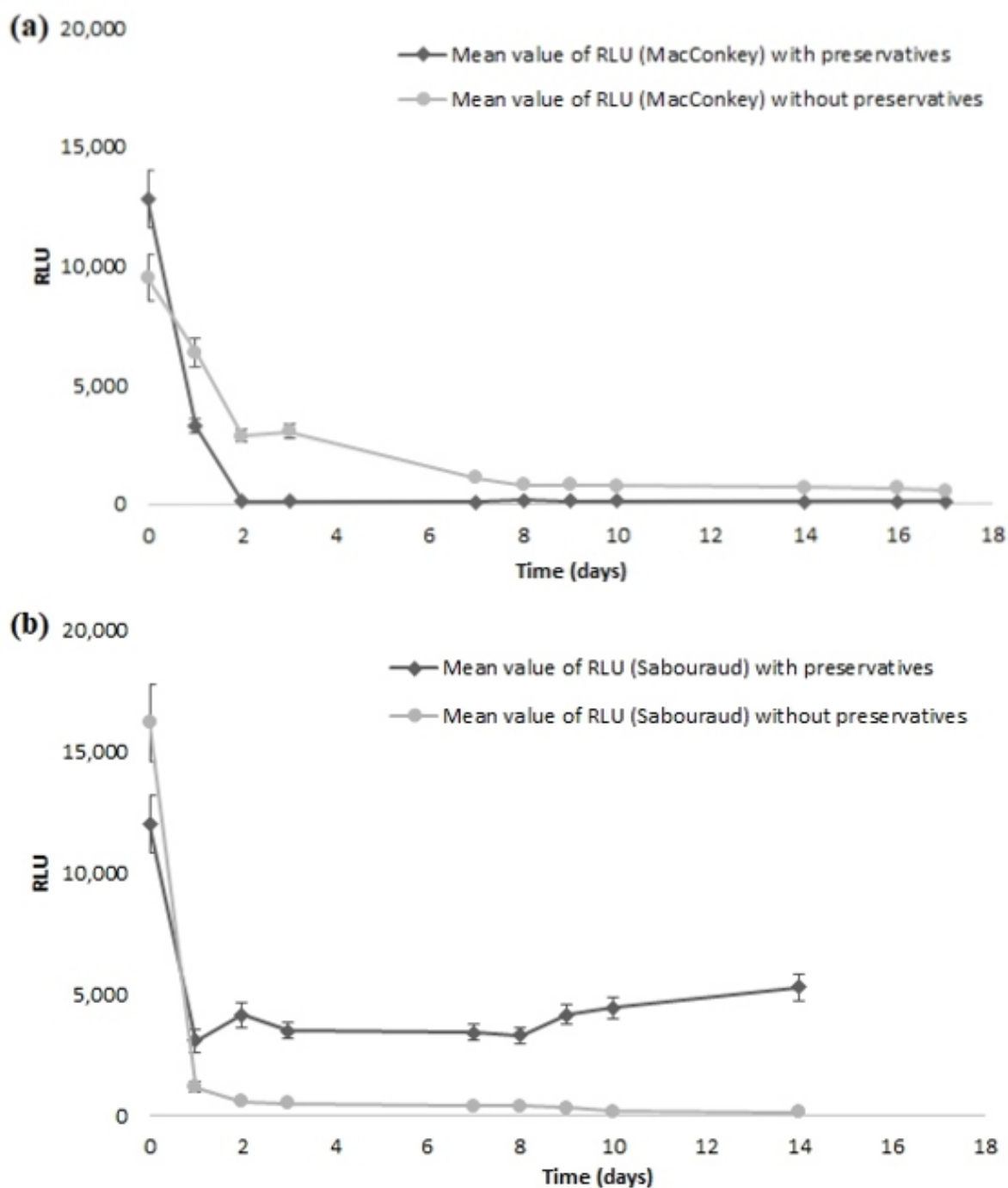


Figure 3: RLU values of Tzatziki, with and without preservatives, after incubation in (a) MacConkey and in (b) Sabouraud, for 24 h at 35 °C.

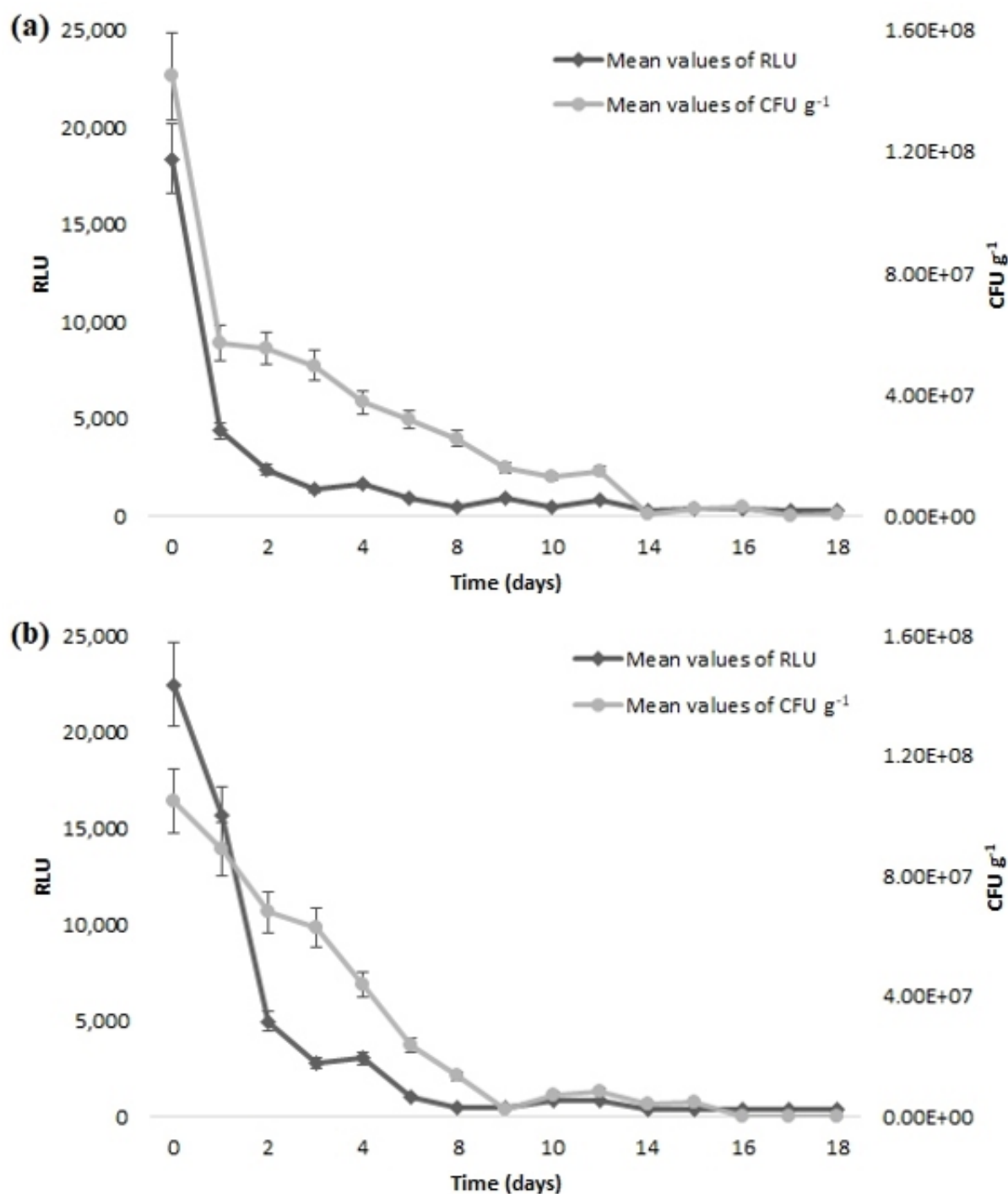


Figure 4: RLU and CFU  $g^{-1}$  counts of Tzatziki, (a) with and (b) without preservatives (Ringer solution)

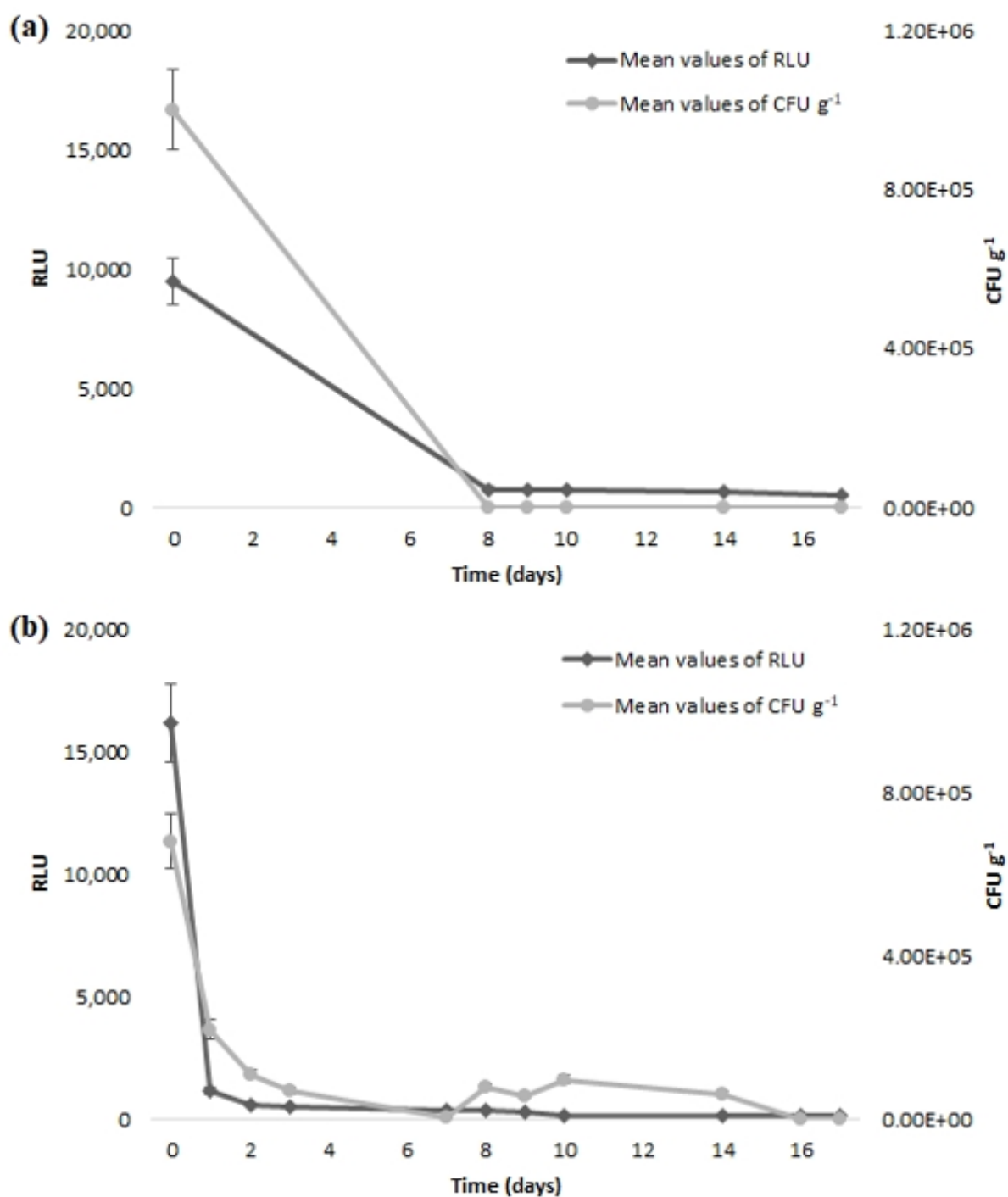


Figure 5: RLU and CFU  $g^{-1}$  counts of Tzatziki with preservatives incubated in (a)  $\mu$ acConkey and in (b) Sabouraud for 24 h at 35 °C



(Kim, Huh, Kyung, & Kyung, 2004). Cucumber is mainly responsible for the “fresh-green” aroma of fresh vegetables, which characterizes Tzatziki. Aroma compounds are plant secondary volatiles, which are produced when cucumber is finely chopped in order to be incorporated into the yogurt. Secondary volatiles, apart from their sensory contribution, can influence the microbial stability depending on their concentration, composition and type of plant material of origin. In some cases, the enzymatically formed secondary volatiles can accumulate to concentrations which are sufficient to decrease the microbial population. There are examples, such as the case of cucumber, where the main characteristic aroma predominates only when the enzymes are allowed to react on decompartmentation of the plant tissue, by cutting and homogenizing (Schrodter, 1984). Green aldehydes and green alcohols are the compounds responsible for the green flavor note. They are produced from linolenic and linoleic acid in wounded plant tissues (Galliard, 1978; Tressl & Drawert, 1973). A large number of enzymes are released after cell membrane damage (Galliard, 1978). Autolysis gives free linolenic and linoleic acid. Lipoxigenase action on the above-mentioned acids gives rise to linolenic and linoleic hydroperoxides. The two hydroperoxides have antimicrobial activity (Brooks & Watson, 1985). Lyase action on the hydroperoxides is responsible for the formation of (*E*)-2-Hexenal, (*E*)-2-Nonenal and (*E,Z*)-2,6-Nonadienal. These compounds are the basis of green natural aroma at low thresholds (0.07  $\mu\text{g L}^{-1}$ ). It has been proved that the above-mentioned aldehydes also possess fungistatic action in the gas phase (Gueldner, Wilson, & Heidt, 1985; Huhtanen & Guy, 1984).

Sulfur-containing volatiles are normally formed during crushing of the plant material. In Allium plants, essential oils possess antimicrobial activity. This anti-yeast activity (Wills, 1956) is probably due to the high allicin content. The effect of allicin on triose-phosphate-dehydrogenase in microbial cell metabolism has been demonstrated by Barone and Tansey (1977). Inhibition of several sulfhydryl metabolic enzymes was observed. The biogenesis of allicin was summarized by Dourtoglou (1986). The precursors and the products possess antifungal activity (Conner

& Beuchat, 1984; Huhtanen & Guy, 1984).

Generally, the preservation of such products is based mainly on their high acidity and on chemical preservatives employed to inhibit the growth of spoilage and pathogenic microorganisms. However, consumers demand reduced levels of additives in foods and consider their use undesirable. Although the safety of these items is supposed to be ensured primarily by their low pH, several pathogens, namely *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp., have been reported to survive or even grow in these foods. Usually, the pathogens in such products come from the raw ingredients as well as due to contamination from the processing environment and packaging operation (Panagou, Nychas, & Sofos, 2013).

That is why good manufacturing practice must be followed in order to minimize any possible cross-contamination. In the marketplace and consumers' homes, Tzatziki salad must be kept refrigerated and handled with stringent hygienic practices to prevent cross-contamination by other food products or the environment.

As indicated by the results, chemical preservatives are not essential for the microbial stabilization of the product. Specifically, after the 2<sup>nd</sup> day, the Tzatziki salad showed its self-stabilization ability, even without the use of preservatives. Also, it was shown that an initial population of *Lactobacillus* spp. of 1.00E+08 CFU g<sup>-1</sup> and RLU measurements lower than 4.00E+04 RLU in Greek traditional Tzatziki salad are considered as a minimum specification for the microbial stability and safety of the product.

Because of the good correlation that is presented between CFU g<sup>-1</sup> measurements and RLU values, the bioluminescence method appears to be an appropriate rapid method to determine Tzatziki microbial stability during industrial production practices.

## 4 Conclusions

In conclusion, Greek traditional Tzatziki salad is a self-stable product due to its high acidity, plant derived natural preservatives and storage under refrigeration, and the rapid bioluminescence method can be used to define the microbial stability of the product from the beginning, on an industrial scale. The method requires little to no sample pretreatment as compared to conventional approaches and it can detect the total bacterial viability quickly and give an excellent aid in nonspecific detection of bacterial contaminants.

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## Moisture Sorption Isotherm and Thermal Characteristics of Freeze-Dried Tuna

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### Abstract

Water activity is considered an important factor in assessing the stability of food. Understanding the relationship between water activity and equilibrium moisture content (moisture sorption isotherm) benefits food processing in terms of modeling of drying and estimation of shelf life. In addition, glass transition helps to quantify molecular mobility which helps in determining the stability of food. The aim of this study was to determine the moisture sorption isotherm and thermal characteristics of freeze-dried tuna. These characteristics will help in determining the monolayer moisture and glassy state of the product, at which food is considered most stable. Moisture sorption isotherm at 20 °C and thermal characteristics (over a wide temperature range i.e. from -90 to 250 °C) of freeze-dried tuna flesh were measured. Isotherm data were modeled by BET (Brunauer-Emmett-Teller) and GAB (Guggenheim-Anderson-De Boer) models. The GAB and BET monolayer water values were determined as 0.052 and 0.089 g g<sup>-1</sup> dry-solids (dry-basis), respectively. In the case of samples at moisture contents above 0.10 g g<sup>-1</sup> (wet basis), DSC (Differential Scanning Calorimetry) thermograms showed two-step state changes (i.e. two glass transitions), one exothermic peak (i.e. molecular ordering) and another endothermic peak (i.e. solids-melting). However, the sample at moisture content of 0.046 g g<sup>-1</sup> showed three-step state changes (i.e. three glass transitions). The multiple glass transition could be explained by the natural heterogeneity of tuna flesh and inhomogeneity due to molecular incompatibility of the different compositions. The moisture content did not affect the first glass transition temperature nor the exothermic peak ( $p > 0.05$ ), whereas the third glass transition temperature decreased (i.e. plasticized) with increasing moisture content ( $p < 0.05$ ). The solids-melting peak temperature decreased, and enthalpy increased with decreasing moisture content ( $p < 0.05$ ).

**Keywords:** Dried fish; Sorption isotherm; Tuna; Thermal transition; Solids-melting

### 1 Introduction

Water interacts with different ingredients present in foods. This interaction is important in determining the physical, chemical and microbial

stability of the foods (Bhandari & Howes, 1999; Rahman, 2006; Sablani, Kasapis, Rahman, Al-Jabri, & Al-Habsi, 2004). Water activity is considered an important property to determine the

stability of foods compared to the total amount of water (Labuza, McNally, Gallagher, Hawkes, & Hurtado, 1972; Roos, 1993; Scott, 1953). Therefore, the water activity concept is commonly used to determine the stability of foods in relation to microbial growth, lipid oxidation, non-enzymatic and enzymatic activities, and the texture of foods (Labrousse, Roos, & Karel, 1992; Shi, Zhao, Chen, Li, & Xue, 2009; Shimada, Roos, & Karel, 1991). In addition, it is used as a tool in food processing operations, such as modeling of drying processes, estimating shelf-life, and choosing suitable packaging material for a food product (Orlien, Risbo, Andersen, & Skibsted, 2003). Water activity is related to the moisture content in the sorption isotherm (i.e. the relationship of water activity and equilibrium moisture content) (Delgado & Sun, 2002).

Recently, it has been observed that the water activity concept is not sufficient to determine stability of food, thus glass transition has been proposed to quantify molecular mobility (Delgado & Sun, 2002; Karel, Buera, & Roos, 1993; Karmas, Buera, & Karel, 1992; Sablani et al., 2004; Slade & Levine, 1988, 1991). Foods are considered most stable when they are stored at or below their glass transition because of the low molecular mobility of the reactants in a highly viscous medium. This reduced mobility could significantly retard microbial and chemical reactions (Meste, Champion, Roudaut, Blond, & Simatos, 2002; Rahman, 2006). As the storage temperature increases above the glass transition temperature, foods become less stable with increased molecular mobility (Goula, Karapantsios, Achilias, & Adamopoulos, 2008; Sablani et al., 2007; Syamaladevi, Sablani, Tang, Powers, & Swanson, 2010; Tonon et al., 2009). The water activity and glass transition concepts could be combined to determine the stability of foods (Rahman, 2009; Rahman & Al-Saidi, 2017; Shi et al., 2009).

Fresh tuna fish has a short shelf life unless it is stored at very low temperatures or dried (Orlien et al., 2003). It is important to measure the thermal characteristics and sorption isotherm in order to determine efficient processing and storage stability of dried tuna (Roos & Karel, 1991). Glass transition of different types of fish and other muscle tissues like chicken and beef are reported in the literature (Brake & Fennema,

1999; Sablani et al., 2007; Tolstorebrov, Eikevik, & Bantle, 2014). Harnkarnsujarit, Kawai, and Suzuki (2015) measured the glass transition and water sorption isotherm of water extract from freeze-dried tuna. Most probably, they attempted to simplify the complex intact structure into a sample of only soluble components. Moreover, their temperature range in Differential Scanning Calorimetry (DSC) was within -80 to 80 °C without measuring solids-melting or solids-decomposition. However, there are negligible data available on the thermal characteristics of dried fish (i.e. whole sample) over a wide range of temperature (i.e. including solids-melting or solids-decomposition) (Suresh, Al-Habsi, Guizani, & Rahman, 2017). The structural collapse during drying processes influenced water uptake during rehydration, which could be related to a glassy state of dried fish (Marques, Prado, & Freire, 2009). The hygroscopicity was also affected by different drying methods and affected the sorption isotherm (Caparino et al., 2012). The aim of this study was to determine the moisture sorption isotherm and thermal characteristics (i.e. -90 to 250°C) of freeze-dried tuna. These characteristics could be used to determine monolayer moisture and glassy state, which is considered the best stability during processing and storage.

## 2 Materials and Methods

### 2.1 Sample Preparation

Fresh yellowfin tuna (*Thunnus albacares*) was purchased from a local fish market and brought (iced) within 30 min to the laboratory. Tuna was harvested overnight by the fisherman and ice-stored in the boat. Tuna was landed in the morning and displayed at the beach fish market (i.e. around 10 hr after harvest). The fillets were prepared and diced at room temperature (i.e. 20 °C) into 3 cm cubes; and placed into several 50 ml plastic containers. The diced cubes were then frozen at -40 °C for 24 h and freeze-dried at room temperature (20 °C) (i.e. sample enter at -40 °C and dried at 20 °C) under vacuum of 200 Pa for 96 h using an Edwards K4 Freeze Dryer (Corawky, Crawley, England).

Freeze-dried tuna was then ground into powder using a KMF grinder (KIKA Werke, Wilmington, USA) running at 6000 rpm, and stored at -20 °C until used for the DSC experiments.

## 2.2 Moisture Sorption Isotherm

The isopiestic method was used to develop the moisture sorption isotherm (Rahman & Hamed Al-Belushi, 2006). In this method, 1 g of freeze-dried tuna powder was placed in an open bottle and stored in an air-sealed glass jar containing a saturated salt solution (for example, lithium chloride) in a 50 ml beaker to maintain a specific relative humidity environment in the jar. Thymol in a 5 ml beaker was also placed inside the jar to prevent the growth of molds during storage. The salts used were lithium chloride, potassium acetate, magnesium chloride, potassium carbonate, magnesium nitrate, sodium nitrate, sodium chloride, potassium chloride and potassium nitrate (water activity: 0.1 - 0.9) and the water activity values were taken from Rahman (1995). The air-sealed glass jars were then stored at 20°C for 3 to 4 weeks until equilibrium was reached. This temperature was close to commonly used room temperature storage. Equilibrium conditions were achieved when there was no loss or gain of mass ( $\pm 0.01$  g) for two consecutive days. Weight was monitored using an analytical balance (OHAUS, model AS200, Florham Park, USA). The moisture content was determined by an oven method according to AOAC (2005), by drying 1 g of the sample in a conventional hot-air oven at 105 °C for at least 20 h until a constant weight was achieved. The moisture content in the sorption isotherm data was expressed in dry basis as commonly presented in the literature. Each water activity data collection was replicated 3 times.

The moisture sorption isotherms were modeled using Brunauer–Emmet–Teller (BET) (Brunauer, Emmet, & Teller, 1938) and Guggenheim–Andersen–de Boer (GAB) equations (Bizot, 1983). BET and GAB equations are as follows:

$$M_w = \frac{M_{bm} B a_w}{(1 - a_w)[1 + (B - 1)a_w]} \quad (1)$$

$$M_w = \frac{M_{gm} C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)} \quad (2)$$

where,  $M_w$ ,  $M_{bm}$ , and  $M_{gm}$  are the moisture content at any water activity, BET-monolayer water content and GAB monolayer water content, respectively (all in g g<sup>-1</sup> dry-solids),  $a_w$  is the water activity,  $B$  is the parameter of BET model, and  $C$  and  $K$  are the parameters of the GAB model. The BET equation has been widely used and been found to give a good fit when water activities are less than 0.45. The GAB model has been found to be successful up to water activities as high as 0.9 (Basu, Shivhare, & Mujumdar, 2006; Al-Muhtaseb, McMinn, & Magee, 2002).

## 2.3 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was used to determine state and phase transitions in freeze-dried tuna (Rahman, Al-Saidi, Guizani, & Abdullah, 2010). The samples containing different moisture (i.e. 0.10, 0.15, and 0.20 g g<sup>-1</sup> sample, wet basis) were prepared from the freeze-dried sample (initial moisture of 0.046 g 100g<sup>-1</sup> sample, wet basis) by spraying the desired amount of distilled water and mixing thoroughly with a spatula. The samples were then stored at 4 °C for 24 h for equilibration. After equilibration, samples were stored at -20 °C for 24 hours until they were used for DSC measurements. The samples were analyzed thermally in a DSC (Q200, TA Instrument, New Castle, DE, USA) and the thermal characteristics were determined. The instrument was calibrated using indium with melting temperature of 156.5 °C and enthalpy of 28.5 J g<sup>-1</sup>.

Samples of 3 to 5 mg of freeze-dried tuna were placed in Tzero aluminum hermetic pans and sealed. The pans containing the samples were cooled from 25 to -90 °C at a rate of 5°C min<sup>-1</sup> and then heated to 250 °C at 10 °C min<sup>-1</sup> under nitrogen (carrier gas) purged at 50 mL min<sup>-1</sup>. An empty pan was used as a reference. Thermograms were obtained in triplicates for the samples with different moisture contents and these were analyzed for exothermic (i.e. ordering in molecules) and endothermic (i.e. solids-melting) peaks and shifts in the thermogram (i.e. glass



transition). The moisture content of samples used for the DSC analysis is expressed in wet basis as commonly presented in the literature.

## 2.4 Statistical Analysis

Each value is presented as mean  $\pm$  standard deviation. A non-linear regression analysis was used to estimate the parameters of the isotherm models (i.e. Guggenheim-Anderson-de Boer, GAB and Brunauer-Emmett-Teller, BET). The significant effect of moisture on the thermal characteristics was determined using ANOVA (Microsoft Excel, 2010).

## 3 Results and Discussion

### 3.1 Moisture Sorption Isotherm

Figure 1 shows the moisture sorption isotherm of tuna flesh at 20 °C. The data were fitted with BET and GAB models and the parameters are presented in Table 1 with their prediction accuracy. The BET and GAB monolayer water values were 0.089 and 0.052 g g<sup>-1</sup> dry-solids, respectively, and samples at BET monolayer water could be considered most stable since deteriorative chemical reactions are minimal (Rahman & Hamed Al-Belushi, 2006; Sablani et al., 2007). However, the GAB model is useful for predicting water activity over the complete range of moisture up to 0.90. In the case of king fish flesh (fat content: 0.039 g g<sup>-1</sup> sample), BET and GAB monolayer water content were found to be 0.036 and 0.052 g g<sup>-1</sup> dry-solids, respectively (Sablani et al., 2007). The same authors analysed fat-free king fish and they found values of 0.048 and 0.086 for the BET and GAB models, respectively. This indicated that fat content marginally increased the monolayer values. Delgado and Sun (2002) reported values of 0.073 and 0.068 g g<sup>-1</sup> dry-solids, respectively for chicken meat (fat content: 0.004 g g<sup>-1</sup> sample). The main variations of the monolayer values depended on the types of proteins. It was reported that fat content had very small or negligible effects as a plasticizer when added to protein (Kalichevsky, Jaroszkievicz, & Blanshard, 1992; Shaw, Monahan, O'Riordan, & O'Sullivan, 2002). Fat free

protein, such as gelatine and collagen showed high BET and GAB monolayers, 0.12 and 0.10 g g<sup>-1</sup> dry-solid, respectively (Timmermann, Chirife, & Iglesias, 2001).

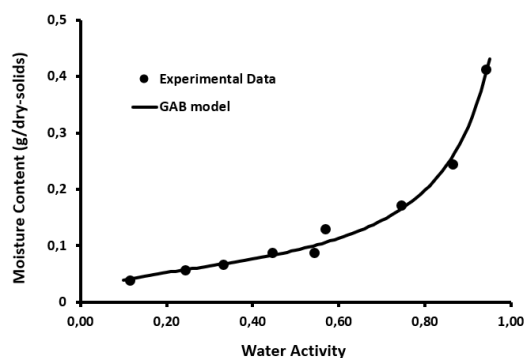


Figure 1: Experimental moisture sorption isotherm of freeze-dried tuna and predicted GAB model

### 3.2 Thermal Characteristics

A typical DSC thermogram for freeze dried tuna is shown in Figure 2 (moisture content: 0.046 g g<sup>-1</sup> sample, wet basis). Three letters A, B and C represent three transitions however, they could not be visualized clearly due to the high exothermic and endothermic enthalpy (i.e. high heat flow). Therefore, these regions were expanded with the appropriate scales and are shown in Figure 3. Figure 2 also shows an exothermic peak (i.e. molecular ordering) and an endothermic peak (i.e. solids-melting). The sample at moisture 0.046 g g<sup>-1</sup> showed three glass transitions, while other samples showed only two glass transitions. This additional shift (i.e.  $T_{gi}$ :  $97.6 \pm 5.7$  °C,  $T_{gp}$ :  $103.2 \pm 8.2$  °C,  $T_{ge}$ :  $104.7 \pm 8.2$  °C,  $\Delta C_p$ :  $400 \pm 120$  J kg<sup>-1</sup> K<sup>-1</sup>) was observed between the first and the third glass transition. Table 2 shows two glass transitions (first and third) as a function of moisture content. At moisture content 0.046 (g 100g<sup>-1</sup> sample), the first and third glass transitions were observed at 28.3 and 148.5 °C, respectively (Table 2). Similarly, Tolstorebrov et al. (2014) observed variation in the glass transition onset temperature from 36.6 to 40.3 °C

in the case of vacuum dried cod, salmon, trout, herring and mackerel at a moisture of  $0.055 \text{ g g}^{-1}$  sample. This transition was close to the values of the first transition observed in the case of tuna. In the case of king fish muscle samples (moisture content:  $0.035 \text{ g g}^{-1}$  sample), Sablani et al. (2007) observed two glass transitions, one at  $-11.0^\circ\text{C}$  and the other at  $74.7^\circ\text{C}$ . In the case of air-dried tuna (moisture:  $0.046 \text{ g g}^{-1}$  sample), (Rahman, Kasapis, Guizani, & Al-Amri, 2003) observed a glass transition at  $67.1^\circ\text{C}$ , as measured by mechanical thermal analysis. Their value was relatively close to the second glass transition  $97.6^\circ\text{C}$ . In the case of water-soluble extract of freeze-dried tuna, one glass transition was observed, and it was plasticized with the increase of water content (i.e. more hydrophilic components) (Harnkarnsujarit et al., 2015). The whole freeze-dried sample was more rigid and amorphous, and the plasticization process was hindered although water content increased. Earlier, it was identified that multicomponent food systems could show multi-glass transitions (Biliaderis, 1991; Hashimoto, Hagiwara, Suzuki, & Takai, 2003).

Table 1: BET and GAB model parameters for moisture sorption isotherm of dried tuna

Model	Parameter	
GAB	$M_{bm}$	$0.052 \text{ g g}^{-1}$ dry solid
	K	0.926
	C	19.36
	$r^2$	0.99
	SE	0.013
	p-value	<0.05
BET	$M_{gm}$	$0.089 \text{ g g}^{-1}$ dry solid
	B	4.038
	$r^2$	0.95
	SE	0.0056
	p-value	<0.05

Moisture content in freeze-dried tuna did not affect the first glass transition temperature ( $p > 0.05$ ) and specific heat change at that transition ( $p > 0.05$ ), whereas the third glass transition temperature decreased ( $p < 0.05$ ) and specific

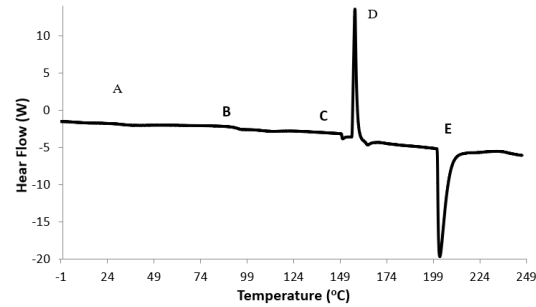


Figure 2: A typical DSC thermogram of freeze-dried tuna at a moisture content  $4.6 \text{ g } 100\text{g}^{-1}$  sample, A: first glass transition, B: second glass transition, C: third glass transition, D: exothermic peak (i.e. molecular ordering, E: endothermic peak (i.e. solids-melting)

heat change increased with the increase of moisture content ( $p < 0.05$ ) (Table 2). Similarly, in the case of king fish, Sablani et al. (2007) did not observe any plasticization (i.e. decrease of glass transition) with water for the first glass transition. In the case of the third glass transition (i.e. in tuna), higher water contents decreased glass transition due to plasticization, and increased specific heat change, which suggested the formation of hydrogen bonding causing the development of disordered structure (i.e. more amorphous domain) (Rahman & Al-Saidi, 2017). In the case of sugar-based foods containing freezable water, two glass transitions were commonly observed, one at low temperature and another just below ice-melting temperature (Rahman, 2004). In the case of tuna, the low glass transition temperature did not plasticize with increasing water content. However, glass transition temperature before melting decreased with the increase of water content (i.e. plasticized). In the case of rice, Cao, Nishiyama, and Koide (2004) observed three transitions, and the second transition showed a significant decrease with increasing moisture, while first and third transitions remained stable with increasing moisture. They hypothesized that the second transition could be the glass transition as plasticization (i.e. decrease of glass transition) occurred at this transition. The multiple glass transitions could be

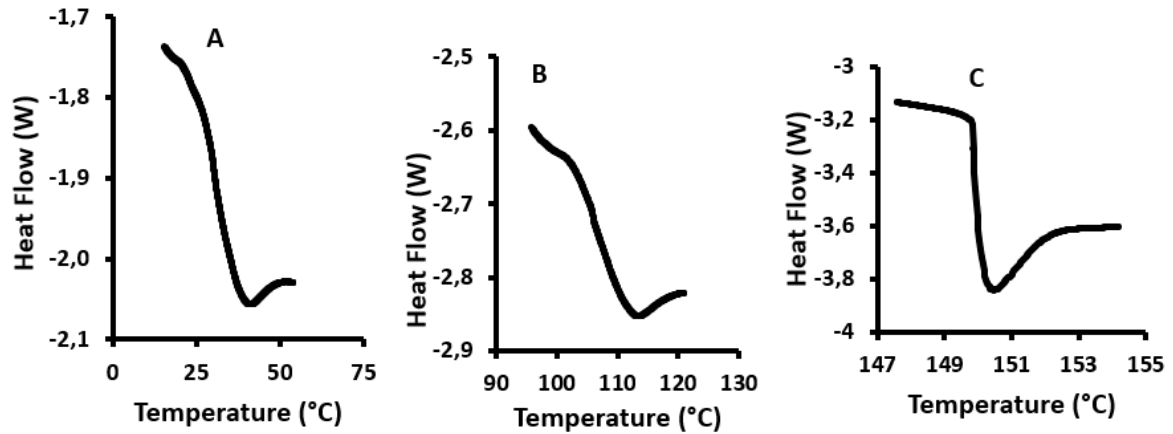


Figure 3: Expansion of the glass transitions as marked in Figure 2. A: first glass transition, B: second glass transition, C: third glass transition

Table 2: First and third glass transitions of freeze-dried tuna

Xw	First Glass Transition				Third Glass Transition			
	$T_{gi}$ (°C)	$T_{gp}$ (°C)	$T_{ge}$ (°C)	$\Delta C_p$ (J kg <sup>-1</sup> °C <sup>-1</sup> )	$T_{gi}$ (°C)	$T_{gp}$ (°C)	$T_{ge}$ (°C)	$\Delta C_p$ (J kg <sup>-1</sup> °C <sup>-1</sup> )
0.046	28.3±0.5	29.9±0.4	34.1±1.1	300±50	148.5±1.2	148.6±1.1	148.8±1.2	510±170
0.100	30.9±0.7	32.1±0.6	35.5±0.9	184±3	138.2±0.1	138.2±0.1	138.4±0.1	530±180
0.150	30.6±0.8	32.5±2.0	35.7±1.3	207±8	134.1±2.3	134.1±2.3	134.2±2.4	630±230
0.200	31.9±2.1	33.8±2.7	35.7±1.6	192±12	131.7±2.5	131.8±2.6	132.0±2.6	760±240
	p>0.05			p>0.05	p<0.05			p<0.05

Note:

Each data point is presented as mean ± Standard deviation and p-value was set at 0.05 for determining significance

Xw: water content (g water/g sample),  $T_{gi}$ : initial glass transition temperature,  $T_{gp}$ : peak glass transition temperature,  $T_{ge}$ : end of glass transition temperature;  $\Delta C_p$ : change in specific heat capacity

Table 3: Characteristics of exothermic and endothermic (i.e. solids-melting) peaks of freeze-dried tuna

Xw	T <sub>ea</sub> (°C)	T <sub>ep</sub> (°C)	T <sub>ee</sub> (°C)	ΔH <sub>e</sub> (kJ kg <sup>-1</sup> )	T <sub>mi</sub> (°C)	T <sub>mp</sub> (°C)	T <sub>me</sub> (°C)	ΔH <sub>m</sub> (kJ kg <sup>-1</sup> )
0.046	155.5±0.4	157.0±0.5	161.4±0.4	36.1±1.1	202.2±1.4	203.2±1.6	217.9±1.2	85±7
0.100	155.5±0.2	157.0±0.2	161.7±0.2	37.1±2.3	188.4±3.6	188.4±3.5	202.7±2.5	151±9
0.150	155.4±0.2	156.9±0.2	160.9±1.4	31.3±3.4	175.8±3.8	181.6±5.0	192.1±3.6	166±6
0.200	155.5±0.4	156.9±0.5	161.1±0.8	34.6±2.6	176.8±0.8	176.7±1.8	189.6±3.0	299±5
	p>0.05			p>0.05		p<0.05		p<0.05

Note:

Each data point is presented as mean ± Standard deviation and p-value was set at 0.05 for determining significance

T<sub>mi</sub>: peak onset temperature, T<sub>mp</sub>: peak maximum temperature, T<sub>me</sub>: peak end temperature, ΔH: change in enthalpy. Other variables are explained in Table 2

explained by the natural heterogeneity of biological materials, such as meat and fish muscle and the inhomogeneity due to molecular incompatibility in complex foods (Orlien et al., 2003).

Table 3 shows the exothermic and endothermic peaks as a function of moisture content. It was observed that the moisture content did not affect the exothermic peak and enthalpy ( $p > 0.05$ ). The average values of the peak temperature and enthalpy were  $155.5 \pm 0.1$  °C and  $34.8 \pm 2.5$  kJ kg<sup>-1</sup>, respectively. Similarly, in the case of freeze-dried broccoli, Suresh et al. (2017) observed an exothermic peak before the solids-melting endothermic. The exothermic peak indicated that there was an order in the molecules at the higher temperature before melting. The endothermic peak can be considered as solids-melting (i.e. softening of the solid phase as proteins denature with the release of heat). The peak temperature decreased and enthalpy increased with the decrease of moisture ( $p < 0.05$ ). Protein denaturation can be noticed as an endothermic peak in the DSC curve in the temperature range of 40-80 °C in case of fresh food samples (i.e. high moisture content) (Hashimoto et al., 2003). The peak temperature increased (e.g., 120 °C) as sample moisture content decreased. It was suggested that protein denaturation significantly affected the glass transition of protein-rich food (Hashimoto, Suzuki, Hagiwara, & Takai, 2004; Sochava & Smirnova, 1993; Wright, Leach, & Wilding, 1977). The increase of enthalpy during solids melting could be explained by the formation of more hydrogen bonds

at higher water content. A similar trend was observed in the case of freeze-dried broccoli (Suresh et al., 2017).

Considering the water activity concept, stability of freeze dried samples could be achieved if stored at 0.089 g g<sup>-1</sup> dry-solids at room temperature (i.e. 20 °C). The two or three state changes (i.e. glass transitions) indicated that different types of physico-chemical changes could occur in the tuna sample. The lower one is important if freeze-dried fish is to be stored at room temperature and higher glass transition is relevant in roasting and frying processes. Further studies need to be conducted for determining the types of chemical reactions that could occur at the different stages of glass transitions.

## 4 Conclusions

Moisture sorption isotherm of freeze-dried tuna was measured and modeled by BET and GAB equations. The BET and GAB monolayer water content were 0.052 and 0.089 g g<sup>-1</sup> dry-solids, respectively. In this work, isotherm was measured at 20 °C (i.e. close to usual sale display, around 18-20 °C). However, further work at extreme conditions, such as 10 ° or 30 °C, could provide enriched information on the effects of temperature on isotherm. For a sample with moisture content of 0.046 g g<sup>-1</sup>, three glass transitions were observed at different temperatures, first one onset at 28.3 °C, second one at 97.6 °C, and third at 148.5 °C. However, samples at higher moisture

content showed only two glass transitions; one at low and the other at higher temperature. The lower glass transition was un-affected by moisture content, whereas higher temperature glass transitions decreased with increasing moisture content. Similarly, solids-melting (i.e. protein denaturation) decreased with increasing moisture content. In addition, specific heat change at the glass transition and enthalpy at solids-melting increased with the increase of water content. The sample used in this study was powder to simplify sample handling and measurement. The thermal transitions may not be changed for powder or intact solid samples, however kinetics of moisture sorption would be faster in the case of powder samples, although final equilibrium conditions would be unaffected. This point needs to be considered when freeze-dried samples are stored.

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## Antioxidant Indices and Amino Acid Composition of Phenolic Containing Lima Beans (*Phaseolus lunatus*) After Simulated Human Gastrointestinal Digestion

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### Abstract

The present investigation was designed to characterize the phenolic profile of Lima beans (*Phaseolus Lunatus*) and also to evaluate the antioxidant indices: total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and amino acid composition at different stages of simulated gastrointestinal digestion (oral, gastric, intestinal). High Performance Liquid Chromatography (HPLC-DAD) analysis revealed the presence of some phenolic compounds (gallic acid, catechin, caffeic acid, rutin, quercitrin, quercetin, kaempferol and apigenin), with a reduced amount (mg/g) after cooking: gallic acid (raw:  $1.96 \pm 0.02$ ; cooked:  $1.82 \pm 0.01$ ); catechin (raw:  $0.83 \pm 0.01$ ; cooked:  $0.73 \pm 0.01$ ); rutin (raw:  $2.61 \pm 0.03$ ; cooked:  $1.74 \pm 0.03$ ); quercitrin (raw:  $5.73 \pm 0.01$ ; cooked:  $5.68 \pm 0.01$ ); apigenin (raw:  $2.09 \pm 0.01$ ; cooked:  $1.79 \pm 0.02$ ), with exception of quercetin (raw:  $2.11 \pm 0.02$ ; cooked:  $5.73 \pm 0.02$ ) and caffeic acid (raw:  $2.08 \pm 0.04$ ; cooked  $2.95 \pm 0.04$ ). The results of the antioxidant indices of *in vitro* enzyme digested lima beans revealed higher values for cooked Lima beans compared to the raw counterpart, with a stepwise increase at the different stages of *in vitro* digestion, with the exception of ferric reducing antioxidant power; TPC (oral digestion:  $65.44 \pm 0.96$ ; gastric digestion:  $134.87 \pm 0.46$ ; intestinal digestion:  $517.72 \pm 4.70$ ; mg/g tannic acid equivalent), TFC (oral digestion:  $199.30 \pm 6.43$ ; gastric digestion:  $1065.97 \pm 1.22$ ; intestinal digestion:  $3691.87 \pm 4.2$ ; mg/g quercetin equivalent), DPPH (oral digestion:  $85800.00 \pm 305.50$ ; gastric digestion:  $99066.66 \pm 115.47$ ; intestinal digestion:  $211354.20 \pm 360.84$ ;  $\mu\text{mol TE/g sample}$ ). The results also revealed a progressive increase in the antioxidant indices and amino acid composition (mg/kg) for both raw and processed lima beans at various stages of the *in vitro* digestion, with the intestinal phase of simulated digestion ranking higher. This implied that the Lima beans contained some essential amino acids and antioxidant molecules that would be readily available after passing through the gastrointestinal tract and could therefore be explored as functional food in the management of free radical mediated diseases.

**Keywords:** Antioxidant Activities; Amino Acid Composition; Phenolic Compounds; *P. Lunatus*; *in vitro* Digestion

## 1 Introduction

Plants are the most predominant harvesters of solar energy and they are valuable primary resources of essential nutrients for human food production (Bain et al., 2013; Fanzo, 2015). In common with most developing nations of the world, the state of nutrition in Nigeria is still characterized by inadequate calorie and protein supplies (Babatunde, Adejobi, & Fakayode, 2010; FAO, 2011). Protein foods and particularly animal protein have continued to be in short supply. Most experts consider protein deficiency as the commonest form of malnutrition in the developing countries, especially in regions where diets are based mainly on roots and tuber crops (FAO, 2013; Trehan & Manary, 2015). The production of protein-rich foods (leguminous seeds and particularly animal products) has been much less efficient. Therefore, in an attempt to widen the narrow food base, food and agricultural scientists are screening lesser known and under-exploited native plants for possible potential sources of food nutrients (Baldermann et al., 2016).

Grain legumes are grouped into two classes; major and minor species. The major species include the industrial legumes such as soybean, groundnut, common beans (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), and pea (*Pisum sativum*). Minor species exist in a wide range of diversity either as cultivated or wild species across various regions of the world and are usually cultivated by the traditional farmers; they are also referred to as neglected, underutilized, under-cultivated or lesser-known legumes (Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2016). The wild species of the minor grain legumes include kersting groundnut (*Kerstingiella geocarpa*) and marama bean (*Tylosema esculentum*). The miscellaneous legumes are the minor grain legumes that have received very little research attention when compared with the major grain legumes such as cowpea and soybean. Leguminous seeds are an important source of proteins, energy and other nutrients in the diets of large population of people around the world; they form an excellent source of lysine, methionine and tryptophan, water-soluble vitamins (riboflavin, niacin and folacin) and of minerals: phosphorus, iron and magnesium (FAO,

2012). Legume seed coats, commonly referred to as hulls, are rich sources of polyphenolics and antioxidants (Iriti & Varoni, 2017; Pastor-Cavada et al., 2016). A number of studies have shown that legumes are rich in antioxidants, some of these legumes include peanut, lentils and soybean (Zou, Chang, Gu, & Qian, 2011), Bambara groundnut (Nyau, Prakash, Rodrigues, & Farrant, 2015), African yam bean, Lima bean (Obboh, Ademiluyi, & Akindahunsi, 2009), Pigeon pea (Obboh et al., 2009).

Lima bean, *P. Lunatus* belongs to the family leguminosae and is mostly cultivated in South America (Yellavila, Agbenorhevi, Asibuo, & Sampson, 2015). Lima beans are sometimes referred to as haba beans, sugar beans, butter beans, Guffin beans, civet beans, Hibbert beans, Pallar beans, Sieva beans, Madagascar beans, and Burma beans. It is one of the underutilized legumes found in Nigeria, though a minor crop; they have been an important source of plant protein to millions of Nigerians and are widely known for their fibre, mineral and protein contents but with a lesser attention to their nutraceutical value (Saleem, Ahmed, & Hasan, 2016; Yellavila et al., 2015).

The role of medicinal food plants in disease prevention has been attributed to the antioxidant properties of their constituents, usually associated with a wide range of amphipathic molecules, broadly termed polyphenolic compounds (Miguel, 2010; Ozcan, akpinar, Yilmaz-Ersan, & Delikanli, 2014). The protective effects of the dry beans in disease prevention may not be entirely associated with dietary fibre, but to phenolic compounds and other non-nutritive compounds (Miguel, 2010; Oomah, Tiger, Olson, & Balasubramanian, 2006). Polyphenols from Lima beans will possibly act as antioxidants, hindering the formation of free radicals that eventually lead to the deterioration of biological molecules. These naturally occurring phenolic compounds are predominantly present in the seed coat and possess anti-mutagenic and antioxidant activities (Attree, Du, & Xu, 2015; Iriti & Varoni, 2017).

The antioxidant compounds and other phytonutrients present in most legumes have been extracted and measured by different methods (Khang, Dung, Elzaawely, & Xuan, 2016; Nyau

et al., 2015; Oboh et al., 2009; Salawu, Bester, & Duodu, 2014). The results of such experiments clearly demonstrate a range of phyto-constituents with potential antioxidant action, but that cannot be translated to be available phyto-constituents when the legumes are degraded by the enzymes and juices of the gastrointestinal tract during passage through it. Some studies have compared the content of bioactive compounds and antioxidant activities in undigested and digested extracts (Bouayed, Hoffmann, & Bohn, 2011; Faller, Fialho, & Liu, 2012; Gimenez, Moreno, Lopez-Caballero, Montero, & Gomez-Guillen, 2013; Tavares et al., 2012). Hence, the present investigation sought to evaluate the antioxidant activities and the amino acid profile of Lima beans after a stepwise simulated human gastrointestinal digestion.

## 2 Materials and Methods

### 2.1 Chemicals

Follin-Ciocalteu's reagent, tannic acid, sodium carbonate, iron (iii) chloride, potassium ferri- cyanide, trichloroacetic acid, aluminium chloride, potassium acetate, 2,2- diphenyl-1-picrylhydrazyl radical and 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma chemical company, USA. All other chemicals were obtained from standard chemical suppliers and were of analytical grade, while the water used was glass distilled.

### 2.2 Sample collection

Lima beans (*P. Lunatus*) were obtained from Oba market in Akure, Nigeria. They were identified and authenticated in the Department of Crop, Soil and Pest Management, School of Agriculture and Agricultural Technology, Federal University of Technology, Akure, Ondo state, Nigeria.

### 2.3 Sample treatment and preparation

The sample was divided into two portions; the first portion of the sample (raw) was sorted, washed, air-dried and milled into a powdered form, prior to enzyme treatments of *in vitro* digestion. While, the second portion was sorted, washed and cooked at 100 °C for 4 h, cooled, air-dried and then milled prior to the enzyme treatments of *in vitro* digestion. All the milled samples were stored in plastic containers at room temperature in the Department of Biochemistry, Federal University of Technology, Akure, Nigeria until used.

### 2.4 *in vitro* enzyme digestion

The *in vitro* digestion using sequential enzymatic steps was based on a slightly modified method as reported by Delgado-Andrade, Alberto Conde-Aguilera, Haro, Pastoriza de la Cueva, and Angel Rufian-Henares (2010). Various stages of the gastrointestinal digestion (oral, gastric and total gastrointestinal digestion) were carried out.

#### Oral digestion

Ten grams of the milled Lima beans were weighed and dispersed in 200 mL of distilled water. 32.5 mg of alpha amylase was dissolved in 25 mL 1mM calcium chloride at pH 7.0, and 1500 µl of the alpha amylase was added to each test tube (simulating pH conditions in the mouth). The tubes were incubated in a shaking water bath set at 37 °C for 40 min and at 80 strokes/ min. The sample was incubated in boiling water at 100 °C for 4 min for enzyme inactivation and centrifuged for 60 min at 4000 x g, then the soluble fraction was kept in the refrigerator for subsequent analysis and the insoluble fraction was discarded. A non-enzymic digest (control) was also done for both raw and cooked samples with the same procedure used for the *in vitro* enzyme digestion but without the addition of enzymes.

#### Oral-Gastric digestion

Ten grams of the milled lima beans were weighed and dispersed in 200 mL of distilled water. 32.5

mg of alpha amylase was dissolved in 25 mL 1mM calcium chloride at pH 7.0, and 1500  $\mu$ l of the alpha amylase was added to each test tube (simulating pH conditions in the mouth). The tubes were incubated in a shaking water bath set at 37 °C for 40 min and at 80 strokes/ min. The pH was adjusted to 2 after 40 min using concentrated HCl. 10 mg pepsin which was dissolved in 5 mL of 0.05 M HCl was added to each tube (simulating pH conditions in the stomach). The tubes were incubated in a shaking water bath set at 37 °C for 40 min and at 80 strokes/ min. The sample was incubated in boiling water at 100 °C for 4 min for enzyme inactivation and centrifuged for 30 min at 4000 x g, then the soluble fraction was kept in the refrigerator for subsequent analysis: the insoluble fraction was discarded. A non-enzymic digest (control) (without enzyme) was also done for both raw and cooked samples with the same procedure used for the *in vitro* digestion.

### Total gastrointestinal digestion

Ten grams of the milled lima beans were weighed and dispersed in 200 mL of distilled water. 32.5 mg of alpha amylase were dissolved in 25 mL 1 mM calcium chloride at pH 7.0, and 1500  $\mu$ l of the alpha amylase was added to each test tube (simulating pH conditions in the mouth). The tubes were incubated in a shaking water bath set at 37 °C for 40 min and at 80 strokes/ min. The pH was adjusted to 2 after 40 min using concentrated HCl. 10 mg pepsin, which was dissolved in 5 mL of 0.05 M HCL, was added to each tube (simulating pH conditions in the stomach). The tubes were incubated in a shaking water bath set at 37 °C for 40 min and at 80 strokes/ min. After 40 min shaking, the pH was adjusted to 6 using NaOH. After 20 min, 50 mL of pancreatin solution (9 gram of pancreatin in 60 mL of distilled water), was added in each test tube and the pH was adjusted finally to 7.5 using NaOH (simulating pH conditions in the small intestine). The sample was incubated in boiling water at 100 °C for 4 min for enzyme inactivation and centrifuged for 60 min at 4000 x g. The soluble fraction was kept in the refrigerator for subsequent analysis and the insoluble fraction was discarded. A non-enzymic digest (control) (without

enzyme) was also made for both raw and cooked samples with the same procedure used for the *in vitro* digestion.

## 2.5 Quantification of phenolic compounds by HPLC-DAD

For analysis of the Lima bean extracts, 10 mL of the each extract was injected into a Phenomenex C<sub>18</sub> column (4.6 mm x 250 mm) packed with 5  $\mu$ m diameter pore size. Mobile phases A and B were Milli-Q water, acidified to pH 2.0 with 1 % of phosphoric acid and methanol, correspondingly, solvent gradient was used as follows: 0-10 min, 5 % B; 10-25 min, 15 % B; 25-40 min, 30 %; 40-55 min 50 % B; 50-65 min 70 % B; 65-80 min, 100 % B, following the method described by Omoba, Obafaye, Salawu, Boligon, and Athayde (2015) with slight modifications. The extracts and mobile phase were filtered through a 0.45  $\mu$ m membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The extract was analyzed at a concentration of 10 mg/mL. The flow rate was 0.6 mL/min and the injection volume was 40  $\mu$ L. The sample and mobile phase were filtered through a 0.45  $\mu$ m membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of reference standards were prepared in the HPLC mobile phase at a concentration range of 0.050 – 0.300 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid; 280 nm for catechin, 327 nm for caffeic acid, and 365 nm for rutin, quercitrin, quercetin, kaempferol and apigenin. The chromatography peaks were confirmed by comparing the retention time with those of the reference standards and by DAD spectra (200 to 700 nm). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10  $\delta$ /S, respectively, where  $\delta$  is the standard deviation of the response and S is the slope of the calibration curve (Boligon et al., 2015).



## 2.6 Determination of total phenolic content

The total phenolic content of the *in vitro* enzyme digested Lima bean and undigested control samples was determined by the Folin-Ciocalteu assay as described by Mole and Waterman (1994). 500  $\mu$ L of Folin-Ciocalteu reagent was added and mixed with a solution containing 100  $\mu$ L of the extract and 2 mL of distilled water. 1.5 mL of 7.5 % sodium carbonate was then added to the solution and the volume was made up to 10 mL with distilled water. The mixture was left to stand for 2 h after addition of the sodium carbonate. The absorbance of the mixture was measured at 760 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The standard used was tannic acid and the results were expressed as mg tannic acid equivalents per gram of the sample.

## 2.7 Determination of total flavonoid content

The total flavonoid content of the *in vitro* enzyme digested Lima bean and undigested control samples were determined using a slightly modified method reported by Meda, Lamien, Romito, Millogo, and Nacoulma (2005). Briefly, 0.5 mL of enzyme digested sample was mixed with 0.5 mL distilled H<sub>2</sub>O, 50  $\mu$ L of 10% AlCl<sub>3</sub>, 50  $\mu$ L of 1mol L<sup>-1</sup> potassium acetate and 1.4 mL water, and incubated at room temperature for 30 min. Thereafter, the absorbance of each reaction mixture was measured at 415 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The total flavonoid was calculated using quercetin as standard by making use of a seven point standard curve (0-100  $\mu$ g/mL). The total flavonoid content of samples was determined in triplicate and the results were expressed as mg quercetin equivalent per gram of the sample.

## 2.8 Reducing power assay

The reducing power of the *in vitro* enzyme digested Lima bean samples and undigested control were determined by assessing the ability of each extract to reduce FeCl<sub>3</sub> solution as described by Oyaizu (1986). Briefly, 1 mL of the sample was

mixed with 1 mL 200 mM sodium phosphate buffer (pH 6.6) and 1 mL 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then 1 mL 10% trichloroacetic acid (TCA) was added. This mixture was centrifuged at 353 x g for 10 min. 2 mL of the supernatant was mixed with an equal volume of water and 0.4 mL of 0.1 % ferric chloride. The absorbance was measured at 700 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The ferric reducing antioxidant power was expressed as mg ascorbic acid equivalent/g of the sample.

## 2.9 DPPH radical scavenging assay

DPPH radical scavenging activity was measured according to the method of Brand-Williams, Cuvelier, and Berset (1995), with some modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at -20°C until needed. The working DPPH solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 units at 515 nm using the spectrophotometer. 0.5 mL of the *in vitro* enzyme digested sample was diluted with 2 mL of methanol to obtain a mother solution. 150  $\mu$ L of the mother solution were allowed to react with 2850  $\mu$ L of the DPPH working solution for 6 h in the dark. Then the absorbance was taken at 515 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). Result was expressed in  $\mu$ mol Trolox equivalent/g sample.

## 2.10 Amino acid profile assay

Amino acid profile was determined according to the method of Obreshkova, Tsvetkova, and Ivanov (2012). In order to ensure that the dried and pulverized undigested (cooked and raw) samples were completely dry, they were dried to constant weight for a period of time in the laboratory. The sample of 10.0 g was weighed into a 250 mL conical flask. The sample was defatted with 30 mL of the petroleum spirit three times with soxhlet extractor that was equipped with thimble. The sample was hydrolyzed three times to ensure complete hydrolysis to achieve total recov-



ery of amino acids. The pulverized and defatted sample was soaked with 30 mL of 1 M potassium hydroxide solution and incubated for 48 h at 110 °C in a hermetically sealed borosilicate glass container. After the alkaline hydrolysis, the hydrolysate was neutralized to get the pH in the range 2.5-5.0. The solution was purified by cation-exchange solid-phase extraction. The amino acids in the purified solution were derivatised with ethylchloroformate by the established mechanism, and subsequently injected into the gas chromatograph (GC) for amino acid analysis. The deactivated enzyme digests of raw and cooked samples (digest incubated in boiling water at 100 °C for 4 min), obtained after the total intestinal phase of the *in vitro* digestion were also injected into the GC for amino acid analysis.

## 2.11 Statistical Analysis

All the analyses were run in triplicate. Results were then computed using Microsoft Excel software (Microsoft Corporation, Redmond, WA) and followed by one-way Anova with Duncan's Multiple Range Test (DMRT) to compare the means that showed significant variation by using SPSS 11.09 for Windows. The significance level was set at  $p < 0.05$ .

## 3 Results and Discussion

### 3.1 Results

#### HPLC-DAD Phenolic Characterization

The HPLC-DAD analysis of phenolic compounds in processed Lima beans (cooked and raw) as shown in Fig. 1, revealed the presence of gallic acid, catechin, caffeic acid, rutin, quercitrin, quercetin, kaempferol and apigenin. The quantitative estimates of these phenolic compounds in Lima beans (Table 1) revealed a slight reduction in the level of some of them (gallic acid, catechin, rutin, quercitrin, apigenin) after cooking, with exception of caffeic acid and quercetin which were higher in the raw Lima beans.

#### Total Phenolic and Flavoid Content

The phenolic contents of raw and processed Lima beans at various stages of *in vitro* digestion were as shown in Fig. 2. The results revealed a slightly higher phenolic content in the enzyme digested cooked beans compared to the raw counterpart. The results also showed a progressive increase in the phenolic content (mg/g) of the digested Lima beans at each stage of *in vitro* digestion with the oral phase of digestion showing the least phenolic content (raw: 45.67, cooked: 65.44) followed by the oral-gastric phase of digestion (raw: 61.76, cooked: 134.87). The highest phenolic content was recorded after the total gastrointestinal digestion (raw: 439.18, cooked: 517.72). The total flavonoid content of raw and processed Lima beans at various stages of *in vitro* digestion is shown in Fig. 3. Similarly, the results revealed higher flavonoid content in the enzyme digested cooked beans compared to the raw sample. The results also showed a progressive increase in the total flavonoid content of the digested Lima beans at each stage of *in vitro* digestion with the oral phase of digestion showing the least total flavonoid content. The higher flavonoid content was recorded after the total gastrointestinal digestion in digested raw and digested cooked Lima beans respectively.

#### Antioxidant Properties

The results of the ferric reducing antioxidant power of raw and processed lima beans at various stages of *in vitro* digestion were as shown in Fig. 4. Conversely, the ferric reducing antioxidant power of the digested raw lima beans was higher than that of the digested cooked lima beans. The DPPH radical scavenging activity of raw and cooked lima beans, at various stages of *in vitro* digestion was as shown in Fig. 5. The results also revealed higher DPPH radical scavenging activity in the enzyme digested cooked beans compared to the raw counterpart. Similarly, the results also showed a progressive increase in the ferric reducing power and DPPH radical scavenging activity of the digested Lima beans at each stage of *in vitro* digestion, with the total phase of the simulated gastro intestinal digestion ranking higher.

Table 1: Phenolic and flavonoid composition of Lima Bean extracts

Compounds	Raw Lima beans mg/g	Cooked Lima beans mg/g
Gallic acid	1.96 ± 0.02 <sup>a</sup>	1.82 ± 0.01 <sup>a</sup>
Catechin	0.83 ± 0.01 <sup>b</sup>	0.73 ± 0.01 <sup>b</sup>
Caffeic acid	2.08 ± 0.04 <sup>a</sup>	2.95 ± 0.04 <sup>c</sup>
Rutin	2.61 ± 0.03 <sup>d</sup>	1.74 ± 0.03 <sup>a</sup>
Quercitrin	5.73 ± 0.01 <sup>e</sup>	5.68 ± 0.01 <sup>d</sup>
Quercetin	2.11 ± 0.02 <sup>a</sup>	5.73 ± 0.02 <sup>d</sup>
Kaempferol	-	0.81 ± 0.03 <sup>b</sup>
Apigenin	2.09 ± 0.01 <sup>a</sup>	1.79 ± 0.02 <sup>a</sup>

Results are expressed as mean ± standard deviations (SD) of three determinations.

Means followed by different letters differ significantly by Tukey test at  $p < 0.05$

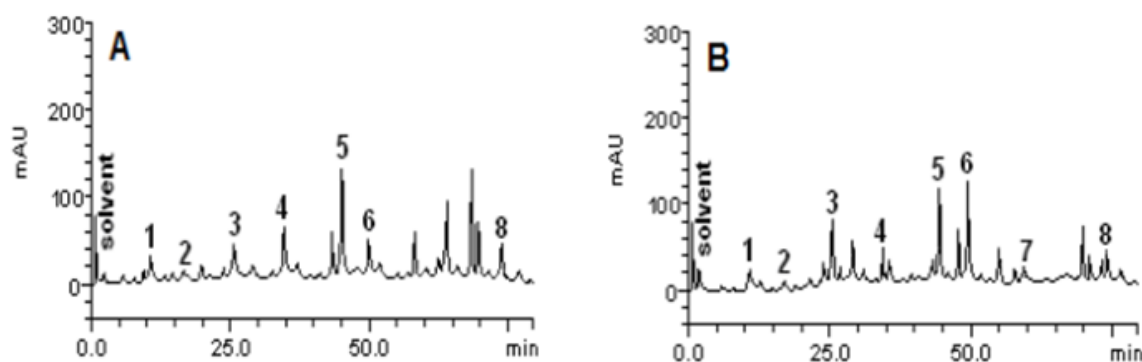


Figure 1: Representative high performance liquid chromatography profile of Lima bean raw (A) and cooked (B) extracts. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), quercetin (peak 6), kaempferol (peak 7) and apigenin (peak 8)

### Amino acid Profile

The amino acid profiles of raw and processed Lima beans at various stages of *in vitro* digestion were as shown in Table 2. The results revealed a varied level of glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, aspartate, lysine, methionine, glutamate, phenylalanine, histidine, arginine, tyrosine, tryptophan and cysteine in the raw and cooked Lima beans respectively. High levels of these amino acids were found after the total intestinal phase of the simulated gastrointestinal digestion in both cooked and raw *in vitro* digested Lima beans. The results also showed that the *in vitro* en-

zyme digested cooked sample had higher amino acid values than the raw counterpart. It was observed that the process of cooking gave significantly higher amino acid contents compared with the raw counterpart.

### Discussion

Legumes are one of the most important classes of food that have been used as a stable food to provide the proteins and energy requirements of man (Brand, Brandt, & Cruywagen, 2004). In addition to the provision of nutrients, legumes also contain valuable bioactive compounds such

Table 2: The Amino Acid Profile of Raw and Cooked *in vitro* Enzyme Digested and Undigested Lima Beans (mg/kg)

Amino Acid	RP	ORD	OGRD	TDR	CP	ODC	OGCD	TCD
Glycine	310±32.4 <sup>f</sup>	0.083±0.002 <sup>a</sup>	0.15±0.03 <sup>c</sup>	290±19.2 <sup>c</sup>	330±19.4 <sup>h</sup>	0.14±0.02 <sup>b</sup>	0.29±0.03 <sup>d</sup>	320±34.2 <sup>g</sup>
Alanine	290±12.5 <sup>f</sup>	0.096±0.004 <sup>a</sup>	0.15±0.05 <sup>c</sup>	270±15.1 <sup>c</sup>	320±17.2 <sup>h</sup>	0.11±0.04 <sup>b</sup>	0.23±0.05 <sup>d</sup>	310±12.5 <sup>g</sup>
Serine	500±21.2 <sup>g</sup>	0.10±0.05 <sup>a</sup>	0.23±0.04 <sup>c</sup>	440±21.3 <sup>c</sup>	520±22.3 <sup>h</sup>	0.17±0.04 <sup>b</sup>	0.46±0.03 <sup>d</sup>	490±43.3 <sup>f</sup>
Proline	120±14.3 <sup>f</sup>	0.012±0.006 <sup>a</sup>	0.037±0.02 <sup>c</sup>	82±4.1 <sup>e</sup>	120±11.4 <sup>g</sup>	0.029±0.001 <sup>b</sup>	0.059±0.002 <sup>d</sup>	87±6.4 <sup>g</sup>
Valine	500±21.5 <sup>f</sup>	0.15±0.03 <sup>a</sup>	0.24±0.05 <sup>c</sup>	460±21.3 <sup>c</sup>	510±17.4 <sup>g</sup>	0.21±0.03 <sup>b</sup>	0.27±0.04 <sup>d</sup>	500±24.3 <sup>f</sup>
Threonine	340±13.3 <sup>g</sup>	0.12±0.04 <sup>a</sup>	0.21±0.01 <sup>c</sup>	320±14.2 <sup>c</sup>	350±15.2 <sup>h</sup>	0.18±0.02 <sup>b</sup>	0.24±0.03 <sup>d</sup>	330±31.3 <sup>f</sup>
Isoleucine	410±14.1 <sup>f</sup>	0.14±0.02 <sup>a</sup>	0.19±0.03 <sup>c</sup>	400±15.3 <sup>c</sup>	500±21.3 <sup>h</sup>	0.16±0.06 <sup>b</sup>	0.23±0.06 <sup>d</sup>	480±32.4 <sup>g</sup>
Leucine	600±15.2 <sup>f</sup>	0.17±0.03 <sup>a</sup>	0.23±0.01 <sup>c</sup>	570±20.4 <sup>c</sup>	650±31.4 <sup>h</sup>	0.19±0.06 <sup>b</sup>	0.26±0.05 <sup>d</sup>	640±18.4 <sup>g</sup>
Aspartate	850±23.6 <sup>g</sup>	0.35±0.01 <sup>a</sup>	0.44±0.04 <sup>c</sup>	710±18.1 <sup>c</sup>	870±28.5 <sup>h</sup>	0.39±0.03 <sup>b</sup>	0.50±0.07 <sup>d</sup>	780±33.5 <sup>f</sup>
Lysine	520±21.3 <sup>g</sup>	0.17±0.05 <sup>a</sup>	0.19±0.03 <sup>c</sup>	410±12.3 <sup>c</sup>	570±21.4 <sup>h</sup>	0.19±0.07 <sup>b</sup>	0.22±0.04 <sup>d</sup>	500±41.2 <sup>f</sup>
Methionine	75±4.7 <sup>f</sup>	0.014±0.003 <sup>a</sup>	0.015±0.006 <sup>ab</sup>	73±4.1 <sup>e</sup>	79±5.5 <sup>g</sup>	0.015±0.001 <sup>ab</sup>	0.15±0.03 <sup>c</sup>	70±8.5 <sup>d</sup>
Glutamate	960±15.6 <sup>f</sup>	0.38±0.04 <sup>a</sup>	0.42±0.03 <sup>c</sup>	940±1.4 <sup>e</sup>	1000±29.7 <sup>h</sup>	0.40±0.05 <sup>b</sup>	0.53±0.02 <sup>d</sup>	930±27.3 <sup>g</sup>
Phenylalanine	420±17.3 <sup>g</sup>	0.15±0.03 <sup>a</sup>	0.22±0.04 <sup>c</sup>	370±21.4 <sup>c</sup>	440±12.3 <sup>h</sup>	0.17±0.03 <sup>b</sup>	0.25±0.04 <sup>d</sup>	400±33.4 <sup>f</sup>
Histidine	270±12.4 <sup>f</sup>	0.25±0.02 <sup>a</sup>	0.31±0.03 <sup>c</sup>	250±14.2 <sup>c</sup>	280±21.4 <sup>g</sup>	0.29±0.02 <sup>b</sup>	0.36±0.06 <sup>d</sup>	250±14.5 <sup>e</sup>
Arginine	400±25.3 <sup>e</sup>	0.13±0.04 <sup>a</sup>	0.17±0.05 <sup>c</sup>	400±13.6 <sup>c</sup>	530±25.2 <sup>h</sup>	0.15±0.01 <sup>b</sup>	0.22±0.03 <sup>d</sup>	470±23.4 <sup>g</sup>
Tyrosine	250±1.3 <sup>g</sup>	0.055±0.003 <sup>a</sup>	0.26±0.03 <sup>c</sup>	230±10.2 <sup>c</sup>	240±22.4 <sup>h</sup>	0.08±0.001 <sup>b</sup>	0.32±0.06 <sup>d</sup>	210±19.4 <sup>f</sup>
Tryptophan	94±7.5 <sup>g</sup>	0.11±0.02 <sup>a</sup>	0.17±0.01 <sup>c</sup>	73±5.1 <sup>e</sup>	110±9.7 <sup>h</sup>	0.12±0.03 <sup>ab</sup>	0.18±0.03 <sup>cd</sup>	82±3.7 <sup>f</sup>
Cysteine	100±9.7 <sup>g</sup>	0.034±0.005 <sup>a</sup>	0.13±0.03 <sup>c</sup>	80±4.3 <sup>e</sup>	100±8.5 <sup>g</sup>	0.047±0.002 <sup>b</sup>	0.14±0.01 <sup>cd</sup>	99±2.9 <sup>f</sup>

Abbreviations: RP = Raw dried and powdered; ORD = Oral raw digested; OGRD = Oral and gastric raw digested; TDR = Total digested raw; CP = Cooked dried and powdered; ODC = Oral cooked digested; OGDC = Oral and gastric cooked digested; TCD = Total cooked digested. Values are given as mean ± SE of independent experiments performed in triplicate.

Values are given as mean ± SE of independent experiments performed in triplicate. Values with different superscripts in the same row differ significantly ( $P < 0.05$ )

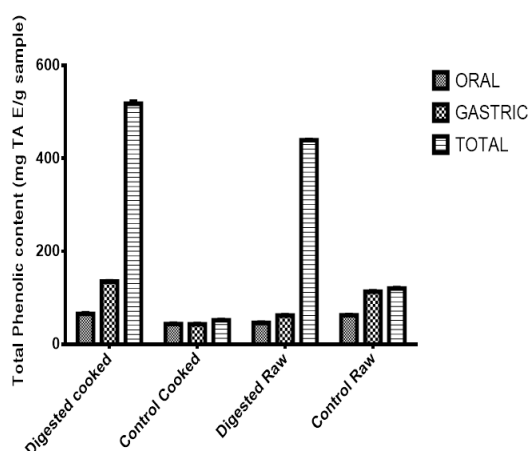


Figure 2: Total phenolic content of *P. Lunatus* extract. The results are expressed as milligrams of tannic acid equivalent (mg TA) per 100 g of dry weight sample. Value are given as mean ± SE of independent experiments performed in triplicate. TA= Tannic Acid; Oral= Oral digestion; Gastric= Gastric digestion; Total=Total intestinal digestion

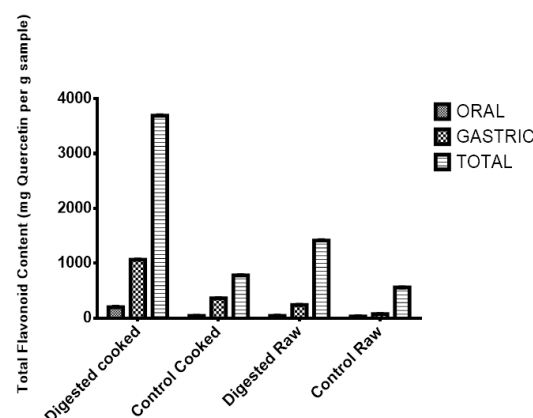


Figure 3: Total flavonoid content of *P. Lunatus* extract expressed as mg of quercetin equivalents (QE) per 100 g of dry weight of the sample. Value are given as mean ± SE of independent experiments performed in triplicate. Oral= Oral digestion; Gastric= Gastric digestion; Total=Total intestinal digestion

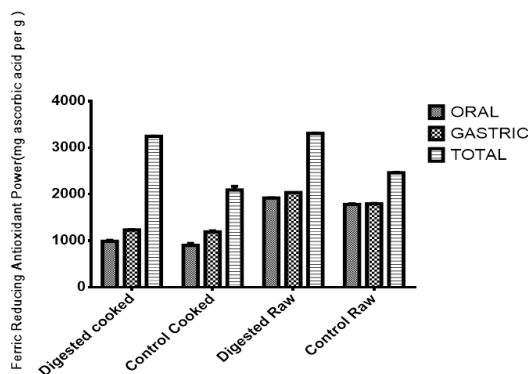


Figure 4: Ferric reducing antioxidant power of *P. Lunatus* extract expressed as milligrams ascorbic acid equivalent per 100 g of dry weight sample. Value are given as mean  $\pm$  SE of independent experiments performed in triplicate. AA= Ascorbic Acid; Oral= Oral digestion; Gastric= Gastric digestion; Total=Total intestinal digestion

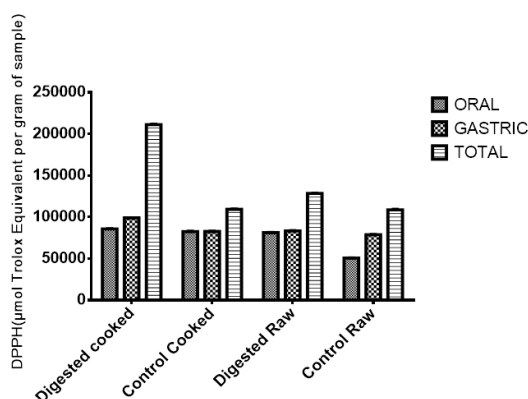


Figure 5: DPPH radical scavenging activity of *P. Lunatus* extract expressed as  $\mu$  mole Trolox equivalents per 100 g of dry weight of the sample. Value are given as mean  $\pm$  SE of independent experiments performed in triplicate. Oral= Oral digestion; Gastric= Gastric digestion; Total=Total intestinal digestion

as enzyme inhibitors, lectins, phytates, oxalates, polyphenols, phytosterols and saponins that may help prevent humans developing various diseases. Phenolic compounds constitute an important group of natural products contributing significantly to the medicinal value of a number of food plants (Huang, Cai, & Zhang, 2010). Studies have focused on the biological activities of phenolic compounds, which have potential antioxidant and free radical scavenging abilities (Lisanti et al., 2016).

Investigations on the phenolic composition of legumes have revealed that legumes are good sources of phenolic compounds with antioxidant activities that can be harnessed in the management of free radical related diseases (Attree et al., 2015; Iriti & Varoni, 2017). A previous report on the phenolic composition of three varieties of the common bean, (*Phaseolus vulgaris* L.), soybeans (*Glycine max* L.), and peas (*Pisum sativum* L.) from Rwanda revealed the presence of 11 different phenolic compounds in these common beans: gallic acid, (+)-catechin, (-)-epicatechin, caffeic acid, o-coumaric acid, chlorogenic acid, quercetin, 4-hydrobenzoic acid, syringic acid, ferulic acid and vanillic acid (Joseph et al., 2014). Catechin was also reported in green beans (Escarpa & Gonzalez, 2000). In the same vein, a study on the phenolic acids' content of fifteen dry edible bean varieties revealed a high amount of caffeic acid in black beans (Luthria & Pastor-Corrales, 2006). The phenolic profiles of *Phaseolus vulgaris* revealed the presence of 13 quercetin-3-O-glycosides, eight kaempferol-3-O-glycosides, two myricetin glycosides and four phenolic acids with potential antioxidant activities (Reyes-Martinez et al., 2014).

The reduction in phenolic contents of the Lima beans during cooking could be due to leaching of phenolic compounds in the soaking water. The slight reduction in the level of some of the phenolic compounds after cooking, was in agreement with previous reports, where cooking lowers the total phenolic content of the food materials that were studied (Gujral, Angurala, Sharma, & Singh, 2011; Han & Xu, 2014).

Several investigations on common beans highlight their antioxidant potential (Akond et al., 2011; Karamac & Amarowicz, 2004). Phenolic compounds are widely distributed in the

plant kingdom and are important in the antioxidant capacity *in vitro* because of their ability to donate hydrogen and form stable radical intermediates (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). The higher total phenolic content of the *in vitro* digested cooked beans compared with those digested in the raw form may have been due to alterations in the chemical structure and composition as a result of heat during boiling (Han & Xu, 2014). Thus, cooking might have enhanced the breakdown of the insoluble fibre matrix of the beans thereby making its polyphenols more accessible for further breakdown by the enzymes of the gastro-intestinal tract (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010).

The higher phenolic content at the intestinal phase of gastrointestinal digestion was in agreement with previous reports (Ryan & Prescott, 2010; Wootton-Beard, Moran, & Ryan, 2011), where a higher phenolic content and antioxidant activity was reported for commercially available juices after the gastric and intestinal phases of gastrointestinal digestion. The higher total flavonoid content of the *in vitro* digested cooked beans compared with those digested in the raw form may have also been due to alterations in the chemical structure and composition as a result of heat during boiling (Han & Xu, 2014), thereby making its constituent flavonoids more accessible for further breakdown by the enzymes of the gastro-intestinal tract (Tagliazucchi et al., 2010). The higher total flavonoid content at the intestinal phase of the gastrointestinal digestion was in agreement with previous reports (Ryan & Prescott, 2010; Wootton-Beard et al., 2011), where a higher polyphenolic content and antioxidant activity was reported for commercially available juices after the gastric and intestinal phases of gastrointestinal digestion.

The decreasing value of the ferric reducing antioxidant power in the cooked lima beans compared to the raw lima beans might have been associated with a quantitative loss of some flavonoids during the heat treatment (Jeong et al., 2004; Salawu, Innocenti, Giaccherini, Akindahunsi, & Mulinacci, 2008). The higher DPPH radical scavenging activity in the cooked beans of the enzyme digested Lima beans compared to the raw counterpart was in agreement with pre-

vious report (Han & Xu, 2014).

The observed progressive increase in the ferric reducing power and DPPH radical scavenging activity of the digested Lima beans at each stage of *in vitro* digestion may have been associated with a significantly positive correlation that usually exists between the polyphenolic content and antioxidant activities (Rahman, Khan, Das, & Hossain, 2016; Ryan & Prescott, 2010; Sadeghi, Valizadeh, Shermeh, & Akaberi, 2015; Wootton-Beard et al., 2011).

High levels of amino acids found at the total intestinal phase of the simulated gastrointestinal digestion in both cooked and raw *in vitro* digested lima beans was in agreement with previous reports (Jin, Zhou, Li, Lai, & Li, 2015), where it was reported that pepsin digestion brings about a marked increase in the free amino acid content of a coconut meat protein hydrolysate by 30.3 % compared to the control and that after digestion with pancreatin, the free amino acid content of a coconut meat protein hydrolysate increased dramatically by 86.7 % compared to the control. Thus, the maximum amounts of amino acids were released during the total gastrointestinal phase of the simulated *in vitro* digestion process as a result of the action of the digestive enzymes ( $\alpha$ -amylase, pepsin, and pancreatin) of the GIT. This observation was in agreement with a previous report (Tagliazucchi et al., 2010), that suggested that digestion might be a determinant factor in the release of nutritionally relevant compounds from the food matrix. The higher amino acid contents in the cooked Lima beans compared with the raw counterpart was in agreement with previous reports (Lombardi-Boccia, Schlemmer, Cappelloni, & Di Lullo, 1998; Oluwaniyi, Dosumu, & Awolola, 2010), where it was reported that the process of boiling generally gives slight but significantly higher amino acid contents respectively compared with the fresh samples.

## 4 Conclusions

The results of this study revealed that Lima beans have a number of health promoting phenolic compounds with antioxidant potential. The results also revealed higher antioxidant indices

after the intestinal phase of the simulated human digestion. Furthermore, the results also established the availability of a number of essential amino acids after the simulated *in vitro* digestion, with that of the total intestinal phase of simulated *in vitro* digestion ranking higher. Therefore, Lima beans could be considered a functional food, with consumption providing a source of nutritionally important amino acids and aiding the prevention of free radical mediated diseases.

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# Moisture Sorption Isotherm and Thermodynamic Properties of Jamun (*Syzygium cumini* L.) Powder made from Jamun Pulp and Seed

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## Abstract

The present work aimed to: i) find the suitable proportion, based on sensory evaluation, of microwave-convective hot air dried jamun (*Syzygium cumini* L.) pulp and seed kernel powder to be mixed for the preparation of jamun powder (JP); ii) generate and model the moisture sorption isotherm (MSI) of JP; and iii) estimate net isosteric heat of sorption ( $q_{st}$ ), spreading pressure ( $\Phi$ ), net integral enthalpy ( $Q_{in}$ ), and net integral entropy ( $S_{in}$ ). To formulate JP, the proportion (w/w, db) comprising 2% kernel and 98% pulp powder was the most desirable. The Peleg model was the best fit to MSI of JP. The  $q_{st}$  decreased following linear relationship from 11.02 kJ. mol<sup>-1</sup> at 5% equilibrium moisture content (EMC) to 0.27 kJ. mol<sup>-1</sup> at 30% EMC. The  $\Phi$  increased with increase in water activity and decreased with increase in temperature from 25 °C to 35 °C, and the values of  $\Phi$  at 45 °C were even higher than at 25 °C. Net integral enthalpy ( $Q_{in}$ ) initially decreased till 6% moisture content in JP and displayed an increasing trend with further increase in moisture content. On the contrary,  $S_{in}$  kept on decreasing continually with increasing moisture content. The moisture zone of 7-11% was considered safe for storage of JP within the temperature range of 45-25 °C.

**Keywords:** Jamun powder; Moisture sorption isotherm; Net isosteric heat of sorption; Spreading pressure; Net integral enthalpy; Net integral entropy

## 1 Introduction

Jamun (*Syzygium cumini* L.), a type of berry with an attractive sweet-sour taste and purple colour, is a fruit native to India and East Indies, but is also found in other countries like Thailand, Philippines and Madagascar. All the fractions of whole jamun fruit, besides their nutritional constituents, contain a lot of phytochemicals and, hence, are widely popular for their medicinal values. Sehswag and Das (2015) reported a broad classification of the phytochemicals such

as anthocyanins, phenolic acids, flavonols, flavanoneols, carotenoids and terpenes in the pulp and skin, and alkaloids, flavonoids, glycosides, phytosterols, saponins, tannins and triterpenoids in the seed. Recent studies on jamun pulp and skin have established many therapeutic properties such as antidiabetic, antioxidant, hepatoprotective, antibacterial and anticancerous. Jamun seed kernel is richer in medicinal properties than pulp and skin, and possesses a range of pharmacological actions (Sehswag & Das, 2015, 2016). However, due to its short seasonal availability

## Nomenclature

$a_m$	Surface area of a water molecule ( $\text{m}^2$ )	MSI	Moisture sorption isotherm
$a_w$	Water activity	$Q_{in}$	Net integral enthalpy ( $\text{kJ. mol}^{-1}$ )
$\Phi$	Spreading pressure ( $\text{J. m}^{-2}$ )	$q_{st}$	Net isosteric heat of sorption ( $\text{kJ. mol}^{-1}$ )
$a_w^*$	Geometric mean water activity at constant $\Phi$	R	Universal gas constant ( $\text{kJ. mol}^{-1}. \text{K}^{-1}$ )
EMC	Equilibrium moisture content (% db)	$r^2$	Co-efficient of determination
GMS	Glycerol monostearate	RH	Relative humidity (%)
JP	Jamun powder	RMSE	Root mean square error
$K_\beta$	Boltzmann constant ( $\text{J. K}^{-1}$ )	$S_{in}$	Net integral entropy ( $\text{kJ. mol}^{-1}. \text{K}^{-1}$ )
LDPE	Low density polyethylene	SD	Standard deviation
LSD	Least significant difference	T	Absolute temperature (K)
M	EMC (% db) of the sample	TCP	Tricalcium phosphate
MCD	Microwave-convective hot air drying	Z	Integration constant
MD	Maltodextrin		
MRE	Mean residual error (%)		

and highly perishable nature, jamun remains underutilized.

Removal of moisture by quick and efficient drying is the most promising way for seasonal and perishable fruits to be made available throughout the year. Dehydrated fruit in powder form can be incorporated within a variety of recipes, like desserts, biscuits and spreads, to add case specific flavor and functionalities without the moisture and volume of fresh fruit. Among different methods, a hybrid technique, microwave-convective hot air drying (MCD) has been used effectively for drying various food materials such as apricot halves (Horuz, Bozkurt, Karatas, & Maskan, 2017), button mushrooms (Das & Arora, 2018), oyster mushrooms (Bhattacharya, Srivastav, & Mishra, 2015), okra (Kumar, Prasad, & Murthy, 2014) and garlic cloves (Sharma & Prasad, 2001). Prolonged exposure in conventional hot air drying leads to undesirable changes in product quality. Combining microwave with convective hot air can signifi-

cantly reduce the drying time and improve product quality (Schiffmann, 1992). Although jamun seed powder (no mention of the presence or absence of seed coat) is available as an ayurvedic medicine for control of diabetic mellitus, technical information on drying of jamun seed and pulp is scanty in the literature. Recently Dey Paul and Das (2017) produced free flowing jamun pulp (along with skin) powder containing maltodextrin (MD, 12.2%), tricalcium phosphate (TCP, 0.4%) and glycerol monostearate (GMS, 1.4%) on a dry basis (db) of pulp-skin, using MCD at 70 °C, 1 watt.  $\text{g}^{-1}$  (W.  $\text{g}^{-1}$ , power density), 0.5 m.  $\text{s}^{-1}$  air velocity, to maximally maintain its antioxidant activity. Monica, Dey Paul, and Das (2016) also observed that MCD (60 °C, 2 W.  $\text{g}^{-1}$ , 0.5 m.  $\text{s}^{-1}$ ) of jamun seed retained maximum antioxidant activity of its kernel. Thus, if pulp powder is mixed with seed kernel powder (bitter in taste probably due to the presence of saponins and tannins) (Kamal, 2014; Kapoor & Iqbal, 2013) in a sensorially acceptable ratio,

the mixture could be used as jamun powder (JP) for food purpose, with more health benefits than that of pulp powder alone.

Moisture sorption tendency, which depends on relative humidity (RH) and temperature, is a major criterion in maintaining quality of fruit powders during handling and storage. Moisture sorption isotherm (MSI) provides the relationship between equilibrium moisture content (EMC) and its water activity ( $a_w$ ) when the food equilibrates in air with different RHs at a certain temperature. On equilibration, the  $a_w$  of food (relates to stability) equals the RH of environment (Al-Muhtaseb, McMinn, & Magee, 2002). Moisture sorption isotherm (MSI) is an essential tool in design of drying, packaging and storage of food. Mathematical modelling enables generation of MSI at any unknown temperature within the temperature range for which the MSIs have already been developed empirically. de Santana et al. (2015) reported MSIs of freeze dried jamun pulp (without skin) containing no additives. Biswal, Mohapatra, Panda, and Dash (2017) modelled the desorption isotherm of fresh jamun fruits. Ferrari, Marconi Germer, Alvim, Vissotto, and de Aguirre (2012) modelled the MSI of spray dried blackberry (*Rubus* species) powder containing maltodextrin and gum arabic. Information on the MSI of powder containing pulp, skin and kernel of jamun, together with any drying aid (to make the powder form) is not available.

Moisture sorption isotherms at different temperatures are used to evaluate thermodynamic parameters like net isosteric heat of sorption ( $q_{st}$ ), spreading pressure ( $\Phi$ ), net integral enthalpy ( $Q_{in}$ ) and net integral entropy ( $S_{in}$ ) (Al-Muhtaseb, McMinn, & Magee, 2004). The  $q_{st}$  (total heat of sorption for binding water vapor on sorbent less than the heat of vaporization of pure water, at the system moisture content) determines moisture-sorbent binding strength, and is required in designing equipment for dehydration processes as well as prediction of  $a_w$  when the food is stored at fluctuating temperature (Chowdhury & Das, 2010). Spreading pressure ( $\Phi$ ), the difference in surface tension between bare sorption sites of the sorbent and sites with adsorbed molecules, indicates the free surface energy of adsorption (Al-Muhtaseb et al., 2004).

Net integral enthalpy ( $Q_{in}$ ) at constant  $\Phi$ , a measure of the strength of moisture-solid binding, is used to determine  $S_{in}$  (McMinn & Magee, 2003). Net integral entropy ( $S_{in}$ ) explains the degree of disorder and randomness of the adsorbed water molecules (Arslan & Togrul, 2005; Igathinathane, Womac, Sokhansanj, & Pordesimo, 2007), and helps to determine the moisture at which the sorbent is stable.

Based on the above knowledge, the objectives of the present work were to: i) find the suitable proportion of pulp (with skin) and kernel powder to be mixed for preparation of JP; ii) generate and model the MSI of JP; and iii) estimate the thermodynamic parameters, including net isosteric heat of sorption ( $q_{st}$ ), spreading pressure ( $\Phi$ ), net integral enthalpy ( $Q_{in}$ ) and net integral entropy ( $S_{in}$ ) for the JP.

## 2 Materials and Methods

### 2.1 Material

Fully matured jamun fruit (raw material), apparently free from any damage, was procured from the local market of IIT Kharagpur, India. About 75% of the fruit was comprised of pulp with adherent skin (hereunder called pulp), with the remaining 25% being the seed. The moisture, protein, fat, ash, crude fiber and carbohydrate content of pulp were analysed and found to be  $83.04 \pm 0.10\%$  (wet basis, wb),  $8.59 \pm 0.07$ ,  $3.23 \pm 0.39$ ,  $4 \pm 0.001$ ,  $2.49 \pm 0.00$  and  $81.71 \pm 0.45\%$  (dry basis, db) respectively. For seed kernel, the respective values were  $15.12 \pm 0.04\%$  (wb),  $5.90 \pm 0.14$ ,  $1.01 \pm 0.01$ ,  $2.34 \pm 0.02$ ,  $2.33 \pm 0.37$  and  $88.41 \pm 0.53\%$  (db) (Sehwag & Das, 2016). MD (dextrose equivalent < 20) and TCP, both from HiMedia Laboratories Pvt. Ltd., Mumbai, India, and GMS from Alfa Aesar, Massachusetts, USA, were used as additives for production of pulp powder. Analytical grade lithium chloride (LiCl), potassium acetate ( $\text{CH}_3\text{COOK}$ ), magnesium chloride ( $\text{MgCl}_2$ ), potassium carbonate ( $\text{K}_2\text{CO}_3$ ), magnesium nitrate  $\text{Mg}(\text{NO}_3)_2$ , sodium nitrate ( $\text{NaNO}_3$ ), sodium chloride (NaCl) and potassium chloride (KCl) were used to adjust RH (described below). All the chemicals were used as received. Glass distilled water was used for



preparation of salt solutions.

## 2.2 Preparation of raw materials

The fruits were washed with potable water and air dried at room temperature. The pulp was separated from the seed with a knife, and stored in low density polyethylene (LDPE) pouches (about 250 g pouch<sup>-1</sup>; pouch film thickness ~ 0.05 mm) at -30 °C. The seeds were washed with water to remove any adhering pulp, and similarly packed and stored.

## 2.3 Preparation of jamun pulp and seed kernel powder

The frozen pulp was put in an empty glass beaker, which was then thawed in a water bath at room temperature (28 °C). The thawed mass was then ground into paste (at same temperature) using domestic grinder (Sumeet Research and Holdings Limited, Chennai, India). The paste was mixed with 12.2% MD, 0.4% TCP and 1.4% GMS on dry basis (db) of the pulp. The mixed paste was dried using a fully controlled miniature conveyer type industrial microwave-convective hot air dryer (Enerzi Microwave Systems Pvt. Ltd., Bangalore, Karnataka, India) having 20-100 kg. h<sup>-1</sup> drying capacity, 2450 MHz frequency and 250-3000 W power. An in-built heater of 6 kW provided hot air (25 °C to 200 °C) circulation. The drying chamber, 2 m x 300 mm x 200 mm, was made of 2 mm thick stainless steel. Drying was carried out at 70 °C, 1 W. g<sup>-1</sup> and 0.5 m. s<sup>-1</sup> air velocity. The composition of the mixture and drying method and conditions were described earlier (Dey Paul & Das, 2017; Paul & Das, 2018). The dried pulp obtained in flake form was ground into fine powder (212 µm passing).

The frozen jamun seeds were thawed, dried using the same dryer but at 60 °C, 2 W. g<sup>-1</sup> and 0.5 m. s<sup>-1</sup> air velocity, and the seed coat was manually removed (Monica et al., 2016). The dried kernels were ground into fine powder (212 µm passing). To judge the functionality, in separate work (not included here), total phenolic content (TPC) in mg gallic acid equivalent (GAE)/g, DPPH antioxidant activity (AA) in mg butylated hy-

droxyanisole (BHA)/g, db, both in 50% aqueous ethanolic extract, and monomeric anthocyanin content (MAC) in mg malvidin-3-glucoside (M3G)/g, db were measured for different portions (triplicate) of the powder before and after mixing. The pulp powder contained 26.19±0.45 TPC, 23.79±0.35 AA and 9.96±0.16 MAC, whereas for kernel powder the values were 53.95±0.26 TPC and 57.97±0.31 AA.

## 2.4 Mixing of jamun pulp and seed kernel powder

Pulp powder and kernel powder, in different proportions, were mixed by tumbling (10 min), followed by wire whipping (10 min) using a hand blender (Anjali Marketing & Research Centre, Mumbai, India). Based on a nine-point hedonic rating of appearance, aroma, taste, mouthfeel and overall acceptability of the mixtures by 27 semi-trained panellists, a suitable proportion was selected (Ranganna, 1986). The selected mixture, referred to as JP, was double packaged in LDPE pouches, and stored at -30 °C for further studies.

## 2.5 Determination and modelling of EMC

The isopiestic vapour transfer technique was used to determine EMC (Chowdhury & Das, 2010). Around one gram of JP was placed in a weighing bottle (in triplicate) and the bottles placed in eight vacuum desiccators, each containing a saturated solution of a salt (section 3.2) to maintain RH (10-90%). The desiccators were placed in an incubator at 25 °C. After equilibration (about five days based on preliminary experiments), the mass of the bottles plus samples were noted. These were then dried overnight at 105±1 °C to determine the dry mass of JP. The EMC (% db) was calculated using Eq. (1).

$$EMC(\%) = \frac{W_{eq} - W_{dry}}{W_{dry}} \times 100 \quad (1)$$

where  $W_{eq}$  and  $W_{dry}$  are the masses of equilibrated and dried powder.

The experiment was similarly carried out at 35

°C and 45 °C.

Five MSI models (Table 1) were used to fit the experimental EMCs using non-linear regression analysis (Microsoft Excel 2013). In the models,  $M$  represents EMC (% db) of the sample, and  $a_w$  is its water activity.  $A$ ,  $B$  and  $C$  are the model specific coefficients. In the GAB model,  $A$ ,  $B$  and  $C$ , respectively, represent the monolayer moisture content, heat of the first layer sorption and heat of the multilayer sorption.

Model fitting parameters, including co-efficient of determination ( $r^2$ ), root mean square error (RMSE) (Eq. 2), mean residual error (MRE %) (Eq. 3) and residual plot (a plot of ( $M_i - M_{pi}$ ) versus  $M_i$  for respective  $a_w$ s) were evaluated. The  $r^2$  was evaluated through regression analysis.

$$RMSE = \left[ \frac{1}{n} \times \sum_{i=1}^n (M_i - M_{pi})^2 \right]^{1/2} \quad (2)$$

$$MRE(\%) = \frac{100}{n} \sum_{i=1}^n \left| \frac{M_i - M_{pi}}{M_i} \right| \quad (3)$$

where  $M_i$  and  $M_{pi}$  are the experimental and predicted values of EMC and  $n$  is the number of experimental runs.

To be a good fit, the model should have a  $r^2$  close to 1, RMSE close to 0, MRE below 10% and randomness in the residual plot where the data points are distributed in a horizontal band around zero (Lomauro, Bakshi, & Chen, 1985).

## 2.6 Net isosteric heat of sorption ( $q_{st}$ )

The  $q_{st}$  (kJ. mol<sup>-1</sup>) was estimated using the Clausius-Clapeyron equation (Eq. 4).

$$\ln a_w = -\frac{q_{st}}{RT} + Z \quad (4)$$

where  $R$  is the universal gas constant ( $8.314 \times 10^{-3}$  kJ. mol<sup>-1</sup>. K<sup>-1</sup>),  $T$  is the absolute temperature in Kelvin (K) and  $Z$  is the integration constant.

Using the best fit model,  $a_w$  values were evaluated, at any particular moisture content, for different temperatures. The slope of the plot of  $\ln a_w$  versus  $1/T$  gave the  $q_{st}$  at that moisture

content. This was repeated for different moisture contents to evaluate the effect of moisture content on  $q_{st}$ . The absolute value of  $q_{st}$  was considered, as the sign of  $q_{st}$  doesn't bear any physical interpretation (Chowdhury & Das, 2010).

## 2.7 Spreading pressure ( $\Phi$ )

According to Igathinathane et al. (2007), spreading pressure,  $\Phi$  (J. m<sup>-2</sup>), was calculated using Eq. (5) as shown below.

$$\Phi = \frac{K_\beta T}{A_m} \int_0^{a_w} \frac{M}{A a_w} da_w \quad (5)$$

where  $K_\beta$  is the Boltzmann constant ( $1.380 \times 10^{-23}$  J. K<sup>-1</sup>),  $A_m$  is the surface area of a water molecule ( $1.06 \times 10^{-19}$  m<sup>2</sup>),  $M$  is the EMC and  $A$  is the monolayer moisture content.

Since the integral cannot be evaluated at  $a_w = 0.0$ , the lower limit in Eq. (5) was considered as 0.05. After substituting the GAB equation (Table 1) in Eq. (5) and integrating, spreading pressure for the range of  $a_w$  from 0.05 to  $a_w$  was calculated using Eq. (6) (Lago, Liendo-Cardenas, & Zapata Norena, 2013).

$$\Phi = \frac{K_\beta T}{A_m} \ln \left[ \frac{1 + BC a_w - C a_w}{1 - C a_w} \right]_{0.05}^{a_w} \quad (6)$$

The integral covering the  $a_w$  range from 0.00 to 0.05 was evaluated (Eq. 7) assuming a linear relationship (Henry's law) between  $M/A$  and  $a_w$  (Fasina, Ajibola, & Tyler, 1999; Igathinathane et al., 2007).

$$\Phi = \frac{K_\beta T M}{A_m A} \quad (7)$$

Totalling the values of Eq. (6) and Eq. (7), the  $\Phi$  corresponding to any temperature and  $a_w$  combination was produced. Spreading pressure versus  $a_w$  was plotted for different temperatures.

## 2.8 Net integral enthalpy ( $Q_{in}$ )

Net integral enthalpy ( $Q_{in}$ , kJ. mol<sup>-1</sup>) was evaluated (Eq. 8) in a comparable methodology to that of the  $q_{st}$  but at constant  $\Phi$  instead of constant moisture content:

$$\left| \frac{d[\ln a_w]}{d[1/T]} \right|_\Phi \approx -\frac{Q_{in}}{R} \quad (8)$$

Table 1: Sorption models used for fitting experimental EMC data of JP

Model	Equation	Reference
Peleg	$M = A(a_w)^B + C(a_w)^D$	Chowdhury and Das (2010)
Guggenheim-Anderson-de-Boer (GAB)	$M = ABCa_w / [(1 - Ca_w)(1 - Ca_w + BCa_w)]$	Ibanoglu, Kaya, and Kaya (1999)
Oswin	$M = A [a_w / (1 - a_w)^B]$	de Santana et al. (2015)
Henderson	$M = [-\ln(1 - a_w) / A]^{1/B}$	de Santana et al. (2015)
Halsey	$M = [-A / \ln(a_w)]^{1/B}$	Chowdhury and Das (2010)

From the plot of  $\Phi$  versus  $a_w$  at various temperatures,  $a_w$  values at any particular  $\Phi$  were obtained through interpolation (Igathinathane et al., 2007). The slope of the plot of  $\ln a_w$  versus  $1/T$  at constant  $\Phi$  produced the  $Q_{in}$ . Next, moisture contents at the interpolated  $a_w$  values were calculated using the best fit MSI equation. Thus, at three temperatures, there were three moisture contents for each  $\Phi$ , and the geometric mean moisture content was derived.  $Q_{in}$  versus geometric mean moisture content was then plotted.

## 2.9 Net integral entropy ( $S_{in}$ )

Net integral entropy ( $S_{in}$ , kJ. mol<sup>-1</sup>. K<sup>-1</sup>) was calculated using the following equation (Eq. 9) (Igathinathane et al., 2007).

$$S_{in} = \frac{-Q_{in}}{T} - R \ln(a_w^*) \quad (9)$$

where  $a_w^*$  is the geometric mean water activity at constant  $\Phi$ . The temperature term  $T$  of Eq. (9) was interpolated linearly for  $a_w^*$  from the  $T$  versus  $a_w$  data for a given  $\Phi$ .  $S_{in}$  was then plotted against moisture content.

## 2.10 Statistical analyses

The arithmetic mean of the replicated values of different observations and the respective standard deviation (SD) were evaluated. Geometric means, in calculations of thermodynamic parameters, were also evaluated. The mean values were used in analysis of variance (F test, one way) relating to parametric variation (treatment effect) followed by evaluation of least significant differ-

ence (LSD at  $P < 0.05$ , in cases of positive F test), paired t-test ( $P < 0.05$ ), and model fitting.

## 3 Results and discussion

### 3.1 Mixing of pulp and kernel powder

In JP, the sensory quality of pulp is most desirable, as the kernel powder possesses bitter taste. The results of sensory evaluation on mixing of pulp and kernel powder are shown in Table 2. It is seen that aroma, taste and overall acceptability of the pulp powder were negatively affected (F test positive) by addition of kernel powder, whereas appearance and mouthfeel remained unaffected. The corresponding LSD value (included in the same table) indicates that deterioration of the pulp powder starts at 6% addition of kernel powder for aroma and 4% addition of kernel powder for both taste and overall acceptability. At 2% addition, none of these qualities were affected. Therefore, 2% kernel addition was finally selected for the production of JP. The produced powder (JP) is shown in Figure 1, which contained TPC, AA and MAC values of  $27.25 \pm 0.19$  (mg GAE/g, db),  $25.04 \pm 0.07$  (mg BHA/g, db) and  $9.77 \pm 0.22$  (mg M3G/g, db) respectively. These values are statistically different (t-test) from those of pulp powder. Thus, addition of 2% kernel powder led to an increase in TPC and AA values of the pulp powder by 4-5%, while the MAC value was less ( $\sim 2\%$ ). Furthermore, the standard deviation of these functionalities was within 0.2-2%. Since low standard deviation is an indication of powder mixing, the JP can be considered to have good overall uniformity.

Table 2: Sensory evaluation of the mixed powder containing different proportions of pulp and kernel powder

Mixed powder constituents (%, w/w, db)		Mean±SD				
Pulp powder	Kernel powder	Appearance <sup>Ψ</sup>	Aroma	Taste	Mouthfeel <sup>Ψ</sup>	Overall acceptability
100	0	7.56±0.70	7.63±0.97 <sup>#</sup>	7.43±0.72 <sup>#</sup>	6.63±1.42	7.67±0.64 <sup>#</sup>
98	2	7.56±0.70	7.74±0.82 <sup>#*</sup>	7.46±0.80 <sup>#</sup>	6.80±0.94	7.56±0.66 <sup>#</sup>
96	4	7.52±0.80	7.26±1.32 <sup>#*</sup>	6.39±0.75 <sup>*</sup>	6.52±1.53	6.50±0.71
94	6	7.20±1.11	6.91±1.58 <sup>*\$</sup>	6.00±0.77 <sup>*</sup>	6.52±1.42	5.82±0.84
92	8	7.13±0.89	6.83±1.39 <sup>\$!</sup>	5.56±0.74	6.22±1.31	5.39±0.83
90	10	7.04±1.29	6.74±1.68 <sup>\$!</sup>	4.93±0.84	6.06±1.30	4.74±0.90
LSD		-	0.71	0.41	-	0.41

<sup>Ψ</sup>, P > 0.05 in F-test; <sup>#</sup>, <sup>\*</sup>, <sup>\$</sup>, <sup>!</sup>, The same superscript indicates no significant difference between the mean values.

Figure 1: Jamun powder (JP) having particle size below 212  $\mu\text{m}$ 

### 3.2 Equilibrium moisture content (EMC) of JP

The mean EMCs±SD of JP for varying RH (with different salts) at 25 °C, 35 °C and 45 °C are presented in Table 3. One-way ANOVA, at any temperature, resulted in a positive F-test ( $p < 0.05$ ) indicating that EMC is significantly dependent

on the RH of the environment ( $a_w$  of the sample). LSD values further confirm that at all three temperatures, EMC increased with increase in RH/ $a_w$ , except for the range of  $a_w$  from 0.113 to 0.215 at 35 °C. Thus, the sorption characteristics show a direct relationship between  $a_w$  and EMC.

### 3.3 Fitting of sorption model

Table 4 summarizes the results of regression analysis for fitting the EMC data to various models, and the model fitting criteria. All five models produced  $r^2$  close to 1 at all three temperatures. Considering MRE%, RMSE and residual plot requirements, the Peleg model was found to be the most suitable. Except for a RMSE value at 25 °C, the GAB model satisfied all the selection criteria at all three temperatures. The Oswin model suited all criteria better at 35 °C. At 25 °C it indicated a MRE >15% and at 45 °C there was a residual plot pattern. The Halsey model was more satisfactory at 45 °C. At 25 °C it indicated a pattern shape and at 35 °C the MRE was  $\approx$  10%. The Henderson model produced a pattern plot at 35 °C and 45 °C, and a >10% MRE at all the temperatures. Hence, the Peleg model can be considered as the best fit, followed by the GAB model for EMCs of microwave-convective hot air dried JP within the temperature range of 25-45 °C. The Peleg model was found to be suitable by de Santana et al. (2015) for freeze dried jamun

Table 3: The mean equilibrium moisture content (EMC) along with standard deviation (SD) of jamun powder (JP) for varying  $a_w$  at 25 °C, 35 °C and 45 °C

Salt solutions used for generation of $a_w$	$a_w$	25 °C	$a_w$	35 °C	$a_w$	45 °C
		EMC (%, db)±SD <sup>Ψ</sup>		EMC (%, db)±SD <sup>Ψ</sup>		EMC (%, db)±SD <sup>Ψ</sup>
Lithium chloride (LiCl)	0.114	4.43±0.36	0.113	3.38±0.37 <sup>#</sup>	0.112	2.82±0.31
Potassium acetate (CH <sub>3</sub> COOK)	0.237	6.09±0.27	0.215	4.73±0.64 <sup>#</sup>	0.197	4.57±0.27
Magnesium chloride (MgCl <sub>2</sub> )	0.327	9.98±0.41	0.320	7.51±0.44	0.311	6.73±0.11
Potassium carbonate (K <sub>2</sub> CO <sub>3</sub> )	0.443	12.84±0.24	0.436	10.72±0.94	0.429	10.51±0.27
Magnesium nitrate {Mg(NO <sub>3</sub> ) <sub>2</sub> }	0.536	16.52±0.47	0.515	14.25±2.49	0.497	12.07±0.26
Sodium nitrate (NaNO <sub>3</sub> )	0.742	30.59±0.53	0.720	27.12±1.65	0.699	24.89±0.42
Sodium chloride (NaCl)	0.752	32.20±0.24	0.748	29.64±1.37	0.745	31.16±0.84
Potassium chloride (KCl)	0.855	43.17±0.19	0.822	41.19±0.64	0.791	40.09±0.08
<b>LSD</b>		<b>0.62</b>	<b>LSD</b>		<b>2.19</b>	<b>LSD</b>
					<b>0.68</b>	

Ψ, Average of three replications ± SD; #, indicates no significant difference between the values.

pulp isotherms.

### 3.4 Moisture sorption isotherms

Figure 2 illustrates the MSIs of JP at 25 °C, 35 °C and 45 °C, as fitted by the Peleg model. Depending on slope, the MSIs can be divided into three zones. At any temperature, the EMC of JP increased relatively slowly at  $a_w$ s within  $\approx 0.1$ -0.5, compared to the region beyond 0.5  $a_w$  where sharp increases were observed. Thus, shape of the isotherms indicates Type III behaviour, which is a characteristic of foods rich in soluble components (Rizvi, 2014). JP contains about 45% reducing sugars (db) with glucose itself amounting to about 19% (db) (obtained in different experiments not included here). The observed trend of moisture sorption can be attributed to the dissolution tendency of fruit sugars in the absorbed moisture (Maroulis, Tsami, Marinou-Kouris, & Saravacos, 1988). A similar kind of sorption trend was also noticed for sugar-rich foods such as tomato pulp powder (Goula, Karapantsios, Achilias, & Adamopoulos, 2008), grapes, apples (Kaymak-Ertekin & Gedik, 2004) and orange juice powder (Sormoli & Langrish, 2015). Since EMC underwent a sharp increase beyond 0.5  $a_w$ , it can be said that, at RH higher than 50%, JP needs special storage conditions to inhibit several possible degradation reactions. da Silva, Meller da Silva, and Pena (2008) also voiced a similar opinion in their study.

In Figure 2, the EMC decreased with increasing temperature (25 °C > 35 °C > 45 °C) at any value of  $a_w$  up to about 0.75, with slopes being higher than that exhibited below 0.5  $a_w$ . The decrease in the total number of active sites as a consequence of temperature induced physical/chemical changes may result in the fall of EMC (Chowdhury & Das, 2012; Muzaffar & Kumar, 2016). At increased temperatures, water molecules because of their activation at higher energy levels gain higher mobility, become unstable and break away from the binding sites, thereby causing a reduction in EMC, as explained by Menkov and Durakova (2007). According to de Santana et al. (2015) and Al-Muhtaseb et al. (2002), the water vapour pressure (WVP) on powder surfaces increases at higher temperatures, allowing the water molecules to get detached from the binding sites. Interestingly for  $a_w$  higher than 0.75, the increase in EMC with  $a_w$  is even higher than for the previously displayed regions. Moreover, a crossover is seen so that the EMCs follow the order: 45 °C > 35 °C > 25 °C. As described by Basu, Shivhare, and Muley (2013) and Labuza and Altunakar (2007), such crossover at high water activity is mainly due to increased solubility of sugar and dissolution of new solutes at higher temperatures. Also, at high water activity the soluble components absorb more water, which is further accentuated by the temperature (Basu et al., 2013).

Table 4: Model coefficients and error terms for different sorption models

Temp (°C)	EMC Model	A	B	C	D	r <sup>2</sup>	RMSE	MRE (%)	Residual Plot
25	Peleg	<b>30.3963</b>	<b>0.9989</b>	<b>36.8762</b>	<b>5.0221</b>	<b>0.9946</b>	<b>0.8827</b>	<b>7.8715</b>	<b>Random</b>
	GAB	11.1246	5.2765	0.8781	-	0.9874	1.4263	9.1236	Random
	Oswin	16.4801	0.5272	-	-	0.9844	1.8427	15.4206	Random
	Henderson	0.0198	1.2322	-	-	0.9960	1.8681	12.4555	Random
	Halsey	38.0501	1.4649	-	-	0.9740	2.4035	19.5301	Pattern
35	Peleg	<b>23.9963</b>	<b>0.9999</b>	<b>67.8792</b>	<b>5.9221</b>	<b>0.9875</b>	<b>0.4133</b>	<b>5.1785</b>	<b>Random</b>
	GAB	9.5502	2.9603	0.9584	-	0.9848	0.3629	4.8470	Random
	Oswin	13.4154	0.7336	-	-	0.9858	0.3076	4.0173	Random
	Henderson	0.0740	0.8542	-	-	0.9956	0.9373	10.6109	Pattern
	Halsey	10.4390	1.0626	-	-	0.9765	0.8959	9.9953	Random
45	Peleg	<b>23.0082</b>	<b>0.9978</b>	<b>123.3597</b>	<b>7.3802</b>	<b>0.9802</b>	<b>0.2312</b>	<b>2.5248</b>	<b>Random</b>
	GAB	7.3339	3.9545	1.0452	-	0.9798	0.2416	2.0775	Random
	Oswin	12.7807	0.8417	-	-	0.9905	0.6595	6.8383	Pattern
	Henderson	0.0478	1.0028	-	-	0.9984	3.0425	10.5552	Pattern
	Halsey	6.6982	0.9085	-	-	0.9837	0.3622	4.4701	Random

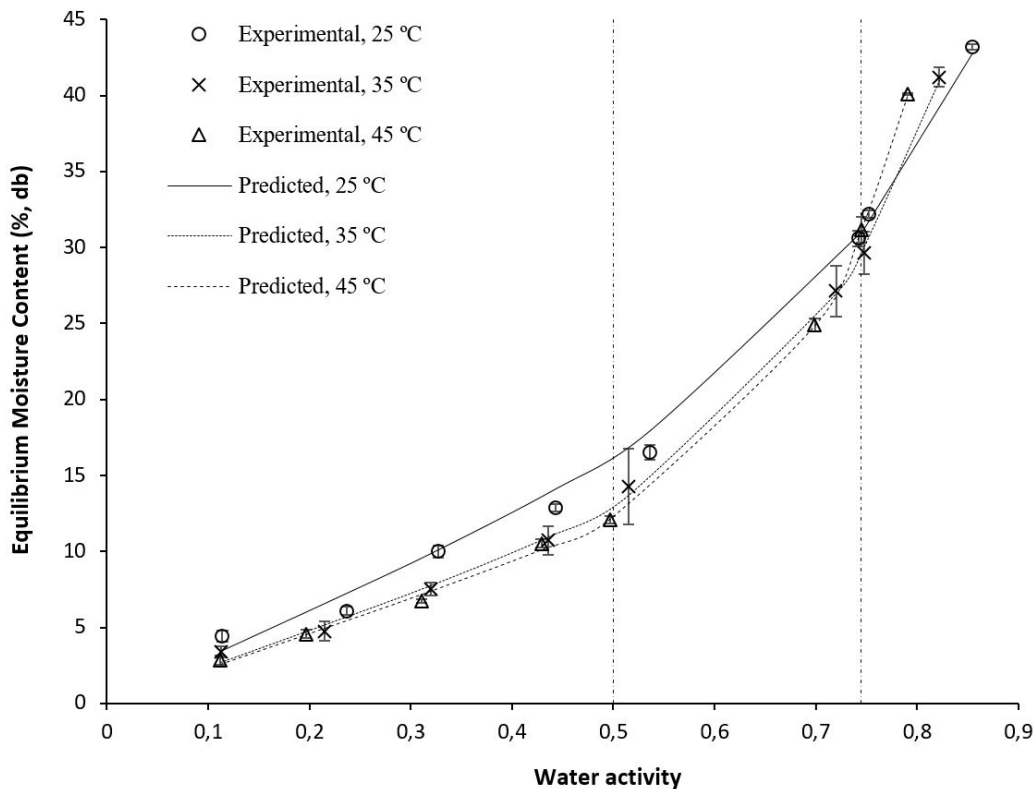


Figure 2: Effect of temperature on moisture sorption isotherm of JP as expressed by the Peleg model



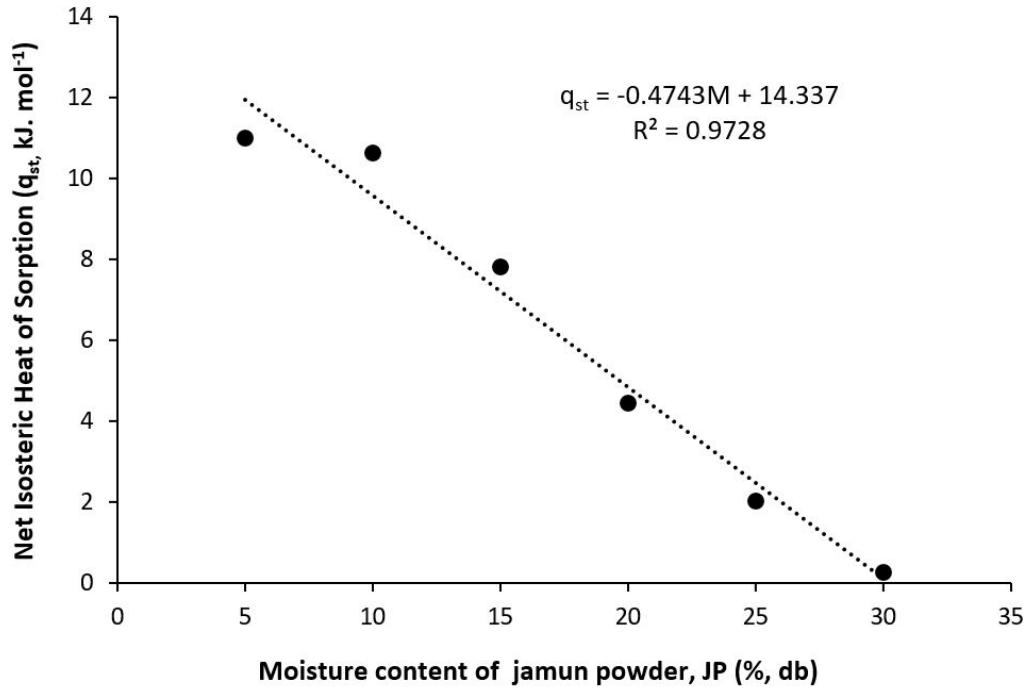


Figure 3: Variation in net isosteric heat of sorption ( $q_{st}$ ) of jamun powder (JP) as a function of moisture content

### 3.5 Net isosteric heat of sorption ( $q_{st}$ )

The absolute value of  $q_{st}$  (Eq. 4) of JP as a function of moisture content is represented in Figure 3. Mathematically, the trend is represented by the following linear relation (Eq. 10), and the resulting  $r^2$  justifies the appropriateness of the fit.

$$q_{st} = -0.4743M + 14.337; r^2 = 0.9728 \quad (10)$$

where  $M$  is the moisture content in %, db. Thus, the  $q_{st}$  decreases with the increase in moisture content from 11.02 kJ. mol<sup>-1</sup> at 5% moisture to 0.27 kJ.mol<sup>-1</sup> at 30%, thus approaching the heat of vaporization of pure water. The linear decreasing trend of  $q_{st}$  was also observed by de Santana et al. (2015) for freeze dried jamun pulp and by Basu et al. (2013) for pectin. It may be worth mentioning that the maximum  $q_{st}$  in this study is

comparable with the value for orange juice powder (9.05 kJ. mol<sup>-1</sup>) calculated by Sormoli and Langrish (2015).

Since higher the value of  $q_{st}$  means higher is the degree of binding, the water molecules become tightly bound to the active sites on the surface of the JP at low moisture contents (Sormoli & Langrish, 2015). As explained by Iglesias and Chirife (1982), sorption occurs initially at the most active sites involving higher interaction energy. As active sites get exhausted, moisture binding starts at less active sites producing less heat of sorption.

### 3.6 Spreading pressure ( $\Phi$ )

The value of  $\Phi$  increases, almost linearly, with increase of  $a_w$  (Figure 4). At any  $a_w$ ,  $\Phi$  decreases for an increase of temperature from 25 °C to 35 °C. However, for further increase to

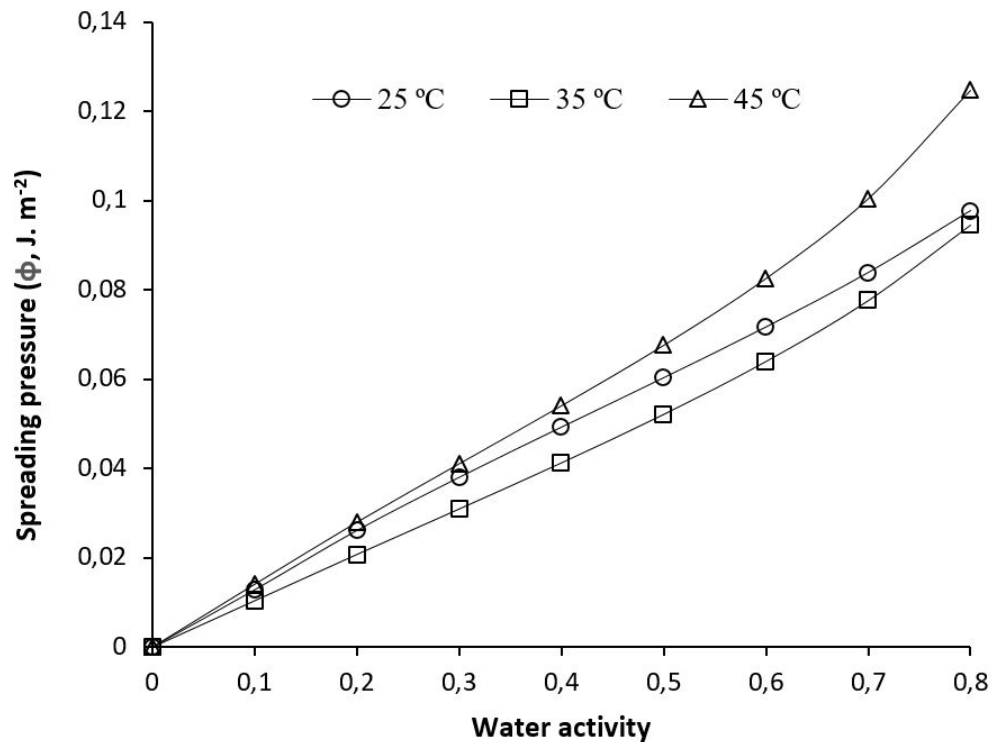


Figure 4: Variation in spreading pressure ( $\Phi$ ) with water activity ( $a_w$ ) at 25 °C, 35 °C and 45 °C

45 °C,  $\Phi$  increased to levels even higher than that at 25 °C and the differences in  $\Phi$  further increased for  $a_w$  from 0.3 onwards. It may be stated that the effect of temperature on  $\Phi$  is case specific, depending on composition of the sorbent. Depending on temperature,  $\Phi$  may be increasing/decreasing/decreasing then increasing/increasing then decreasing (Aviara & Ajibola, 2002; Lago et al., 2013; Al-Muhtaseb et al., 2004; Togrul & Arslan, 2007). Similar observations, as for the present study, were reported for high amylose starch powder (Al-Muhtaseb et al., 2004) at 30-60 °C, and walnut kernel (Togrul & Arslan, 2007) at 25-45 °C.

Spreading pressure is the driving force for diffusion of moisture in a porous solid (Skaar & Babiak, 1982). Torres, Moreira, Chenlo, and Vazquez (2012) explained that high spreading pressure values indicate a high affinity of water

molecules for binding sites. This may lead to swelling in a high RH environment (discussed in section 3.7).

### 3.7 Net integral enthalpy ( $Q_{in}$ )

Net integral enthalpy ( $Q_{in}$ ) of bound water molecules initially decreases up to about 6% moisture content, and then increases for higher moisture content (Figure 5). The decreasing portion probably arises from the monolayer covering of JP (Fasina et al., 1999). At low moisture contents, water is preferentially adsorbed on the most accessible locations on the exterior surface of the solid. Following this, as less favourable sites are filled up, the  $Q_{in}$  then gradually declines till there is formation of multi layers of water (Al-Muhtaseb et al., 2004). With still higher moisture content gained in a higher RH atmo-

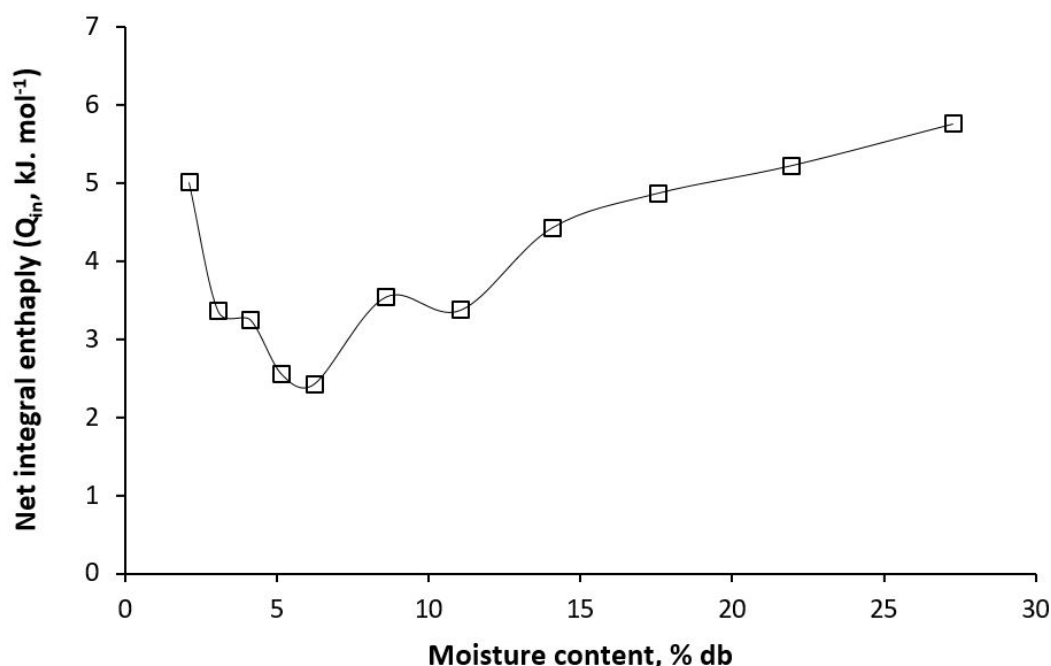


Figure 5: Variation in net integral enthalpy ( $Q_{in}$ ) of jamun powder (JP) with moisture content

sphere, there is probably swelling of the powder with concomitant exposition of higher and higher energy binding sites that require increasingly higher  $Q_{in}$  involvement. Such a decreasing, followed by increasing, profile has been documented for mango skin (Ferreira de Souza et al., 2015) and orange peel (Villa-Velez, Ferreira de Souza, Ramos, Polachini, & Telis-Romero, 2015). An increase in  $Q_{in}$  with increasing moisture content was also reported in studies on cassava (Aviara & Ajibola, 2002) and potato and sweet potato flakes (Lago et al., 2013). In the case of walnuts kernels, the  $Q_{in}$  increased slightly with increasing moisture content and then remained constant (Togrul & Arslan, 2007).

### 3.8 Net integral enthalpy ( $S_{in}$ )

Net integral entropy ( $S_{in}$ ) of the adsorbed water molecules was found to decrease continually with moisture content of JP (Figure 6). The loss in entropy, in general, indicates the loss of ro-

tational freedom or randomness of the adsorbate molecules. On exposure to low RH/ $a_w$ , the easily available sites on the surface become filled, where in addition to ligand-substrate binding, lateral interactions in the adsorbed molecules also contribute to reduction in randomness (Aviara & Ajibola, 2002). At increasingly higher RH, there is attainment of a higher EMC leading to structural transformation arising from solubilisation of ingredients and swelling of the powder which ultimately opens more and more sites to bind water molecules (McMinn & Magee, 2003; Zhang, Bai, Zhao, & Duan, 2016). In Figure 6, the maximum values of  $S_{in}$  were found at the moisture content of around 1% (db) and can be considered as the zone of most energetic water molecules in the material. Following this, up to about 8% db moisture content,  $S_{in}$  decreased but remained in a positive zone. With >8% moisture, the values were all negative. Similar profiles of  $S_{in}$ , covering both positive and negative zones, have been reported for moisture sorption of mango skin (Fer-

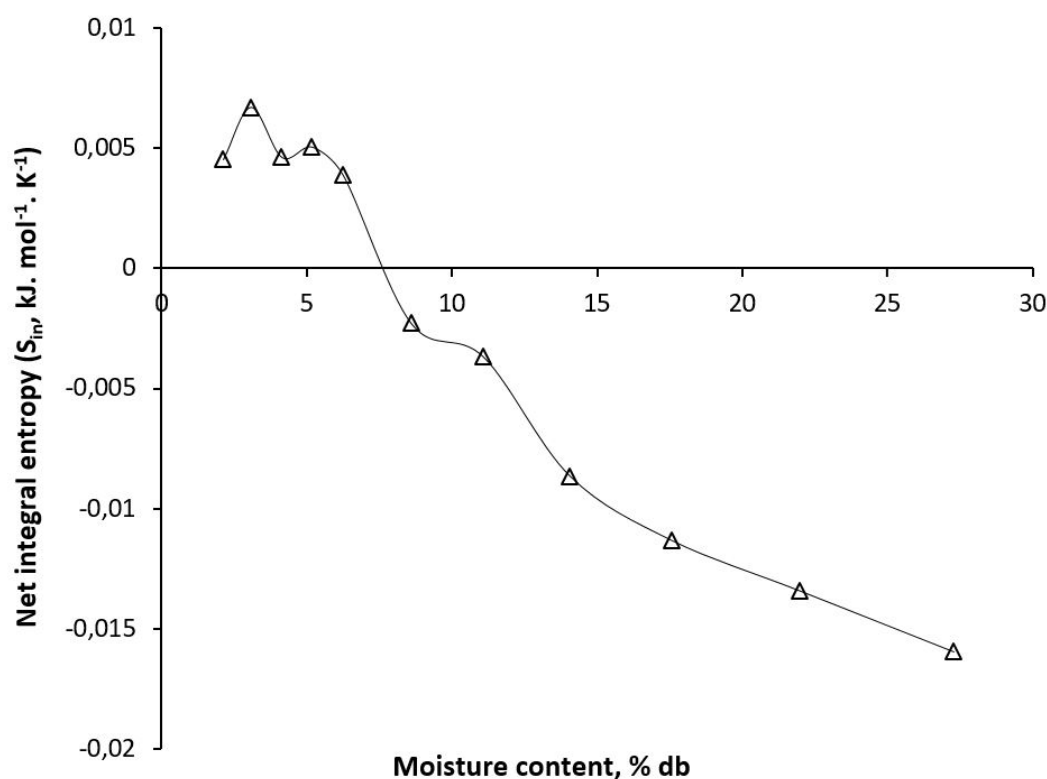


Figure 6: Variation in net integral entropy ( $S_{in}$ ) of jamun powder (JP) with moisture content

reira de Souza et al., 2015) and gari (Aviara & Ajibola, 2002). For mango skin adsorption, the negative value was also comparable to JP. Iglesias, Chirife, and Viollaz (1976) explained that negative values of  $S_{in}$  might be attributed to the existence of chemical adsorption and/or structural modifications of the adsorbent, while Rizvi (2014) attributed it to the fact that the products contain more polar groups, which bind water more strongly.

The minimum integral entropy zone corresponds to maximum stability of food products, since in this zone the water molecules are well accommodated and less available to take part in deteriorating reactions. In the case of JP where  $S_{in}$  is gradually decreasing, probably due to structural modification (as mentioned above), the minimum integral entropy zone can be considered corresponding to the monolayer moisture content

(Ferreira de Souza et al., 2015). Thus from Table 4, the safe moisture content may be in the zone of 7-11% (GAB monolayer value) for the temperature range of 45–25 °C.

## 4 Conclusions

Jamun seed kernel powder could be added to jamun pulp powder at a level of 2% (db) without affecting the sensorial acceptance of the pulp powder in the mixture, finally named as jamun powder (JP). The EMC of JP decreased with increasing temperature (25 °C > 35 °C > 45 °C) at any constant value of  $a_w$  up to about 0.75, and the Peleg model could satisfactorily describe the moisture sorption isotherms within 25–45 °C. Over the range of EMC from 5–30%, the  $q_{st}$  decreased linearly from 11.02 kJ. mol<sup>-1</sup> to 0.27 kJ. mol<sup>-1</sup>. The  $\Phi$  increased with increasing  $a_w$ , and

with respect to temperature it followed the order: 45 °C > 25 °C > 35 °C. Net integral enthalpy ( $Q_{in}$ ) displayed a decreasing trend till 6% moisture content of JP, followed by an increasing pattern thereafter for higher moisture contents. Net integral entropy ( $S_{in}$ ) was observed to decrease continuously with the increase in moisture content, and the monolayer moisture content 7–11% may be regarded as the safe moisture content zone for JP within the temperature range of 45–25 °C.

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