The effect of extrusion on the nutritional, functional and structural properties of gluten-free extruded snacks fortified with whey protein concentrate and cowpea flour

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Declaration

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- H.N.N Dilrukshi, Damir D Torrico, Margaret A. Brennan, Charles S Brennan. Effect of extrusion processing on antioxidant and glyceamic properties of cowpea and whey protein concentrate fortified rice-based extruded snacks. 16th ICC Cereal and Bread Congress 29-31 March 2021 Vienna Austria
- H.N.N Dilrukshi, Damir D Torrico, Margaret A. Brennan, Charles S Brennan. Healthy novel gluten-free ready-to-eat formulation based on cowpea and whey protein concentrate Riddet Institute Conference 7-9 April 2021 New Zealand
- H.N.N Dilrukshi, Damir D Torrico, Margaret A. Brennan, Charles S Brennan. Effect of extrusion processing on predictive glycaemic response of gluten-free snacks based on cowpea and whey protein concentrate International Conference on Food Digestion 6-7 May 2021 Ireland
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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

The effect of extrusion on the nutritional, functional and structural properties of gluten-free extruded snacks fortified with whey protein concentrate and cowpea flour

by

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Rising health concerns along with changes in lifestyles and diets have increased the demand for ready-to-eat snacks with increased the protein, fibre, and bioactive compounds through extrusion processing across the globe. Therefore, this study focused on determining the technical feasibility of adding varying levels of cowpea and whey protein concentrate (WPC) to rice flour to produce extruded snacks and assess the impact of extrusion processing and fortifications of cowpea and WPC on the nutritional and physicochemical properties. Further, the effect of fortifications of cowpea and WPC on textural and sensory properties of rice-based snacks was evaluated. This research also extended to investigate more nutritional aspects of cowpea flour during germination under different LED lights. Rice flour was blended with cowpea flour and WPC at weight ratios of 90:10:0, 80:15:05, 70:20:10, 60:25:15, and 50:30:20 to prepare composite flours. Rice flour was used as a control. The effects of different levels of cowpea flour and WPC additions on the nutritional components, pasting properties, *in vitro* starch and protein digestibility of rice-based blends were investigated. Results indicated that the peak, breakdown, setback, and final viscosities were significantly lower, and

the pasting temperature was significantly higher with cowpea and WPC fortifications compared to the control. The observed total reduction in reducing sugars being released during the *in vitro* starch digestion for the extruded samples was in the range of 14-41% at the inclusion levels of 10-30% CP and 5-20% of WPC, respectively. An increase in the ratio of cowpea flour and WPC increased the protein and fibre contents and decreased the starch content of raw blends. The increased protein content had an impact on protein digestibility.

Raw blends were extruded using a Clextral twin-screw extruder with co-rotating and intermeshing screws. The processing conditions used a die temperature of 111.5 °C, a screw speed of 252 rpm, a feed rate of 7.98 kg h^{-1}, a water rate of 90.67 kg h^{-1}, and a die diameter of 3 mm. Nutritional, physicochemical, and textural properties of the extrudates were evaluated. The protein and fibre contents of the extrudates significantly increased ($p<0.05$) with cowpea (10-30%) and WPC (5-20%) fortifications compared to those of the control. Increased cowpea and WPC contents decreased lightness and increased the bulk density and hardness of the extrudates. The crispiness associated with the number of peaks during the compression test increased with higher levels of cowpea and WPC. Correlation analysis revealed that the protein and fibre contents were significantly correlated to hardness, crispiness, bulk density, water absorption index and colour properties of the extrudates. The essential and non-essential amino acid contents increased proportionally to the increases of the cowpea and WPC fortifications in the extrudates.

Extrusion increased the oligosaccharides (2-3-fold) and resistant starch (1-3-fold) contents, whereas the insoluble fibre content was not significantly affected. Extrusion increased the protein digestibility ($p<0.05$) and amino acid composition in the snacks. Extruded and raw samples enriched with cowpea and WPC had an increase in the total phenolic content (TPC) and antioxidant activity. Extrusion significantly reduced the TPC, and

antioxidant properties of the extruded snacks compared to their raw counterparts. The observed total reduction of released reducing sugars during the *in vitro* digestion was in the range of 14-41% at the inclusion levels of 10-30% cowpea flour and 5-20% of WPC, respectively. After the *in vitro* digestion process, the antioxidant activity of the cowpea and WPC fortified snacks was more than 2-fold higher compared to their extruded counterparts.

The effects on sensory properties of cowpea and WPC fortification in rice-based ready-to-eat extruded were investigated. Six samples of extruded snacks were evaluated by the 70 consumers for acceptability using a 9-point hedonic scale. The sensory properties were assessed using the check-all-that-apply (CATA) method and the just-about-right (JAR) scale. The cowpea and WPC fortified samples had higher scores for overall liking than the control sample made with 100% rice flour. The 15-25% cowpea and 5-15% WPC fortified samples had the highest JAR frequencies in the penalty analysis for colour and texture attributes. According to Cochran's Q test, panellists were able to discriminate different textural attributes, being the terms soft and crunchy terms significant for the extrudates. Overall, the analysis of all sensory attributes demonstrated that the formulation of 15% cowpea and 5% WPC had a higher acceptance by the consumers involved in this study. A principal component analysis (PCA) revealed that there were positive associations between crispiness and L* value, and hardness b* and a* values, respectively.

The final experiments were extended to investigate more extensively about nutritional properties of cowpea flour during germination under different LED light conditions. Light quality and intensity are vital for plant development and pigment biosynthesis. The effects of a dark condition and various light-emitting diodes (LEDs), such as red, blue, and white fluorescent lamps were evaluated on the nutritional components, mineral content, levels of phenolic compounds and antioxidant activities of cowpea sprouts. The protein and total

dietary fibre concentrations of the cowpea sprouts increased significantly with the application of blue and red LED lights compared to the dark condition. The phytic acid, trypsin inhibitory activity, raffinose series oligosaccharides and starch fractions of cowpea sprouts germinated under the light conditions were not significantly different from those germinated under the dark conditions. The total phenolic and antioxidant properties of cowpea sprouts under the white and red LED lights were significantly higher when compared to that of the blue and dark environments. The red LED grown cowpea sprouts had the highest mineral content followed by the white, blue, and dark conditions, respectively.

These results show the potential beneficial use of cowpea and WPC, as functional ingredients to improve the nutritional profile and reduce the glycaemic index of rice-based extruded snacks. In the cowpea sprouts study, the application of red LED light and white fluorescent light sources improved the nutritional, total phenolic content, and antioxidant properties of cowpea sprouts. This experiment confirmed the recent findings on the beneficial effects of LED light on plant growth and quality of the crops, including the accumulation of phytonutrients in sprouts. Incorporation of natural phytonutrients rich foods in the human diet in different ways exert beneficial effects for human health.

Keywords: Ready-to-eat extruded snacks, Cowpea, Whey protein concentrate, Rice flour, Nutritional composition, Physicochemical composition, Sensory attributes, Bioactive components, Antioxidant properties, Germination

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Chapter 1

Introduction

Ready-to-eat foods are a group of products that are ready for consumption without prior preparation or cooking. The global market for these ready-to-eat snack products was valued at USD 439.9 billion in 2018, and this value is expected to grow over 6.2% compound annual growth rate from 2019 to 2025 (Grand View Research, 2019). Consumers' dietary preferences are shifting towards ready-to-eat foods replacing the traditional home-cooked meals due to urbanisation and modified lifestyles (Sarkar et al., 2018). Consumers are attracted to these food products due to their convenience, ease of preparation, attractiveness, reasonable price, taste, appearance, and texture (Brennan et al., 2013b). Most of the available ready-to-eat snacks are made of refined cereal flours, which are relatively high in sugar and salt, and are regarded as energy-dense but nutritionally poor foods (Brennan et al., 2013b). Consumption of energy-dense processed food with high sugars and fat combined with sedentary lifestyles constitute the major risk factor of non-communicable diseases such as diabetes mellitus type 2, cardiovascular diseases, obesity, and cancer (Arora et al., 2020; Sarkar et al., 2018). Rising health concerns along with changing lifestyles and diets have spurred the demand for various snacking options such as high protein and fibre, allergen-free and vegan products. To fill this gap, several food products have emerged by combining different sources of cereals, legumes, vegetables, fruits, and food industry by-products (Dalbhagat et al., 2019; Grasso, 2020 & Sharma et al., 2021a).

White rice (*O. sativa L*.) has been used in making gluten-free cereal-based snacks due to its flavour, hypo-allergenicity, bland taste, colour, and good processing characteristics (di Cairano et al., 2021). However, white rice flour is high in calories from starch, and low in

protein, fibre, and bioactive compounds. Therefore, it is recommended to fortify rice-based products with other food ingredients such as waste materials from food processing (Sampaio et al., 2021), as well as legume material (Sharma et al., 2021b), to improve their nutritional profile. Thus, the development of novel healthy ready-to-eat snack products fortified with legumes could help to increase pulse consumption and allow consumers to obtain health benefits from the bioactive compounds of these snacks.

Cowpea (*V. unguiculata* L. Walp.), a legume, contains 24.2% protein, 63.5% carbohydrates, 14.4% dietary fibre and 2.5% of fat on a dry basis and it is the fourth most widely produced pulse around the world after the dry bean (*Phaseolus vulgaris*), Chickpea (*Cicer arietinum*) and dry pea (*Pisum sativum*) (FAO, 2018). Cowpea is an important source of bioactive compounds which benefit human health in various ways. Polyphenols (comprised of phenolic acids and flavonoids) are the most important type of bioactive compounds in cowpea. The type and amount of phenolic compounds and flavonoids accumulated in the seed are influenced by cowpea phenotypes (Ojwang et al., 2012). Cowpea protein peptides, having a molecular mass below 3 kDa, and phenols contribute to the antioxidant properties of cowpea by scavenging free radicals and preventing oxidative stress (Marques et al., 2015). Cowpea contains phytic acid which is an anti-nutrient that lowers the bioavailability of minerals and inhibits protein digestibility; however, as it has antioxidant and anti-cancer properties, it is also beneficial for health (Turner et al., 2002). Oligosaccharides including α-galactosides, raffinose, stachyose and verbascose can also be found in cowpea (31.7 mg/g), which are causative agents of flatulence in humans. However the presence of these oligosaccharides favours the growth of beneficial microflora such as bifidobacteria in the human gut, which makes cowpea a functional ingredient (Sreerama et al., 2012a). Cowpea seeds contain a high amount of starch which may be of use in the food industry (Oyeyinka et al., 2021). In addition, cowpea flours are rich in resistant starch and dietary fibre, which

help to reduce calorie intake and improve glucose regulation in diabetes patients and can facilitate weight control for the obese (Oboh & Agu, 2010).

Legume crops have recently attracted tremendous attention owing to their excellent growth characteristics and potential nutritional benefits. During germination, some bioactive components, such as phenolics, flavonoids and antioxidants are increased whereas levels of anti-nutritional compounds, including tannins, phytic acid and oxalate, are diminished by germination (Chen et al., 2017; Cornejo et al., 2019). Plant growth and development is affected by light, including light intensity, quality, and photoperiod. LEDs have various advantages compared to conventional lighting sources including high energy conversion efficiency, low thermal energy output, longer life, and controllable emission spectrum. There is evidence that LED light can improve the antioxidant properties of sprouted seeds (Liu et al., 2016).

On the other hand, whey protein concentrate (WPC) is widely used as a food ingredient to improve protein content. Whey protein has many properties that are beneficial to human health such as anti-carcinogenic, antimicrobial, blood pressure-lowering, appetite suppressing, hypocholesterolaemic and dental plaque and caries inhibition (Brnčić et al., 2011). WPC is one of the highest-quality ingredients for possible extrudate enrichment (Brnčić et al., 2011). WPC contains the essential amino acids, leucine (11.8%) and lysine (9.5%), valine (4.7%), threonine (4.6%), methionine (3.1%) and phenylalanine (3.0%) (Huth et al., 2004). Besides these nutritional aspects, whey proteins possess rheological properties suitable for the extrusion process, as they have high solubility and high gel and foam-forming ability (Gong et al., 2021).

Different functional, physicochemical, and sensory properties of expanded snacks produced from cereal/legume mixes by the extrusion technology have been reported and researchers

have studied the influence of extrusion conditions (feed moisture, barrel temperature, screw speed) on the properties (expansion, density, texture, colour, flavour) of expanded snacks (Félix-Medina et al., 2021a; Philipp et al., 2017; Sharma et al., 2017a and Shevkani et al., 2019a). However, there is no scientific data on combining cowpea and WPC in rice-based snacks. The research in this project illustrates the possibility of developing high protein and fibre, rice-based extruded snacks with increased antioxidant effects and a reduced glycaemic impact. The results show the impact of such additions on the physical and textural properties as well as on the sensory aspects. Additionally, the effect of extrusion processing on the bioactive components of extrudates was evaluated. Also the effects of LED lights on the germination and nutritional properties of cowpea was evaluated. The incorporation of cowpea and WPC in rice-based extruded snacks has tremendous potential for the development of nutritious gluten-free snacks which benefit human health and well-being.

1.1 Aims

The main aim of this study was to increase the protein and fibre contents in rice-based ready-to-eat snacks. To achieve this objective, cowpea and WPC were selected and used in different combinations in a rice-based formula. Nutritional, physico-chemical and functional properties of extrudates were analysed. Additionally, the bioactive compounds in raw formulations and extrudates were evaluated to illustrate the effect of extrusion processing on these compounds. The sensory evaluation was performed to understand the consumer acceptance of extrudates. The final experimental study was focused on cowpea, the legume used to increase the protein and fibre content in extrudates; the combined effect of light and germination on nutritional and anti-nutritional factors in cowpea (*V. unguiculate L.*) was studied.

The main aims of the research can be summarised into five key points.

1. To improve the nutritional profile of extrudates including protein, fibre and bioactive compounds by combining rice, cowpea and WPC
2. To determine the nutritional, physicochemical and functional properties of extrudates
3. To determine the effect of extrusion processing on bioactive components, glycaemic response, and antioxidant properties of extrudates
4. To understand the consumer acceptance of extrudates
5. To evaluate the levels of phenolic compounds, flatulence factors, trypsin inhibitory and antioxidant activities in cowpea sprouts with various types of LEDs, including red, blue, and white fluorescent lamps and darkness.

1.2 Hypotheses

- There is a significant difference in nutrient density (contain more protein, fibre, and bioactive compounds) Whey-cowpea fortified rice-based extruded snacks than rice-based extruded snack.
- Physical, structural, and sensory properties are significantly different in Whey-cowpea fortified rice-based extruded snacks than rice-based extruded snack.
- There is a significant difference in nutritional and anti-nutritional factors in cowpea sprouts exposure to different LED lights during the germination period.

1.3 Thesis Outline

Project title: The effect of extrusion on the nutritional, functional and structural properties of gluten-free extruded snacks fortified with whey protein concentrate and cowpea flour (Fig 1.1)

- **Chapter 1**: Introduction and thesis outline
- **Chapter 2**: Literature review
- **Chapter 3**: Material and Methods
- **Chapter 4**: Whey protein concentrate and cowpea fortification of rice flour: Effects of protein and starch digestibility and starch pasting properties
- **Chapter 5**: The effect of cowpea and whey protein concentrate on nutritional, physicochemical and textural properties of ready-to-eat extruded rice snacks
- **Chapter 6**: Effects of extrusion processing on the bioactive constituents, *in vitro* digestibility, amino acid composition, and antioxidant potential of novel gluten-free extruded snacks fortified with cowpea and whey protein concentrate
- **Chapter 7**: Effect of fortifying rice-based extruded snacks with cowpea and WPC on *in vitro* starch and protein digestibility, phenolic content, and antioxidant activities
- **Chapter 8**: Consumer perception of ready-to-eat gluten-free snacks containing cowpea flour and whey protein concentrate
- **Chapter 9**: The influence of light emitting diodes on the antioxidant activities, phenolic and bioactive compounds in cowpea sprouts
- **Chapter 10**: General Discussion and Conclusions
- **References**

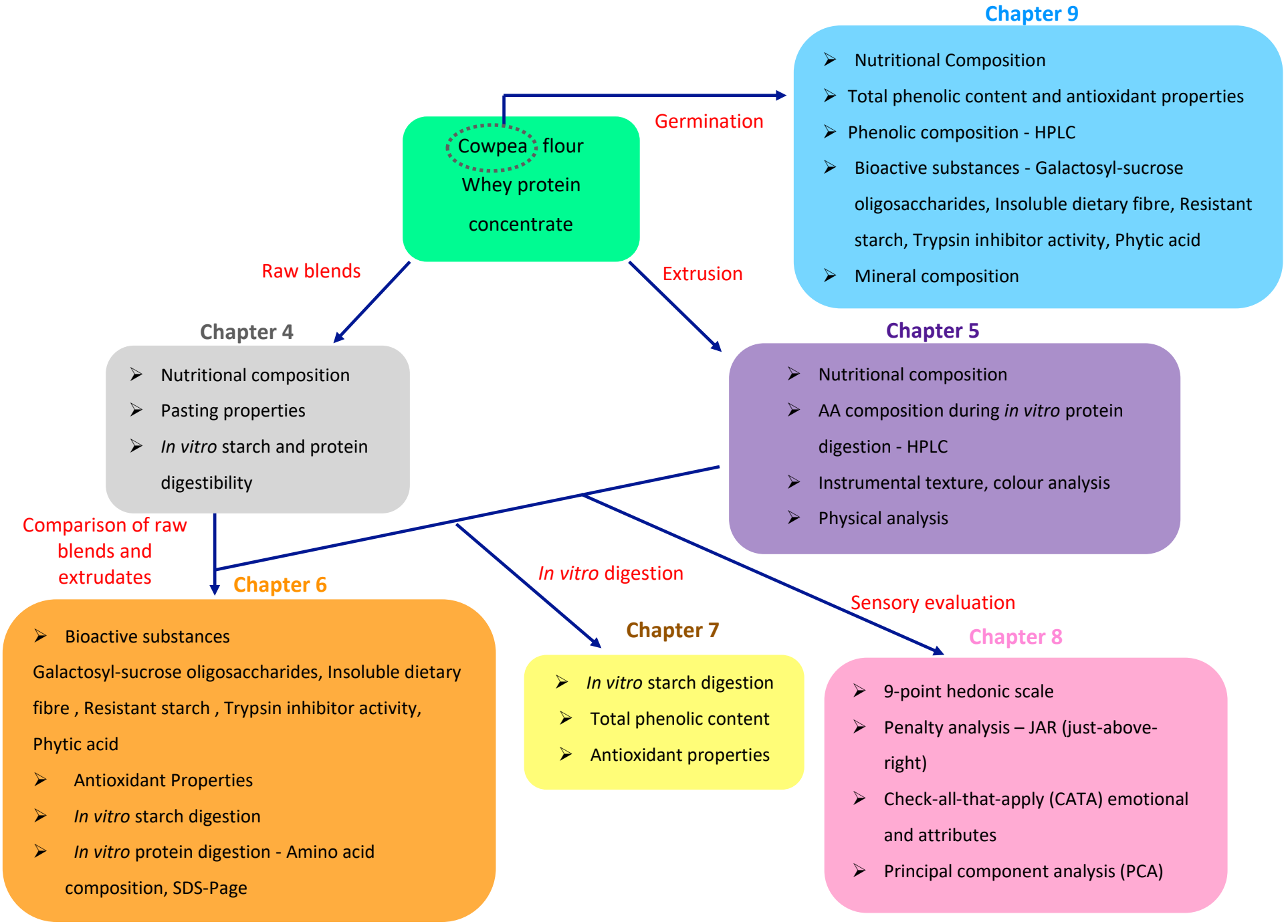

Figure 1-1 Thesis Outline

Chapter 2

Literature Review

2.1 Ready-to-eat Gluten-free Snack Products

Recent trends show that the demand for convenience food is rising dramatically across the globe. The global healthy snacks market size is expected to reach USD 32.88 billion by 2025, expanding at a compound annual growth rate of 5.2% during the forecast period (Grand View Research, 2019). Cereal-based products are the most important segment of the ready-to-eat food market. Originally, most of these cereal-based ready-to-eat products were developed from whole grain sources and were flaked from steamed grains (Brennan et al., 2013b). However, most of the recent products are developed from refined cereal flours that are rich with easily digested carbohydrates, salts and fats and ranked as high glycaemic index foods (Patil et al., 2016).

Increasing consumer awareness regarding health and wellness, together with public health campaigns and improved nutritional guidelines, have resulted in a growing interest in improving the nutritional quality of cereal-based ready-to-eat snack products (Grasso, 2020). This movement towards healthy snacking has challenged the food industry to develop snacks with a high nutritional value by incorporating bioactive compounds. In recent years, there have been many studies focused on combining different food sources to develop healthy snack formulations such as the use of legumes and pulses, namely lentil, faba bean, lupin, pea, chickpea, cowpea (Ciudad-Mulero et al., 2020; Martin et al., 2020a; Philipp et al., 2017 and Yadav et al., 2019); fruits such as orange and apple pomace (Huang & Ma, 2016; Leyva-Corral et al., 2016); and dehydrated vegetables (Bisharat et al., 2013).

Cereals such as wheat, rye, barley, rice have been important for human beings, and these cereals are used as ingredients in a variety of food items around the world. These cereals are also used extensively in developing healthy novel ready-to-eat snack products by combining different plant and animal origin sources. However, the gluten protein present in some cereals has some adverse effects on some genetically predisposed individuals, such as gluten intolerance, coeliac disease, barker's asthma, wheat allergy, and wheat exercise-induced anaphylaxis (Sajid Mushtaq et al., 2021). Almost, 1% of the world population is affected worldwide by these autoimmune disorders, primarily in Europe, North America, and Australia (Roszkowska et al., 2019). In the prevailing scenario, the food industry has a requirement for more gluten-free products due to the recommendation of strict adherence to the complete absence of gluten in food products in the gluten-sensitive population. Due to this increasing demand, the gluten-free market is expected to grow at a compound growth annual rate of 9.2% during 2020–2027 (Grand View Research, 2019). In recent years, extensive work has been done by researchers towards the development of gluten-free extruded food products, using rice, corn, pseudocereals, millet and different legumes, as described in Table 2.1.

Table 2-1 Gluten-free extruded snack

Ingredients	Properties	References
Rice and chickpea	The optimum mild extrusion conditions obtained by numerical optimization for the development of snacks were 102 °C barrel temperature, 281 rpm screw speed, 18.3% feed moisture and rice to chickpea flour ratio as 90:10.	(Altaf et al., 2021)
Corn and rice bran	The addition of rice bran to corn-based extruded snacks increased dietary fibre, lipid, protein, and mineral content while it decreased available carbohydrate and energy value at 15% rice bran. Rice bran inclusion improved textural properties causing a reduction in hardness and increase in the crispness of extrudates.	(Renoldi et al., 2021)
Rice and dried mushroom	Seasoned mushroom-containing snacks with 65% and 75.83% had higher hedonic scores and positive emotions.	(Tepsongkroh et al., 2020)
Rice and turmeric powder	6% substitution of broken rice grains by turmeric powder was reported with 7.76 % protein, 75.3 % available carbohydrates, 4.78 % lipid, 5.84 % dietary fibre, and 174.75 mg gallic acid equivalent per100 g and 6.52% of DPPH scavenging capacity.	(Ribeiro Oliveira et al., 2020)

Table 2-1 Continue

Rice, bean and carob fruit	Carob fruit and bean fortified extrudates were observed with double amount of protein, 10-fold fewer lipids and a similar amount of carbohydrates than a commercial extruded rice. The extrusion process did not modify the protein content, although reduce the soluble protein (80–89%) and the fat (>81%) contents compared to the non-extruded formulations. Extrusion caused a slight increase (1–6%) in total starch content with a slight reduction of the amylose/amylopectin ratio in extrudates.	(Arribas, et al., 2019a)
Rice starch, pea protein and fibre	Increased specific mechanical energy inputs, bulk densities and air cell densities were observed with the addition of protein and fibre in extrudates. A statistically significant increased expansion was observed in extrudates at intermediate protein and fibre contents, compared to the pure rice starch.	(Beck et al., 2018)
Protein isolates/concentrates from faba bean, lentil, blue lupin and white lupin and flours of quinoa, amaranth and buckwheat	The sectional expansion was decreased with increasing protein content from 30% to 50%, and the density and specific hardness were increased of the extrudates. Satisfactory texture and sensory properties were observed in lupin protein-based extrudates. Regardless of pseudocereal type, extruded mixtures of pulses and pseudocereal (70% protein) exhibited a smaller sectional expansion compared to pulses as single ingredients (30%, 50%).	(Martin et al., 2020a)

Table 2-1 continue

Kiwicha	The optimal condition was extrusion temperature of 190 °C and 14 % initial moisture and extrudates showed 7.17 of sectional expansion index, 61.5% water solubility index, 101.2 µmol trolox/gAC-DPPH, 364.2 µmol trolox/gAC-ABTS and 34.5mg GAE/100g total phenolics.	(Basilio-Atencio et al., 2020)
Rice, grain oat, white sorghum, amaranth, quinoa, chickpea, lentil, pinto and black beans	Higher apparent viscosities were obtained under cold conditions for extruded flours compared to raw flours. Extrusion improved the hydration properties and water solubility index. The oil binding capacity, protein solubility and foaming properties were affected depending on the extruded material. Extruded whole grain flours presented comparable or even better techno-functional properties compared to extruded refined rice flour.	(Espinosa-Ramírez et al., 2021a)
Maize semolina, pomace from common flax and golden flax	Expansion ratio and lightness were decreased with the increasing pomace content in the extrudates, and mechanical properties were increased. There were no statistically significant differences between the snacks without pomace addition and those with 5% flax pomace for all sensory attributes. The amount exceeding 10% of flax pomace made the product unacceptable by the panellists.	(Tomaszewska-Ciosk & Zdybel, 2021)
Corn	Reduced radial expansion ratio, increased bulk density and enhanced hardness of the corn extrudates were found with the use of fibre materials.	(Han et al., 2018)

Table 2-1 continue

Sorghum	Increased supplementation with sorghum flour in chickpea blends was resulted in increased total phenolic contents and antioxidant capacities, reflecting the higher antioxidant capacity of sorghum as compared to chickpea.	(Bekele et al., 2021)
White sorghum	Functional properties of extruded sorghum flour were significantly affected. Increased peak gelatinisation temperature, degree of gelatinisation (%) and starch crystallinity (%) were observed with increasing feed moisture content while it decreased the gelatinisation temperature ranges, starch gelatinisation enthalpy and amylose-lipid complex (%) formation.	(Jafari et al., 2017)
Sorghum and defatted soy meal flour	A significant increase in moisture, crude protein, fat, ash, fibre, Ca, and Fe content was observed with increasing the addition level of defatted soy meal flour to sorghum flour with a decrease in carbohydrate content. Extrusion processing significantly reduced tannin and phytic acid content by about 61- 86%, and 24 - 45%, respectively.	(Tadesse et al., 2019a)

2.2 Use of Legumes in Gluten-free Extrusion Cooking

Legumes are important as a sustainable and inexpensive meat alternative in human food culture. Food legumes include lentils, peas, soybeans, broad beans, lupins, mung beans, green beans, and peanuts. Legumes represent an important source of gluten-free food for a healthy diet since they are rich in proteins (20-45%) with essential amino acids, complex carbohydrates (60%), dietary fibre (5-37%), unsaturated fats, vitamins, and minerals (Maphosa & Jideani, 2017). The consumption of legumes is associated with health benefits such as hypoglycaemic, hypocholesterolemic, antiatherogenic and anti-carcinogenic properties (Cotacallapa-Sucapuca et al., 2021). The United Nations General Assembly declared 2016 as the International Year of Pulses based on the vital role of legumes in the human diet across the globe (FAO, 2021).

Legumes are incorporated into cereal-based extruded snack products to improve the nutritional profile in terms of protein, fibre, and bioactive compounds. Extrusion cooking technology seems to be the most suitable processing technique to produce these snacks due to its ability to reduce anti-nutritional factors such as tannins, phytic acid, trypsin inhibitors and lectins (Sajid Mushtaq et al., 2021). In addition, extrusion cooking can increase the digestibility of starch and protein (Brennan et al., 2013b). Extrusion processing conditions: temperature, screw speed, moisture level, and the content of legume flour, its refining degree and particle size, as well as the type of legume, influence the expansion performance and final quality of the extrudates (Sajid Mushtaq et al., 2021). Numerous recent studies covering legume supplementation (with ingredients such as bean, lentil, pea, chickpea, and faba bean) in gluten-free cereal-based extruded snacks are shown in Table 2.2.

Table 2-2 Use of legumes in gluten-free extrusion processing

Legume with other ingredients	Legume content g/100 g	Processing conditions	Remarks	Reference
Chickpea, tomato powder, potato starch, locust bean gum, yeast, strained yoghurt, red pepper	A 100 g of chickpea blend was composed of 50 g chickpea flour	Co-rotating twin-screw extruder, die temperature (130–150 °C) and screw speed (300–400 rpm)	Regression analysis showed that extrusion parameters and gum content had a significant effect on the physical properties of extrudates, but textural properties were mostly affected by changes in temperature and screw speed. Anti-nutritional factors were significantly reduced while starch and protein digestibility increased after extrusion.	(Yağcı et al., 2020a)

Table 2-2 Continue

Chickpea and sorghum	50:50, 60:40, and 70:30 (w/w) chickpea–sorghum blends	co-rotating, twin-screw extruder, Moisture levels - 16, 18, and 20%), Barrel temperatures - 120, 140, and 160 °C	Extrusion increased the total phenolics contents of chickpea–sorghum blends. Increased antioxidant capacity and total phenolics content of chickpea–sorghum blends were observed with increasing extrusion temperature, whereas increasing feed moisture content decreased antioxidant capacity and total phenolics content. The 50:50 chickpea: sorghum blend extruded at 160 °C and 16% exhibited the highest antioxidant capacity and total phenolics.	(Bekele et al., 2021)
Rice and chickpea	20-60 g /100g	Twin-screw extruder Barrel temperature 105 - 120 °C Screw speed 185-445 ppm	The optimum mild extrusion conditions obtained by numerical optimisation for the development of snacks were 102 °C barrel temperature, 281 rpm screw speed, 18.3% feed moisture and rice to chickpea flour ratio as 90:10.	(Altaf et al., 2021)

Table 2-2 Continue

Lentil, corn, and rice	Native flours (corn, rice, and lentil) and corn, rice and blend flours (50:50)	110, 120, and 130 °C	The extrusion process improved the glycaemic index compared to the native flour. Corn-lentil flours reported higher antioxidant properties and lower GI index in comparison with rice-lentil blends. Extrusion increased the total phenol and antioxidant activity, with a temperature-dependent effect (130 °C ≥ 120 °C ≥ 110 °C).	(Rico et al., 2021)
Lentil, lupin, faba bean, quinoa, amaranth, buckwheat	30, 50 and 70% of protein in pulse-starch mixtures	co-rotating twin-screw extruder 140 or 160°C, Screw speed -300 rpm	Mixtures of pulse protein isolates/concentrates, particularly lupin protein isolates, and pseudocereal flours were used to increase the nutritional value of the protein-rich extrudates, while reducing the hardness of the snacks even at protein concentrations of 70%.	(Martin et al., 2020b)

Table 2-2 Continue

Red lentil flour	Moisture content – 18 -22 %	co-rotating twin-screw extruder screw speeds - 150 rpm and 200 rpm feed rate – 3 kg/h Moisture – 18-22%	Decreased extrudate density from 0.25 to 0.41 kg/m^3 to 0.12 to 0.26 kg/ m^3 with N_2 injection at 300 kPa (across all feed moistures and screw speeds studied) compared to conventional and substantially altered the extrudates' microstructure. An increase in feed moisture from 18% to 22% increased extrudate hardness and decreased crispiness.	(Luo et al., 2020)
Bean, dry amaranth grain, maize grain, and fresh, orange-fleshed sweet potato (OFSP)	Bean:maize:OFSP:Amaranath 50-85:10-45:5-15:0-10	twin-screw extruder Moisture – 15%, Screw speed – 45 Hz temperature - 142 °C	A bean-based extruded snack containing 82% beans, 10% maize, 5% OFSP, and 3% amaranth was found to exhibit the most desirable nutritional and acceptability properties.	(Natabirwa et al., 2020a)

Table 2-2 Continue

Black bean Blue corn, sweet chard	66% blue corn, 33% black beans and 1% chard	A twin-screw extruder Moisture – 22 – 35%, Screw speed - 99-157 rpm, temperature - 102-142 °C	The highest expansion index, water absorption index and solubility index, and hardness were obtained at high screw speed and low extrusion temperature. At low extrusion temperature and moisture content, the highest TAC was generated. The predicted optimal conditions were obtained at 133 rpm, 25% moisture, and 122 °C.	(Neder-Suárez et al., 2021a)
common bean, white maize	250 g (175 g white maize +75 g common bean) and were conditioned with purified water until they reached moisture contents of 18 g H_2O/100 g	single-screw laboratory extruder, barrel temperature - 164 °C and screw speed - 187 rpm	Snack from a maize-bean mixture (70/30) was reported with an acceptable nutritional balance, antioxidant properties, and bioactive compounds (phenolics, flavonoids and polyunsaturated fatty acids). The snack also showed an acceptable balance of amino acids with a chemical score of 74.09 and *in vitro* protein digestibility was 77.21%.	(Félix-Medina et al., 2021b)

Table 2-2 Continue

Cowpea	13-27g/100g	The temperature - 160-180 °C, Screw speed - 160-200 rpm, feed rate - 16 rpm (70 g/ min), feed moisture - 16-24%	The increase in feed moisture content and decrease in die temperature significantly increases the density and hardness of extrudates and decreases the expansion, water absorption index, water-soluble index and organoleptic scores. The overall best quality product was obtained when using cowpea flour with 16% moisture content, under 180 °C die temperature and 200 rpm screw speed.	(Jakkanwar et al., 2018b)
Cowpea and plantain	plantain: cowpea: 90:10, 80:20, 70:30, 60:40 and 50:50	Twin-screw extruder, Temperature - 60 °C and 140 °C, Screw speed - 600 rpm, Feed rate - 6.3 kg/h	Pasting properties of the extrusion blends were significantly different *(P<0.05)* depending on the blend ratios. The level of cowpea added affected the paste, hardness properties and the expansion height of the extruded products	(Oduro-Yeboah et al., 2014)

Table 2-2 Continue

Soybean and maize	Soybean flour (5%, 10%, 15% and 20%, w/w)	A single-screw extruder, Temperature - 150 °C screw speed - 170 rpm feed moisture - 5-24%	Increasing the soybean flour and feed moisture content resulted in several changes of the snacks including higher moisture content, lower expansion, and volume, reduced crispiness, reduction in L-and a-values but an increase in b-value and formation of more wrinkly and thicker air bubbles cell walls. The addition of <20% soybean flour and feed moisture content resulted in snacks with acceptable nutritional and physical properties.	(Sharifi et al., 2021)
Soybean and sorghum	0, 10 and 20% defatted soy meal flour	Barrel temperature (135, 150 and 165 °C) and feed	A significant increase *(P<0.05)* in moisture, crude protein, fat, ash, fibre, Ca and Fe content were observed with increasing the addition level of defatted soy meal flour to sorghum flour, but carbohydrate content was decreased. Barrel	(Tadesse et al., 2019b)

Table 2-2 Continue

		moisture content (15, 18 and 21%)	temperature of 165 °C was obtained for the highest reduction of 86% tannin content and 45% of phytic acid content. The mean sensory values of all extrudates were indicated all extrudates were well-liked by the judges.	
Lupin	Moisture content in lupin bean flour 35%	Laboratory extruder Temperature – 130 °C, pressure - 20 Mpa	The extruded products showed higher protein content (55.7 g/100 g) and digestibility (68.1%), along with limited heat damage (8.7 mg furosine/100 g protein).	(Córdova-Ramos et al., 2020)

2.3 Nutritional Changes in Legume Fortified Gluten-free Ready-to-eat Snacks During Extrusion

Extrusion processing is a versatile processing technique that combines different unit operations such as mixing, shearing, kneading, cooking, compressing, and forcing a molten material, under high pressure, through a narrow opening (die). The material expands as it leaves the opening due to a sudden decrease in pressure, causing the water to instantaneously be converted into steam. Extrusion cooking uses high temperatures, short time-process, which causes structural, physicochemical and nutritional changes of raw materials apart from physical alteration (Alam et al., 2016). A considerable amount of literature has been published on the effect of extrusion on nutritional components in extrudates. The extrusion process positively affects starch gelatinisation, reduction of anti-nutritional components, decreased lipid oxidation and improved soluble dietary fibre content. Numerous studies have attempted to develop gluten-free extruded products from rice, pseudocereals such as quinoa, buckwheat and amaranth, corn, sorghum, and legumes such as beans, lentils and chickpeas.

Rice (*O. sativa L*.) has favourable characteristics such as flavour, bland taste, colour, and better processing characteristics to be used in extruded snack processing (di Cairano et al., 2021). It is recommended to fortify rice-based products with other food ingredients, such as waste materials from food processes (Sampaio et al., 2021) and legume material (Sharma et al., 2021b), to improve their nutritional profile. Corn also referred to as maize is composed of 72% carbohydrate (25% amylose and 75%., amylopectin), 8.84% protein, and 4.5% fat and is also a rich source of vitamins E and K, thiamine, niacin, riboflavin, pantothenic acid, minerals and phytochemicals, such as carotenoids, phenolics, and phytosterols (Garzón et al., 2017). Pseudocereals such as quinoa, buckwheat and amaranth have been mentioned as “the grains of the twenty-first century.” These contain proteins with essential amino acids,

fibre, starch, vitamins, minerals and are also rich in phytochemicals such as polyphenols, phytosterols, saponins, betalains, and phytosterols (Hidalgo et al., 2018). Sorghum (*Sorghum bicolor* L.) is the sixth most planted crop in the world with a nutritional profile of carbohydrate (55.2%–72.2%), protein (8.6%–18.9%), ash (1.1%–2.4%), and fibre (9.3%–25.2%), minerals such as Ca, K, Na, Fe and Zn. This is despite the fact that sorghum contains anti-nutritional factors such as tannins, phytic acids, and oxalate in relatively higher concentrations as compared to other cereal crops (Keyata et al., 2021).

Pulses contain between 60–65% carbohydrates, a little bit lower than cereals which have 70–80%. The main storage carbohydrate in pulses is starch, and the starch granules are composed primarily of amylose and amylopectin. The amylose/amylopectin ratio has been found to be a determinant of the functional properties of starch-based materials, such as viscosity, gelatinisation, solubility, mechanical properties, oxygen permeability, water binding capacity and gelling properties (Zhu et al., 2017). Shevkani et al., (2019b) reported that the starch with an amylose/amylopectin ratio of approximately 1:3 or 1:4 provides a suitable material to obtain extrudates with expanding and crispy. Starch fractions are defined as rapidly digestible starch, slowly digestible starch, and resistant starch (Kingman, & Cummings, 1992). Resistant starch resists luminal digestion by human pancreatic amylase (α-1,4-glucan-4-glucanohydrolase) in the small intestine and acts as dietary fibre (Sajilata et al., 2006). Therefore, it contributes to reducing the glycaemic load in starch-based foods. Carbohydrate undergoes major changes during extrusion processing. Extrusion processing usually results in a complete gelatinisation of starch granules. The molecular fragmentation of starch polymers during extrusion cooking, allows the substrate to be more easily hydrated and thus more accessible to amylolytic enzymes. The extrusion also leads to the loss of structural integrity and the partial solubilisation of starch molecules which increases the starch digestibility in extrudates (Arribas et al., 2019b).

Several studies have investigated the effect of the extrusion process on the carbohydrate fraction in legume fortified gluten-free extrudates and a significant increase in the total content of available carbohydrates after extrusion cooking, Yağcı et al., (2020a) reported that the *in vitro* starch digestibility of chickpea-based extrudates increased about by three times after extrusion process fermented chickpea-based extrudates. Research has also shown that pinto bean fortification in brown rice extrudates reduces rapidly digestible starch, and increases resistant starch (Sumargo et al., 2016). Morales et al., (2015a) reported a significant increase in starch digestibility, around 5–17% in the extruded lentil flours processed on a twin-screw extruder. Wani et al., (2021) reported dried green pea fortified extruded snacks lowered the glycaemicglycaemic index levels in comparison to extrudates of base material (corn and rice). Conversely, Berrios et al. (2010) reported that the amount of total available carbohydrates in the extruded lentil, chickpea and dry pea flours was not significantly different compared to their corresponding raw counterpart. The extrusion process quantitatively and qualitatively modifies the starch fraction and supplementation of ingredients rich in protein and fibre are reported to improve the nutritional quality of the extruded snacks (Dalbhagat et al., 2019).

Legumes are rich in proteins; chickpea (19-27%), lentil (23-31%), pea (14-31%), cowpea (24-28%), kidney bean (17-27%), navy bean (19-27%), pinto bean (18-25%) and lupin (32-44%) are commonly used as ingredients in extruded snacks to improve the protein content. Albumins and globulins are the two predominant types of proteins in these legumes. Generally, globulins account for greater than 50% of the total protein, whereas albumins are usually less than 20% (Hall et al., 2017). Legumes and cereals are considered complementary since legumes are rich in the amino acid lysine but relatively low in the sulphur amino acids (methionine and cysteine), which are higher in cereals. During extrusion, the material is subjected to high temperatures (100–180 °C) and high shear at relatively low

levels of moisture content (10–40% on a wet weight basis) which causes both intermolecular and intramolecular changes. On the other hand, high temperature positively affects protein aggregation and negatively affects the interaction of proteins. During the extrusion process, proteins are restructuring including association, aggregation, coagulation, and denaturation is driven by covalent cross-linking, electrostatic interaction, and hydrophobic interaction (Chen et al., 2021). Several studies have revealed that extrusion is an effective process to improve the protein quality and digestibility of legume-based foods. Extrusion temperatures of less than 140 $^{\circ}$C have been reported to have the effect of increasing protein digestibility in different extruded pulses (beans, lentils, and lupin) (Córdova-Ramos et al., 2020; Nosworthy et al., 2018a, 2018b). During extrusion processing, thermal degradation of some anti-nutritional factors; inositol hexaphosphate were hydrolysed to penta-, tetra- and triphosphates; trypsin inhibitors, condensed tannins and polyphenols were also significantly reduced; and the denaturation of proteins resulted in increased protein digestibility in legume fortified extrudates (Alonso et al., 2000 and Arribas et al., 2017). A previous study reported that the lysine, tryptophan, and methionine contents of corn extrudates were increased after extrusion processing (Gong et al., 2018). In contrast, Omwamba & Mahungu, (2014) found a 9.1% loss of lysine in rice, sorghum, and soybean blends after extrusion processing. This loss in amino acids could be due to the Maillard reaction which occurs between amino acids and reducing sugars at high extrusion temperatures.

According to the European Food Safety Authority (EFSA), dietary fibre is composed of mainly non-starch polysaccharides, cellulose, pectins, hydrocolloids, fructo-oligosaccharides and ‘resistant starch’ (Hijová et al., 2019). There are two main types of dietary fibre: soluble and insoluble based on water solubility. The soluble fibres are composed of cell wall components (cellulose, lignin, some hemicelluloses, and resistant starch) and the insoluble

fibre consists of non-cellulosic polysaccharides (non-digestible oligosaccharides, arabinoxylans, beta-glucans, some hemicelluloses, pectins, gums, mucilages, and inulin) (Menis-Henrique et al., 2020). Diets rich in dietary fibre are associated with many health benefits including a reduction in abdominal adiposity, chronic inflammation, development of depression, cardiovascular disease and colorectal carcinoma and improve gut microflora and insulin sensitivity (Barber et al., 2020).

Extrusion is a useful processing technique to increase the dietary fibre level in gluten-free products, by controlling the extrusion conditions (temperature, feed rate, and screw speed) and the suitable blend of raw ingredients. A large and growing body of literature has investigated the effect of the extrusion process on dietary fibre composition in extruded snacks. The extrusion process causes a considerable redistribution of the insoluble dietary fibre (IDF) to soluble dietary fibre (SDF) in the "hard-to-cook" extruded legumes and the extent of these changes depends upon the extrusion conditions (Arribas et al., 2017).

Arribas et al. (2019b) reported that a reduction in the total dietary fibre (TDF) (10–39%) and IDF (19–95%) contents were observed in bean fortified rice-based snacks after extrusion. The reducing effect of the extrusion process on the TDF of the chickpea-based extrudates was reported by Yağcı et al. (2020a). The observation of significantly increased SDF content, and decreased IDF content, in lupin extrudates could be explained by the reduction in particle size, degradation of dietary fibres and increased enzymatic digestibility (Zhong et al., 2021). High temperatures (>150 °C) in extrusion processing destroy the glycosidic bonds between the fibre molecules and lower the shear load in the barrel, this is reflected in a reduced torque and the conversion of the large-molecule IDF into the small-molecule SDF (Qiao et al., 2021 and Zhong et al., 2021). High screw speed (> 180 rpm), feed rate (> 17Hz), and moisture content > 40% (Qiao et al., 2021 and Zhong et al., 2021), reduces

the effect of degradation on dietary fibre into SDF in sweet potato residues (Qiao et al., 2021). Conflicting findings have been reported regarding the dietary fibre content in corn extruded snacks where soluble dietary fibre content was reduced after extrusion (Witczak et al., 2020).

Resistant starch is defined as the fraction of dietary starch, which escapes digestion in the small intestine. Resistant starch is indigestible by body enzymes. It is sub-divided into 4 fractions: RS_1, RS_2, RS_3, and RS_4. These are also called type I, II, III, and IV starches (Sajilata et al., 2006). During extrusion processing, simultaneous application of heat and moisture changes starch properties and leads to producing the retrograded starch due to its interaction with non-starch components. High barrel temperature causes melting of crystallites and the formation of reorganized crystallites which are more stable to extrusion processing (Gulzar et al., 2021). Resistant starch has been found to be increased in cereal-based snacks after extrusion processing (Escobar-Puentes et al., 2019; Gulzar et al., 2021; Raigond et al., 2015; Simons et al., 2015). Conversely, It has been reported that significant amount of resistant starch was not detected in extruded bean, chickpea, lentil, pea and cereal/legume blends after extrusion processing (Alam et al., 2016; Alonso et al., 2000; Arribas et al., 2017).

Vitamins are essential organic elements required in small quantities for human physiological functions including growth and development. They are classified as water-soluble (vitamin c and several members of vitamin B) and fat-soluble (vitamin A, D, E and K). They occur naturally in food items. One possible adverse effect of extrusion processing is the destruction of vitamins due to the conditions such as temperature, moisture content, pressure, the flow rate of raw materials (Fellows, 2017). The vitamins A, E, C, B_1, and folic acid are more sensitive to the extrusion process than the vitamins of the B-complex group

(B_2, B_6, B_{12}, niacin, Ca-pantothenate, and biotin) which are very stable. The least stable vitamins during the extrusion process have been shown to be vitamin E and, ascorbic acid (Riaz et al., 2009). Pro-vitamin A has been found to be significantly retained in the maize-based extruded snacks containing amaranth powder compared to those without it under the extrusion processing temperature of 140.7 °C, feed moisture at 20%, the extruder screw turned at a speed of 400 rpm (Beswa et al., 2016a). Extrusion processing of orange-fleshed sweet potato and bambara groundnut extrudates showed a significant *($p<0.05$)* reduction in pro-vitamin A content from 0.90–20.73 to 0.54–17.33 mg/100 g when the extrusion conditions were 10.15 kg/hr of feed rate, 30 rpm of screw speed and the first and second zones temperatures were set at 100 and 130 °C, respectively (Honi et al., 2018). The extrudates formulated with red chief lentils (*Lens culinaris* L.) showed a significant decrease (83–94%) in the tocopherols after extrusion processing (Morales et al., 2015a). A combination of high temperatures with low moisture content reduced the ascorbic acid content and folate in bean fortified snacks (Gulati & Rose, 2018). Conversely, vitamin B_2, B_6, B_{12}, niacin, pantothenic acid, and biotin (water-soluble vitamins) were largely stabled during the high-temperature conditions of the extrusion process (Singh et al., 2007).

2.4 Effect of Extrusion Processing on Physicochemical Properties of Gluten-free Extrudates

Fortification of cereal-based extruded products with legume flours affects the physical properties such as bulk density, colour, texture, water absorption and water solubility indexes of extrudates.

Bulk density and expansion ratio are the quality attributes of extruded snacks that are correlated in a negative relationship. The formation of air pockets within the expanded structures are due to a sudden reduction in pressure at the die face which causes rapid flash

off internal moisture from extrudates. Several authors have studied the physical quality characteristics of cereal-based products with extruded legume flours. A systematic decrease in expansion and an increase in bulk density were reported when adding legumes to extruded cereals (Espinosa-Ramírez et al., 2021b; Pietrysiak et al., 2020 and Yağcı et al., 2020b). The whole bean flours, including faba bean, lima bean, nuna bean, and lentils showed the highest expansion at the lowest extrusion temperature studied (<120 °C) (Ek et al., 2021 and Pietrysiak et al., 2020). The moisture content of the feed influences the product expansion and density. Soybean flour with 20% moisture had lower expansion and volume compared to flours of 5-10% moisture after extrusion at 150 °C and screw speed of 170 rpm (Sharifi et al., 2021). Yağcı et al. (2020a) reported that the increase in both temperature and screw speed with chickpea-based extrudates caused a reduction in apparent density but an increase in porosity values, yielding more puffed (expanded) extrudates. Amaranth fortification in maize-based extruded snacks reduced the expansion ratio from 2.1 to 3.3 of the control sample (Beswa et al., 2016b). Several researchers have reported that the expansion index of extrudates has also been associated with their chemical composition, interactions among macromolecules including starch, proteins and lipids by formation of complexes which restrict swelling of the extrudates and more compact structures to be formed (Espinosa-Ramírez et al., 2021a; González-Calderón et al., 2021 and Neder-Suárez et al., 2021b).

Colour is an important feature that determines the consumer preference of extruded products. It is affected mainly by the composition of raw formulation and the extrusion process parameters used in extrusion processing. Maillard reaction, caramelisation, and pigment degradation are characterised as non-enzymatic browning reactions which directly affected the colour of the extruded product (Sajid Mushtaq et al., 2021). The effect of

extrusion processing can be determined using the difference in colour values of extruded products and their raw formulation counterparts. Espinosa-Ramírez et al., (2021a) reported that the extrusion caused a decrement in legume flour (chickpea, lentil, black and pinto bean) lightness (*L**) and increments in *a** and *b** parameters whereas amaranth flour showed a different behaviour with no significant differences in *L** and *b**, and lower *a** values when the extruded flour as compared to its raw counterpart. The barrel temperature, feed moisture, and feed rate were the most affected parameters on the lightness (L*) and the redness (a*) of the yellow maize extrudates; the yellowness (b*) was only slightly dependent on extrusion variables (Alam et al., 2016). The feed moisture and die temperature significantly influenced the colour parameters on sorghum-based extrudates. With increasing feed moisture content, the lightness was increased while redness and total colour values decreased in sorghum-based extrudates (Jafari et al., 2017). High extrusion temperature, increasing the die temperature and low moisture content significantly decreased brightness while redness and yellowness increased in the extrudates. This could be due to the oxidation of pigments. The colour of the extrudates turned slightly darker as the level of red lentil bran was increased in the formulation due to the dark colour of red lentil compared to corn flour (Kesre & Masatcioglu, 2022).

The incorporation of new ingredients in ready-to-eat snacks has an important effect on textural attributes that affect eating quality and determines consumer acceptance. The texture analysis of ready-to-eat snacks is performed through penetration, compression, textural profile analysis, and acoustic procedures (Shah et al., 2017). Hardness is determined by measuring the peak force required for a probe to penetrate and break the extrudates. Textural parameters, including, hardness, crispiness and brittleness are all affected by the extrusion parameters of moisture, temperature, and screw speed in chickpea-based

extrudates (Yağcı et al., 2020a). Philipp et al., (2017) reported that fortification of pea protein in rice starch-based extrudates caused an increase in hardness. However, the results showed that the decreasing of both screw speed and temperature resulted in increased hardness and a reduction in the crispness of extrudates. Similar results were reported on gluten-free snacks fortified with mung bean, amaranth, quinoa, kaniwa as hardness increased with an increasing proportion of legumes at feed moisture content (14–18%), screw speed (400–550 rpm) and barrel temperature (130–170 °C) (Ramos Diaz et al., 2017 and Sharma et al., 2017b). Previous studies have reported that the addition of fibre rich material increases the hardness of extrudates due to increased water retention and changes in the protein-starch interactions or the starch-fibre interactions (Brennan et al., 2013b; Robin et al., 2015 and Yu et al., 2017). However, increased red lentil bran addition levels in corn-based extrudates caused slight decreases in the hardness values and slight increases in the crunchiness values which were not found to be significant as compared to control samples (Kesre & Masatcioglu, 2022). The incorporation of legumes in gluten-free extruded products influenced textural properties, resulting in less expansion and increased hardness of the extrudate (Sajid-Mushtaq et al., 2021).

The water absorption index (WAI) measures the ability to absorb water by the starch granules, and it indirectly measures the amount of intact and fully gelatinised starch granules on the gel formation capacity of macromolecule level (Yağci & Göğüş, 2008). The water solubility index (WSI) determines the total degradation of starch granules, which is the proportion of low molecular weight soluble polysaccharides released from starch (Ding et al., 2006). During extrusion, starch granules are shattered, due to the shear forces that are applied, this allows water molecules to enter the starch granules. Higher values of WSI reflect the extensive dextrinization of the starch molecules and the presence of large fragments of

gelatinised starch molecules resulted in higher WAI values (Cuj-Laines et al., 2018). Researchers have shown that rice-based extrudates with increased WAI values were related to the use of high moisture content, which induces plasticising effect thereby retards shearing and lowers starch gelatinisation and degradation (Chaiyakul et al., 2009; Ding et al., 2021 and Sharma et al., 2017b). Sumargo et al. (2016) found that the samples with higher bean flour content had both lower WAI and higher WSI. The blending of legume flour (yellow pea, chickpea, and lentil) to rice flour has been shown to result in a significant increase in WSI, due to the dilution of starch content in extruded snacks (Bouasla et al., 2017). Ramos Diaz et al., (2013) developed gluten-free extrudates from a combination of amaranth, quinoa, and kaniwa flour. It has been reported that the amount of amaranth and kaniwa had a significant effect on WAI and WSI. Extruded snacks with high amaranth flour showed lower WSI and WAI, while those with an increased amount of quinoa showed lower WSI. This could be due to the increasing contents of quinoa, amaranth, and kaniwa may reduce the extent of carbohydrate hydrolysis, the formation of the amylose protein complex and amylose-lipid complex.

2.5 Effect of Extrusion Processing on Anti-nutritional Factors of Gluten-free extruded snacks

Legumes are particularly rich in proteins, starch, fibre, minerals such as calcium, magnesium, iron, zinc, and potassium whereas cereals such as rice, wheat and maize are rich sources of carbohydrates, vitamins, minerals, fats, oils, and proteins (Martín-Cabrejas, 2019). Anti-nutritional compounds are naturally occurring non-nutritional compounds in edible seeds and when ingested, they may inhibit the absorption of some nutrients through decreasing digestibility and bioavailability in the human body, especially proteins, vitamins, and minerals (Nikmaram et al., 2017). Phytate, phenolic compounds (such as tannins), lectin, enzyme inhibitors (such as trypsin inhibitors, α-amylase inhibitors, and chymotrypsin

inhibitors), saponins and oxalate are some of the anti-nutritional factors present in abundance in the edible seeds (Boukid et al., 2019; Nikmaram et al., 2017; Singh et al., 2017a).

Phytate can form insoluble complexes with minerals such as iron, zinc, magnesium, and calcium and in turn cause severe mineral ions deficiency in the human body (Thompson, 1993). Tannins affect protein digestion, causing pancreatic hyperplasia and metabolic disturbance of sulphur and amino acid utilisation (Serrano et al., 2009). Trypsin inhibitors impair protein digestion, causing pancreatic hyperplasia and metabolic disturbance of sulphur and amino acid utilisation (Adeyemo & Onilude, 2013). Lectin interferes with the digestion and absorption of nutrients in the gastrointestinal tract and is highly resistance to proteolysis and stable over a wide range of physiological pH. However, these anti-nutritional factors have been shown to be helpful in the prevention of diabetes, heart disease, kidney stone, anti-cancer activity and controlling plasma cholesterol and triglycerides (Nikmaram et al., 2017).

In a study by Rathod & Annapure, (2016), it has been reported that a reduction of trypsin inhibitors, phytate and tannin levels of up to 99.54, 99.30, and 98.83%, respectively in extruded lentil products obtained at 18% feed moisture content, 160 °C die temperature and 200 rpm of screw speed. A reduction of 96% Trypsin inhibitors, 57% of phytic acid was observed in sorghum-soya blends and the feed moisture, barrel temperature and ingredient composition had a significant effect on the reduction of the above-mentioned anti-nutritional factors (Kumar et al., 2018). Batista et al., (2010b) investigated the effect of extrusion on cowpea (*V. unguiculata L. Walp*) and observed a complete inactivation of α-amylase and lectin, while both trypsin inhibitor activity and phytate content were reduced by 38.2% and 33.2%, respectively. Wani & Kumar, (2016b) investigated the effect of

extrusion on ready-to-eat snacks prepared from fenugreek (*Trigonella foenum-graecum*), oats, dried green pea, rice and cornflour.

Extrusion processing of green pea fortified gluten-free extrudates at 110 °C, 200 rpm of screw speed and 12% moisture level reduced the anti-nutritional factors of tannin and phytic acid content by up to 0.38 and 5.02 mg/g, respectively. The effect of extrusion temperature (125 °C) and screw speed (900-950 rpm) has been shown to decrease inositol phosphates, lectin and protease inhibitors in rice-legume (carob fruit and pea) blends (Arribas et al., 2019c). The reduction of inositol phosphates (5.7 – 30.9%) and lectin (50 – 97%) contents, and complete elimination of protease inhibitors was reported in the final product. The prominent reduction of anti-nutritional factors such as phytate, trypsin inhibitors, lectins, protease inhibitors and tannin during the extrusion process could be explained by the thermal degradation of these molecules and the formation of insoluble complexes at high cooking temperatures (Nikmaram et al., 2017). Screw speed, barrel temperature feed moisture content and extrusion pressure are considered as the most important processing variables are necessary to achieve a satisfactory reduction of anti-nutritional factors.

2.6 Sensory properties of Legume-based Extruded Snacks

On a commercial scale, the cereal-based extrudate's most critical aspect is ensuring the new product's sensory qualities when cereals replace either partially or wholly with legumes or other food sources. Colour, taste, flavour, aroma and texture (i.e. hardness, crunchiness, crispness, etc.) are the unique sensory attributes of the extruded products. Colour differences are observed as a result of the original colour of the legume flour and/or due to the non-enzymatic browning reactions; thermally-induced Maillard reactions(Kesre & Masatcioglu, 2022). The cereal-based extruded snacks usually result in a "beany" flavour,

which is considered "unpleasant" by consumers; high pea flavour incorporations resulted in harder and crunchier texture, darker colour and a less uniform surface in rice-flour-based extrudates (Philipp et al., 2017). Investigation of extrudates' instrumental hardness and crispiness/crunchiness correlated with consumer perception (Qiao et al., 2021; Renoldi et al., 2021; Sharma et al., 2017; Tomaszewska-Ciosk & Zdybel, 2021). Protein-rich extrudates prepared from blue lupin, white lupin, lentil protein isolates and faba bean concentrate had weak aroma impressions. However, it needs to be mentioned that various flavours can be added to the products to enhance acceptability (Martin et al., 2020).

On the other hand, Shadan et al. (2017) reported the highest rating rate for flavour for extrudates developed from Corn: Rice: Chickpea Green gram. Expanded snacks developed from native or preconditioned protein isolates and concentrates from pulses (lentil, lupin and faba bean as single ingredients or in combination with protein-rich flours of pseudocereals) resulted in satisfactory texture and sensory properties (Martin et al., 2020). Cowpea-based extrudates developed at 200 rpm with 16% moisture and a die temperature of 180 °C were reported to be most acceptable using sensory evaluation and statistical analysis (Jakkanwar et al., 2018). Bean-based protein-riched puffed snacks were found to be as crunchy and moderately acceptable with high nutritional quality (Natabirwa et al., 2020).

2.7 Germination of Legumes

Legumes are rich source of protein, fibre, low in fat and free of saturated fat and cholesterol (FAO 2016). They are also a great source of B-complex vitamins, iron, zinc, calcium and magnesium, contain phyto- nutrients, such as enzyme inhibitors, phytoestrogens, saponins and phenolic compounds and are gluten-free, a property that makes them a great choice for people with celiac disease or gluten sensitivity (FAO 2016; Rebello, Greenway, and Finley 2014).

The seed germination process consists of a series of physiological and biochemical processes. During seed germination, the radicle and hypocotyl elongate, the cotyledon expands, and macromolecules are converted into small molecules, increasing the content of bioactive compounds and reducing anti-nutritional factors (Chen et al., 2016; Ohanenye et al., 2020)

As one of the most critical environmental factors, light is the energy source for photosynthesis. Light quality (wavelength), light quantity (intensity), photoperiod (duration) and direction are vital components of light conditions (Rashidi et al., 2021). Recently, several studies have been published that demonstrated crop growth and quality, including increasing the bioactive compounds in sprouts (Chen et al., 2016; Gong et al., 2018; Ohanenye et al., 2020; Zhang et al., 2020).

Phenolic compounds consist of a rich group of secondary plant metabolites. The amount and composition of phenolic compounds are important quality parameters of sprouts, and the synthesis of the phenolic compounds can be stimulated by germination under different LEDs compared to dark conditions (Vastakaite & Virsile, 2015). Numerous studies have highlighted the increased phenolic compounds and antioxidant properties in soybean, Chinese kale and pea sprouts, and *Brassica oleraceavarieties* (Liu et al., 2016; H. K. Liu et al., 2016; Vastakaite & Virsile, 2015; Zhang et al., 2020). The LED blue light significantly increased the total phenolic content compared to the LED red light of Chinese kale sprouts (Qian et al., 2016). Therefore, the use of LED light during seed germination has enormous benefits for human health.

2.8 Future Trends of Legume-based Extruded Snacks

The global market size for extruded snacks was worth around $ 48.3 billion, projecting a compound annual growth rate (CAGR) of 4.4% in the year 2019 (Grand View Research, 2019). In particular, the gluten-free market is expected to grow at a compound growth

annual rate of 9.2% during 2020–2027 (Grand View Research, 2019) due to the increasing number of people suffering from coeliac disease and gluten-related disorders, and those who decide to follow a gluten-free diet without any medical need. Rapidly expanding global gluten-free food markets, along with increasing demand for low glycaemic index foods, and the rising of consumer awareness regarding healthy ready-to-eat snacks will continue to drive new product development activities focus on nutritional composition, health benefits, and production of gluten-free foods. In this scenario, legumes will be fortified in gluten-free ready-to-eat snacks even more widely in future since the global pulse flour market size is projected to reach USD 64.96 billion by 2025 with a CAGR of 14.5% (Grand View Research, 2019). Legumes provide a good source of protein, fibre, vitamins, minerals, and bioactive compounds in which exhibit their potential of partial or entire substitution with cereal flours in terms of developing extruded healthier snacks.

The incorporation of legume flours into cereal-based ready-to-eat snacks is a challenge since their incorporation into a formulation may have a detrimental effect on the physicochemical and sensory attributes of the extrudates. Nevertheless, extrusion processing of cereal and legume blends preserve a valuable amount of macro and micronutrients, including bioactive compounds such as polyphenols, and dietary fibre. There are several legume-added extruded snacks on the market and increasing research into the use of these products will continue raising in an environmentally friendly and sustainable manner.

2.9 Conclusion

The development of novel healthy ready-to-eat snack products fortified with legumes could increase pulse consumption and allow consumers to obtain health benefits from the bioactive compounds that are added to the nutritional profile of these snacks. However, the incorporation of legume flours may present a challenge since their inclusion may interfere with the physicochemical and sensory attributes of the extrudates. A wealth of research has been conducted on investigating the incorporation of legume flours into gluten-free based cereals has highlighted the effects of legume incorporation on extrudate structure and quality/sensory attributes of ready-to-eat snacks. However, more work needs to be carried out to identify the most appropriate combination of cereal and legume flours to improve sensory and quality attributes and increase consumer acceptance. In this regard, innovations in the field of cereal/legume blends to obtain desired texture and sensory quality in legume-based extrudates are equally important.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Raw materials

Commercial white rice (*O. sativa L.*), cowpea (*V. unguiculata L. walp*.) and whey protein concentrate (WPC) were used as ingredients for the development of the extruded products. Rice flour and cowpea seeds were acquired from Yogiji's Food Mart (Christchurch, New Zealand), and WPC with a nutritional composition of 79.8% protein, 6.1% carbohydrates, and 6% fat was obtained from the Kiwi Nutrition and Health Ltd (Wellington, New Zealand). Whole cowpea seeds were ground into a fine flour using a laboratory mill (Model 3310 Perten Instruments, Winnipeg, Canada) with a 1 mm screen size. Mixtures with different ratios of rice, cowpea and WPC were prepared to obtain six formulations as shown in Table 3-1. All formulations were mixed using a Delta 5L planetary mixture (Southern Hospitality, Christchurch, New Zealand) and then stored in polythene bags until extrusion and analysis.

Table 3-1 The proportion of rice flour/ cowpea flour/ WPC in the formulations

Flours % (W/W)	RF	$R_{90}C_{10}$	$R_{80}C_{15}W_{05}$	$R_{70}C_{20}W_{10}$	$R_{60}C_{25}W_{15}$	$R_{50}C_{30}W_{20}$
Rice	100	90	80	70	60	50
Cowpea	0	10	15	20	25	30
WPC	0	0	5	10	15	20

sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

3.1.2 Preparation of Extruded products

Extruded products were produced using a twin-screw extruder (Clextral BC21 twin-screw co-rotating, self-wiping extruder Clextral; Firminy Cedex, France). The internal barrel width was 46 mm, the height was 25 mm, the screw diameter was 24.7 mm, and the die diameter was 3 mm. The extrusion processing conditions are shown in Table 3-2. The extrudates were collected and dried at 103 °C for 10 min. The samples were stored in sealed plastic bags at room temperature. Prior to chemical analysis, the extrudates from the stored samples were milled using a laboratory grinder (Autogrinder, M-EM0415, Sunbeam Crop Ltd., Auckland, New Zealand).

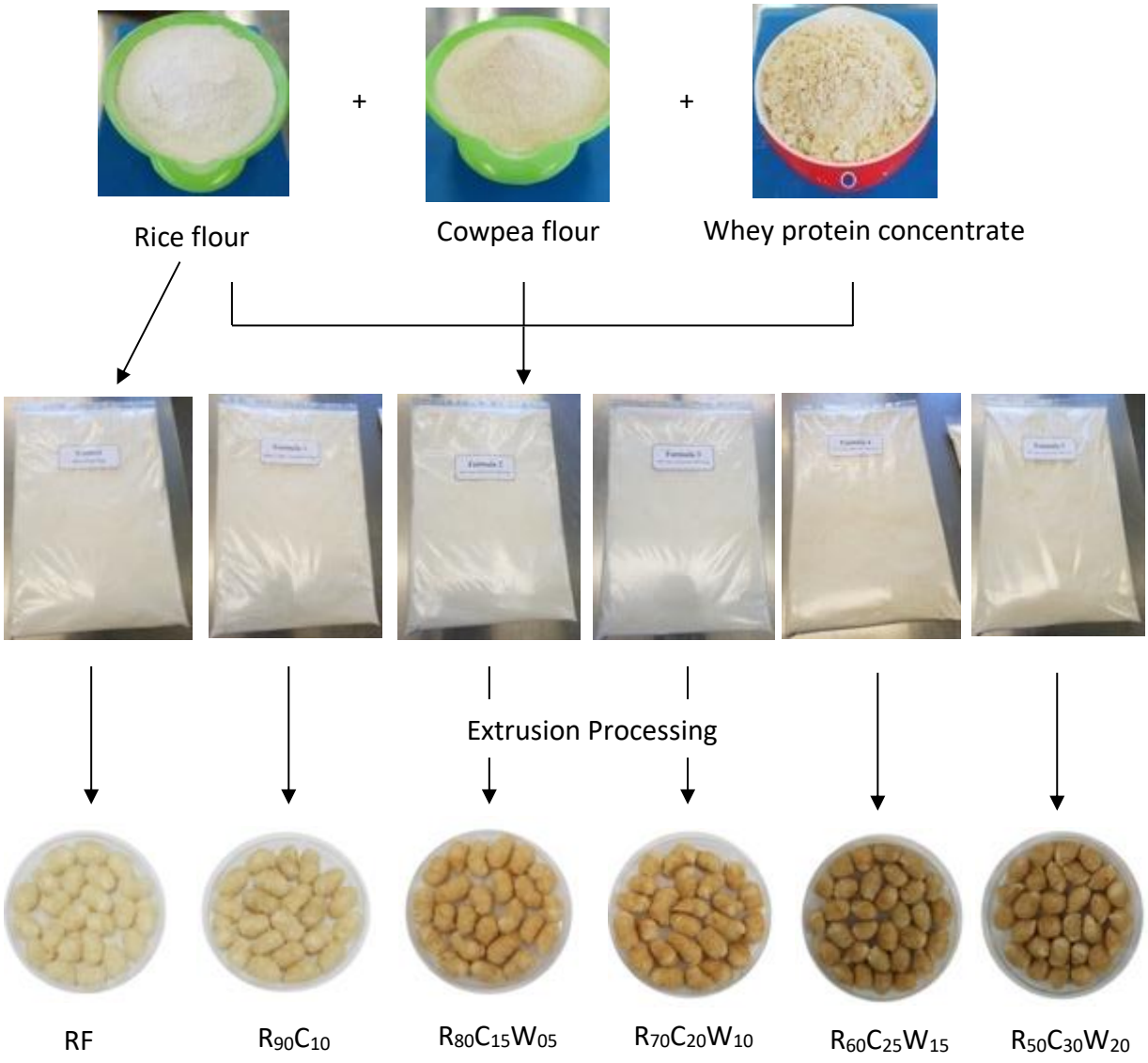

Figure 3-1 Preparation of extruded snacks

Table 3-2 Processing parameters of extrusion cooking

Sample ID	Screw speed (rpm)	Feed rate (kg/hr)	Water rate (kg/hr)	Torque (Nm)	Die temperature °C	Power KW
RF	253	7.99	91	78	115	2.00
$R_{90}C_{10}$	253	7.99	91	80	115	2.01
$R_{80}C_{15}W_{05}$	252	7.99	90	72	111	1.74
$R_{70}C_{20}W_{10}$	251	7.98	90	68	111	1.82
$R_{60}C_{25}W_{15}$	252	7.99	91	72	109	2.1
$R_{50}C_{30}W_{20}$	252	7.97	91	77	108	2.14

sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

3.1.3 Cowpea seeds germination and growth in LED chambers

A different batch of cowpea seeds were washed with distilled water and soaked in water for 12 hours at room temperature (24^0C). The imbibed seeds were evenly distributed in a germination tray and were washed every 12 hours under dark conditions. After one day, the sprouted cowpea seeds were grown under different light qualities, including red (634 nm), blue (464.5 nm) LED lamps and white fluorescent lamps, flux rate of 16 µmol/m²/s in a plant growth chamber (BDW40, Conviron Inc, Canada), Lincoln University, New Zealand. The cowpea sprouts grown under dark conditions were used as the control. A 12-hour light period and 12-hour dark photoperiod (except for control), constant 26 °C, and relative humidity of 80%. Type T (copper-constantan) thermocouple probes were used to monitor the temperature. Logger net software, version 3.3.1 (Campbell Scientific, Logan, Utah) was used to record and monitor digital signals from the temperature at ten-minute intervals. On the sixth day, the sprouts were collected, freeze-dried, milled and stored at –20 °C for further analysis.

3.2 Methods

3.2.1 Moisture content

The moisture content was determined using the method given by Approved Methods of the

(a)

(b)

Figure 3-2 (a) Plant growth chamber, (b) Thermocouples used during seed germination

American Association of Cereal Chemists (AACC-2010). The clean coded aluminium pans were dried in an oven at 105 °C for 30 min, cooled in a desiccator and weighed using an analytical balance (ARC120; OHAUS Corp., Parsippany, NJ, USA). A ground sample (5 g) was placed in the pan and placed in an oven at 105 °C overnight. The pans were allowed to cool in a desiccator. The weight of the pan plus contents after drying was recorded and moisture content was calculated according to the equation below:

$$Moisture\ (\%) = \frac{Weight\ of\ the\ fresh\ sample - Weight\ of\ the\ dried\ sample}{Weight\ of\ the\ fresh\ sample}\ x\ 100$$

$$-\ Equation\ 1$$

3.2.2 Protein content

The protein contents of the rice and cowpea raw flours, whey protein concentrate, and extruded snack samples were determined using the Dumas method (Mæhre et al., 2018). Samples (200 mg) were weighed in triplicate and loaded individually into the Dumas machine to measure the total nitrogen. The nitrogen content was determined using an Elemental analyser Model Vario MAX CN Hanau, Germany. The protein percentages (dry basis) were calculated by the following formula:

$$\%\ Protein\ in\ rice\ flour = N\ x\ 5.95\ \text{- } Equation\ 2$$

$$\%\ Protein\ in\ Cowpea\ flour = N\ x\ 5.30 -\ Equation\ 3$$

$$\%\ Protein\ in\ whey\ Protein\ Concentrate = N\ x\ 6.38\ \text{- } Equation\ 4$$

$$\%\ Protein\ in\ Extruded\ snack = N\ x\ 6.25\ \text{- } Equation\ 5$$

3.2.3 Ash content

Ash content was determined by the AOAC official method for ash determination (AOAC, 2006a). Ground sample (5 g) was placed into a pre-weighed crucible and placed in a furnace at 550 °C overnight. The crucible was cooled down in a desiccator and then the weight was recorded. Ash content (%) was calculated based on the following equation:

$$\text{Ash content } (\%)\ = \frac{\text{Ash weight}}{\text{Sample weight}}\ \text{X } 100\%\ \text{- } Equation\ 6$$

3.2.4 *In vitro* starch digestibility and glycaemic response

The *in vitro* digestion method adopted by Foschia et al., (2015) was used to evaluate carbohydrate digestibility of the raw and extruded snack samples. The crushed samples (2.5 g) were suspended in 30 mL of reverse osmosis (RO) water and placed on a pre-heated 15 place magnetic heated stirring block (IKAAG RT 15, IKA WERKE Gmbit & Co., Staufen,

Germany) and held at 37 °C with constant stirring. Stomach digestion was initiated by adding 0.8 mL 1M HCl and 1 mL of 10 % pepsin (Acros Organics, New Jersey, USA CAS:901-75-6) solution in 0.05 M HCl with continued stirring and incubated at 37 °C for 30 min. An aliquot (1 mL) was taken (time 0) and added to a 15 mL falcon tube with 4 mL ethanol. Amyloglucosidase (0.1 mL) (3260 u/mg, Megazyme Inc. Wicklow, Ireland) was added to the digestion pot to prevent end-product inhibition of pancreatic α-amylase.

Small intestine digestion was mimicked by the addition of enzyme solution 5 mL of 2.5% Pancreatin (EC: 232-468-9, CAS: 8049-47-6, activity: 42362 FIP-U/g, Applichem GmbH, Darmstadt, Germany) solution in 0.1 M sodium maleate buffer pH 6) with constant stirring at 37 °C for 120 min and aliquots withdrawn after 20, 60 and 120 min and added to 15 mL falcon tubes with 4 mL ethanol. The samples were stored at 4 °C until analysis of reducing sugar content using the 3,5-dinitrosalicylic acid (DNS).

For the measurement of the reducing sugars, all test tubes containing the sample aliquots were centrifuged at 1000 RCF for 10 minutes. Clean, dry glass test tubes were placed in a stainless-steel test tube stand, then 0.05 mL of a sample aliquot from each replicate was placed in individual glass test tubes. A 0.05 mL reagent blank (RO water), 0.05 mL of 5 mg/mL glucose standard and 0.05 mL 10 mg/mL were placed in separate tubes. Then, 0.25 mL of enzyme solution (1 % Invertase- 300U/mg in 50 % glycerol, stored at -20 °C and 1% amyloglucosidase) was added to each glass tube and all the tubes were kept for 20 min at room temperature before 0.75 mL of the DNS (reagent) was added to each tube, the tubes were covered and heated for 10 min in a boiling water bath. The glass tubes were then cooled before adding 4 mL of RO water and the absorbance was read at 530 nm. The spectrophotometer was adjusted to zero using a RO water blank. Reducing sugar release was

calculated as mg /g sample and plotted against time and area under the curve was calculated by dividing the graph into trapezoids.

3.2.5 *In vitro* protein digestibility

a) Raw mixtures

The *in vitro* protein digestion was conducted according to the method described by Chen et al., (2013). Raw blend suspensions (4% w/v) were adjusted to pH 2.0 with 1.0 M HCl and the suspension with pepsin (1031 U/mg) was incubated at 37 °C for 1 h. Then the pH was adjusted to 7.0 with 1.0 M NaOH. Then pancreatin (4 units/mg, protein basis) (350 U/mg) was added and the suspension was incubated at 37 °C for 2 h. Aliquots of raw blends digests were removed at 0 h, 1 h pepsin digestion, and 2 h pancreatin digestion for further analysis. The digests were submerged in a boiling water bath for 5 min to stop the digestion and those were centrifuged at 10000g for 10 min at 4 °C, and the supernatant was collected. After digestion, the remaining protein was determined by using the Bradford method. The per cent digestibility was calculated as the difference between protein content at 0 min and after 180 min as a percentage of original protein content.

b) Extruded snacks

The *in vitro* digestibility of extruded snacks was measured by the method described by Natabirwa et al. (2020b). A protein suspension (50mL) was prepared in distilled water (6.25 mg of protein/mL), adjusted to pH 8 with a solution of 0.1 N HCL and /or 0.1 N NaOH, and placed on a magnetic heating stirring block at 37 °C. The decrease in pH was measured after the addition of the multi-enzyme solution (1.6 mg/mL trypsin -2000 U/mg, 3.1 mg/mL chymotrypsin - 40 U/mg and 1.3 mg/mL peptidase - 5 U/mg) at every min for 10 min using a digital pH meter (S20 Seven Easy^{TM}, Mettler Toledo, USA).

The per cent *in vitro* protein digestibility was calculated using the following equation:

$$In\ vitro\ protein\ digestibility\ (\%) = 210.46 - 18.10\ pH_{10\ min}\ \text{- } Equation\ 7$$

where $\mathrm{pH}_{10\ \mathrm{min}}$ represents the change in pH after 10 min.

3.2.6 Determination of amino acid composition using high performance liquid chromatography (HPLC)

Quantitative determination of the amino acid content of the raw formulations and extrudates was performed according to the method described by Heems et al. (1998). The freeze-dried ground sample (0.10g) was hydrolysed with 5.0 mL of 6N HCl and 10.0 µL of 0.5 M amino-butyric acid as an internal standard at 110 °C for 20 hours. For the HPLC procedure, separation and quantification of amino acids were performed as pre-column derivatisation with O-phthaldialdehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids on an Agilent HPLC1100 series (Agilent Technologies, Walbronn, Germany) equipped with a binary pump and auto-sampler with a thermostat that kept the temperature at 4 °C. The software Agilent OpenLab was used to analyse the resulting chromatograms. The separation column 150 X 4.6mm, C18, 3u ACE-111-15460 was kept at 40 °C (Winlab, Leicestershire, UK).

The mobile phase was composed of two eluents: solvent A (0.01 M Na_2HPO_4 with 0.8% Tetrahydrofuran, adjusted to pH 7.5 with H_3PO_4) and solvent B (50% methanol, 50% acetonitrile). The flow rate was 0.7 mL/min, the pump gradient was 0% B to 40% B from 0 to 14 min, 50% B at 20min, 100% B from 24 min to 29min, changed to 0% B at 30 min, and equilibrating at 0% B until 36min. Detection utilised a fluorescence detector with an excitation of 335 nm and emission of 440 nm for primary amino acids. At 22 min, the detector was switched to excitation 260 nm, emission 315 nm to detect secondary amino acids such as proline. Known concentrations (between 5 µM to 200 µM) of mixed amino acid standards

were analysed in parallel to generate calibration curves for the quantification of unknown samples.

3.2.7 Determination of Phenolic compound composition using high performance liquid chromatography (HPLC)

The freeze-dried samples (2.0 g) were extracted with 20 mL of 70% methanol solution overnight on a pre-heated 15 place magnetic heated stirring block (IKAAG RT 15, IKA WERKE G6mbit & Co., Staufen, Germany) and held at 37 °C with constant stirring. After overnight extraction, sample extracts were centrifuged (ROTINA 380, Hettich LAB TECHNOLOGY, Tuttlingen, Germany) at 2500 RCF or 10 min. Supernatants were collected in plastic tubes and stored at -20°C until analysis. The extracts were filtered through 0.22 µm microfilter paper, before the HPLC analysis was performed.

HPLC analysis was carried out according to the method described by Gómez-Alonso et al. (2007). Agilent Technologies 1100 series HPLC machine equipped with quaternary pump and diode array detector (DAD) was used with an ACE 3µ C18-PFP 150X4.6mm (Advanced Chromatography Technologies, Aberdeen, Scotland) as a separation column. The fraction containing monomeric phenolics (10 µl) from each wine sample was injected into the HPLC column while kept at 20 °C temperature. The mobile phase is composed of three solvents: A (0.05M $NH_4H_2PO_4$, pH=2.6), B (100% acetonitrile) and C (0.2M H_3PO_4). The total flow rate was 0.8 mL/min, and the solvents programme is shown in Table 3.3. The detection and quantification of monomeric phenolics were recorded at 280, 320, 360 and 520 nm using the photodiode array detector (DAD). The identification of phenolic compounds was carried out by comparing their retention times and the spectra with the standards. The quantification of phenolic compounds was calculated by using the individual calibration curve of each standard.

Table 3-3 The solvent programme used in mobile phase

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
2	100	0	0
5	93.6	6.4	0
17	2.8	11.2	86
22	3.6	14.4	82
29.5	4.2	16.8	79
55	6.6	26.4	67
70	10	40	50
75	10	40	50
78	36	64	0
81	36	64	0
86	100	0	0
90	100	0	0

A :(0.05M $NH_4H_2PO_4$, pH=2.6), B: (100% acetonitrile) and C:(0.2M H_3PO_4).

3.2.8 Determination of minerals using inductively coupled plasma – optical emission spectrometry (ICP-OES)

A dry ground sample (0.2 g) was weighed into a microwave vessel. Trace element grade nitric acid (69%) (2 mL) and 30% hydrogen peroxide (2 mL) were added to the sample and well mixed. Then the vessel was loaded into the microwave digester (CEM MARS Xpress, CEM Corporation, Matthews, NC, USA). Samples were subjected to rapid heating (to 90 °C over 15 min, held for 5 min and then to 180 °C over 10 min, hold for 15 min) and elevated pressures. Mineral contents were determined in the digested samples with a spectrophotometer (Agilent 5110, Mulgrave, Victoria, Australia) by ICP-OES (Inductively

Coupled Plasma Optical Emission Spectrophotometer) according to the IEC (International Electrical Conference method).

3.2.9 Total phenolic content (TPC) and antioxidant capacity

a) Preparation of sample extracts for assays

A sample (2 g) was weighed into a 50 mL sample bottle and 20 mL of 70% methanol was added to each sample. These were stirred overnight on a multi-stirrer at 20 °C. After overnight extraction, sample extracts were centrifuged at 2500 RCF for 10 min. Supernatants were collected in plastic tubes and stored at -20 °C until analysis.

b) Total phenolic content (TPC)

The TPC of supernatant obtained from the samples and *in vitro* gastrointestinal digested samples was measured using the Folin-Ciocalteu method as described by Wang et al.(2020a). Freshly prepared 2.5 mL of 0.2 N Folin-Ciocalteu reagent and 7.5% Na_2CO_3 was added to 0.5 mL of extract and incubated in a water bath for 30 min at 40 °C. The absorbance of the reaction mixture was measured at 760 nm using the V-1200 model (Schimadzu, Maryland, USA). Gallic acid was used as a standard to determine the TPC of the samples as mg of gallic acid equivalents (GAE)/g sample.

c) DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant capacity of the samples was measured by the DPPH (2,2-diphenyl-1picrylhydrazyl) assay as described by Floegel et al. (2011). A 0.5 mL aliquot of sample extract was mixed with 1 mL of freshly prepared 0.1mM methanolic DPPH (CAS: 1898 66-4, Sigma Aldrich, St. Louise, MO, USA) solution and incubated in the dark at room temperature for 30 min. The reaction mixture absorbance was measured at 517 nm. To calculate the DPPH radical scavenging capacity, Trolox (CAS: 53188-07-1, ACROS Organics™, Morris, NJ, USA) was used as a standard and results were expressed as µmol Trolox equivalent (TE) per g sample.

d) ABTS Radical Scavenging Capacity

The antioxidant capacity of the samples was measured by the ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salts) assay as described by Elfalleh et al. (2009). A 9.5 mL aliquot of 7 mM ABTS stock solution was mixed with 245 µL of 100 mM potassium persulfate ($K_2S_2O_8$) stock solution and made up to 10 mL with RO water. This ABTS radical solution was covered with foil to protect from light and kept overnight for reaction in the dark at room temperature. On the day of analysis, ABTS radical solution was diluted with phosphate buffered saline (PBS) up to absorbance of 0.70 ± 0.02 at 734 nm. A 300µL aliquot of sample extract and 3 mL of diluted ABTS radical solution was incubated for 6 min at room temperature. The reaction mixture absorbance was measured at 734 nm. To calculate the ABTS radical scavenging capacity, Trolox (CAS: 53188-07-1, ACROS Organics™, Morris, NJ, USA) was used as a standard and results were expressed as µmol Trolox equivalent (TE) per g sample.

e) Ferric Reducing antioxidant power (FRAP) Assay

The antioxidant capacity of the samples was measured by the FRAP assay as described by Langley-Evans.(2000). Fresh FRAP reagent was prepared by mixing 300 µM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $feCl_3$ at ratio of 10:1:1 (V/V/V). 250 µL of sample extract and 2.5 mL of FRAP reagent was incubated for 2 h at 37 °C. The reaction mixture absorbance was measured at 593 nm. To calculate the FRAP capacity, iron sulphate (Fe_2SO_4) was used as a standard and results were expressed as µmol Fe^{+3} per g sample.

3.2.10 Total starch content

Total starch was determined according to AOAC Official Method 996.11 (Arribas et al., 2017), using the Megazyme total starch analysis kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The sample (100 mg) was weighed into corning culture tubes and 0.2 mL of 80 %

v/v aqueous ethanol was added and tubes were placed on a vortex mixer to completely wet and disperse the sample. Cold 1.7M sodium hydroxide solution (2 mL) was added into each tube and the tubes were stirred on a vortex mixer for 15 s. The tubes were placed in a rack over a magnetic stirrer and stirred for 15 min. During this time, intermittently, the contents of the tube were stirred vigorously on a vortex mixer 2-3 times to ensure that there were no lumps in the slurry. Sodium acetate buffer (600 mM, pH 3.8, 8 mL) was added, and tubes were stirred on a vortex mixer. Undiluted thermostable α-amylase (0.1 mL) was added immediately into one of the sample tubes. Then 0.1 mL amyloglucosidase (3,300 U/mL) was added into the same tube. Sodium acetate buffer (100 mM, pH 5.0, 0.2 mL) was added to the second tube and both tubes were stirred on a vortex mixer for 3 s. The tubes were incubated at 50 °C for 30 min and allowed to cool to room temperature. Sample and sample blank (2.0 mL from each solution) were transferred to microfuge tubes and centrifuged (ROTINA 380, Hettich LAB TECHNOLOGY, Tuttlingen, Germany) at 13,000 RCF for 5 min. An aliquot of the supernatants (1.0 mL) was transferred to a tube containing 4 mL of 100 mM acetate buffer (pH 5.0) and contents were mixed. Duplicate 0.1 mL aliquots of each sample and single 0.1 mL of sample blank were transferred to the glass test tubes. Then 3.0 mL of GOPOD reagent was added to each tube and incubated at 50 °C for 20 min, including the D-glucose controls and reagent blanks. The absorbance was read at 510 nm, against the reagent blank.

$$Starch\ \% = \ \Delta A\ x\ F\ x \frac{EX}{0.1}\ x\ D\ x\ \frac{1}{1000}\ x\ \frac{100}{W}\ x\ \frac{162}{180} - Equation\ 8$$

where:

ΔA = Absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank

F= Factor to convert absorbance values to µg glucose

EV = Sample extraction volume

0.1 = volume of sample analysed

D = further dilution of sample solution

W= weight of the sample

3.2.11 Resistant starch content

Resistant starch content was determined using the Megazyme resistant starch assay procedure K- RSTAR 05/19 (Megazyme International Ireland Ltd, Wicklow, Ireland) based on the method described by AOAC (2002.02). A ground sample (100 ± 5 mg) was weighed into a screw cap tube (Corning® culture tube; 16 x 125 mm) and 4.0 mL of pancreatic α-amylase (10 mg/mL) containing AMG (3 U/mL) was added to each tube. The tube was mixed on a vortex mixer and incubated in a shaking water bath at 37 °C with continuous shaking (200 strokes /min) for 16 h. The tubes were removed from the water bath and 4.0 mL of ethanol (99% v/v) was added into each tube. The capped tubes were centrifuged at 1500 RCF for 10 min. The supernatants were decanted, and the pellets were re-suspended in 2 mL of 50% ethanol. A further 6 mL of 50% ethanol was added, mixed, and centrifuged again at 1500 for 10 min. The supernatants were decanted, and these suspension and centrifugation steps were repeated once more. The supernatant was removed, and the tube was inverted to drain all excess liquid.

A magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH were added to each tube and the tubes were stirred for 20 min in an ice/water bath over a magnetic stirrer. Then 8 mL of 1.2 M sodium acetate buffer (pH 3.8) was added while stirring on a magnetic stirrer followed by 0.1 mL of amyloglucosidase (3300 U/mL), they were mixed well and incubated in a water bath at 50 °C for 30 min with intermittent mixing on a vortex mixer. The contents of the tube

were quantitatively transferred to a 100 mL volumetric flask and adjusted to 100 mL with distilled water. An aliquot (approx. 15 mL) was placed in a falcon plastic tube and centrifuged at 1500 RCF for 10 min. The supernatant (0.1 mL), blank (0.1 mL distilled water), and D-glucose standard (0.1 mL) were placed into glass test tubes (16 x 100 mm), 3.0 mL of GOPOD reagent was added and it was incubated at 50 °C for 20 min. The absorbance of each solution was measured at 510 nm against the reagent blank. Resistant starch (%) was determined with the following equation (AOAC, 2002):

$$Resistant\ Starch\ \left(\frac{g}{100g}\right) = \Delta E\ x\ F\ x\ \frac{10.3}{0.1}\ x\ \frac{1}{1000}\ x\ \frac{100}{W}\ x\ \frac{162}{180}\ - Equation\ 9$$

Where:

ΔA = absorbance (reaction) read against the reagent blank

F = conversion from absorbance to micrograms

100/0.1 = volume correction (0.1 mL taken from 100 mL)

1/1000 = conversion from micrograms to milligrams

W = dry weight of sample analysed

100/W = factor to present resistant starch as a percentage of sample weight

3.2.12 Soluble, Insoluble and Total Fibre Analysis

Total dietary fibre (TDF) content was determined as a sum of soluble and insoluble fibre using a total dietary fibre assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland) and measurements were recorded for soluble (SDF) and insoluble fibre (IDF) composition as described by Arribas et al. (2017). The sample (1.00 ± 0.005g) was weighed in duplicate into a 400 mL tall form beaker and 40 mL MES-TRIS buffer (0.05 M, pH 8.2) was added to each beaker. The sample was evenly dispersed on a magnetic stirrer to prevent lump formation.

Heat-stable α -amylase (3,000 Ceralpha Units/mL) solution (50 µL) was added and incubated in a shaking water bath at 98–100 °C for 30 min with continuous agitation. The beakers were removed from the hot water bath and cooled to 60 °C and scraped down with a spatula and if necessary, rinsed with 10 mL water. Protease (100 µL) (350 Tyrosine Units/mL) was added and incubated in a water bath at 60 ± 1 °C, with continuous agitation for 30 min. Then 5 mL 0.56 N HCl was added to each beaker after adjusting the pH 4.1- 4.8. Then 200 µL amyloglucosidase (200 pNP β-maltoside Units/mL) were added while stirring on a magnetic stirrer and incubated in the water bath at 60 °C for another 30 min. The resulting solution was filtered through a sintered glass crucible prepared with a celite bed and washed twice with 10 mL water at 70 °C. The filtrate and water washings were transferred to a 600 mL tall form beaker for SDF determination. The remaining residue was washed twice with 10 mL of 95 % ethanol and twice with 10 mL acetone, then crucible was used to determine IDF.

The saved filtrate and water washings were weighed in a pre-tared beaker and added four volumes of 95 % ethanol at 60 °C for determination of SDF. The precipitate was allowed to form for 1h. The precipitate was filtered through the crucible as described above. The precipitate was washed with two 15mL portions of each of 78% ethanol, 95 % ethanol and acetone. Both IDF and SDF crucibles were dried overnight in an oven at 103 °C and weighed before being analysed for protein and ash.

$$\textbf{Dietary fibre}\ (\%) = \frac{(\mathbf{R_1} + \mathbf{R_2})/2 - \mathbf{p} - \mathbf{A} - \mathbf{B}}{(\mathbf{m_1} + \mathbf{m_2})/2}\ \mathbf{X\ 100} -\ \boldsymbol{Equation}\ \mathbf{10}$$

where:

R_1 = residue weight from 1 from m_1

R_2= residue weight 2 from m_2

m_1 = sample weight 1

m_2 = sample weight 2

A = ash weight from R_1

p= Protein weight from R_2

B= blank

$$\mathrm{B} = \frac{\mathrm{BR}_1 + \mathrm{BR}_2}{2} - \mathrm{BP} - \mathrm{BA}$$

BR = blank residue

BP= blank protein from BR_1

BA = blank ash from BR_2

3.2.13 Raffinose series oligosaccharides

Galactosyl-sucrose oligosaccharides were determined using a Megazyme test kit K-RAFGL 04/18 (Megazyme International Ireland Ltd, Wicklow, Ireland) (Escobedo et al., 2019). A sample of 0.5 ± 0.01 g was weighed into a glass test tube (18 x 150mm) and 5 mL of ethanol (95% V/V) was added prior to incubate in a water bath at 84 - 88 °C for 5 min. The tube contents were quantitatively transferred to a 50 mL volumetric flask and volume was adjusted to the mark with sodium acetate buffer (50 mM, pH 4.5). The sample was allowed to extract for 15 min and mixed thoroughly. The 5 mL of solution from this sample was transferred to a glass tube and chloroform (2 mL) was added, mixed vigorously on a vortex mixer for 15 seconds and centrifuged at 1000 RCF for 10 min. This upper solution (0.2 mL) was treated separately with 0.2 mL of each sodium acetate buffer (50 mM, pH 4.5) (solution A), invertase (solution B) and α-galactosidase + invertase (solution C). All solutions were incubated at 50 °C for 20 min. The GOPOD reagent (3 mL) was added to solutions A, B and C

as well as to the reagent blank and the D-glucose controls. All these tubes were incubated at 50 °C for 20 min. The absorbance of all solutions was read against the reagent blank at 510 nm:

Absorbance: $\Delta A = \text{GOPOD absorbance for A}$

$\Delta B = \text{GOPOD absorbance for B}$

$\Delta C = \text{GOPOD absorbance for C}$

Calculations were made according to the following equations:

D-Glucose, millimoles/100 grams = $\Delta A \text{ X F X } 50$

Sucrose, millimoles/100 grams = $(\Delta B - \Delta A) \text{ X F X } 50$

Raffinose series oligosaccharides (RSO), millimoles/100 grams = $(\Delta C - \Delta B) \text{ X F X } 50$

Where:

$\Delta A = \text{GOPOD absorbance for 0.2 mL of samples} + \text{acetate buffer}$

$\Delta B = \text{GOPOD absorbance for 0.2 mL of samples} + \text{invertase}$

$\Delta C = \text{GOPOD absorbance for 0.2 mL of samples} + \alpha - \text{galactosidase} + \text{invertase}$

$$F = \frac{0.556\ (\mu moles\ of\ glucose)}{GOPOD\ absorbance\ for\ 0.556\ \mu moles\ of\ glucose} \text{ - } Equation\ 11$$

3.2.14 Phytic acid content

Phytic acid (phytate) was measured using phytic acid/Total Phosphorus Assay Kit (Megazyme International Ireland Ltd, Wicklow, Ireland) (Escobedo et al., 2019).

A. Sample extraction

A Sample of 1 g was weighed into a 75 mL glass beaker and 20 mL of HCl acid (0.66M) was added. Then the beaker was covered with an aluminium foil and vigorously stirred overnight. The extract (1 mL) was transferred to a 1.5 mL microfuge tube and centrifuged at 13,000 RCF for 10 min. The resultant extract supernatant (0.5 mL) was immediately transferred to a fresh

1.5 mL microfuge tube, and it was neutralised by adding 0.5 mL of sodium hydroxide solution (0.75M). The neutralised sample extract was used in the enzymatic dephosphorylation reaction procedure described in section B.

B. Enzymatic dephosphorylation reaction

The proportions of solutions and procedure used for enzymatic dephosphorylation reaction was illustrated in the table 3-4.

Table 3-4 The enzymatic dephosphorylation reaction procedure

Pipetted into 1.5 mL microfuge tubes	Free Phosphorus	Total Phosphorus
Distilled water Solution I (buffer) Sample extract Suspension 2 (phytase)	0.62 mL 0.20 mL 0.05 mL -	0.60 mL 0.20 mL 0.05 mL 0.02 mL
Mixed by vortex and incubated in a water bath set at 40 °C for 10 min. The next reaction was started after 10 min by addition of;		
Distilled water Solution 3 Suspension 4 (ALP)	0.02 mL 0.20 mL -	- 0.20 mL 0.02 mL
Mixed by vortex and incubated in a water bath set at 40 °C for 15 min. The next reaction was started after 15 min by addition of;		
Trichloroacetic acid (50% W/V)	0.30 mL	0.30 mL
Tubes were centrifuged to terminate the reaction at 14,200 RCF for 10 min. The supernatant was pipetted for colorimetric determination of phosphorous.		

C. Colorimetric determination of phosphorous

The colour reagent (0.5 mL) was added to the 1 mL of sample or phosphorous standard and mixed by a vortex. Tubes were incubated in a water bath set at 40 °C for 1 h. After 1 h tubes were mixed by vortex again and 1 mL of solution was transferred to a semi-micro cuvette and the absorbance was taken at 655 nm within 3 h.

D. Preparation of phosphorous calibration curve

Table 3-5 The standard phosphorous solutions preparation

Pipetted into 13 mL polypropylene tubes	STD 0 (0 µg)	STD 1 (0.5 µg)	STD 2 (2.5 µg)	STD 3 (5 µg)	STD 4 (7.5 µg)
Distilled water	5.00 mL	4.95 mL	4.75 mL	4.50 mL	4.25 mL
Phosphorous standard solution 5	-	0.05 mL	0.25 mL	0.50 mL	0.75 mL
Total volume	5.00 mL	5.00 mL	5.00 mL	5.00 mL	5.00 mL

$$M = \frac{P\ (\mu g)}{\Delta A_{phosphorous}}$$

$$Mean\ M = \frac{M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4}}{4}\ \mu g/\Delta A_{\ phosphorous}$$

$$C = \frac{phosphorous\ (g/100g)}{0.282}\ \text{- } Equation\ 12$$

Where:

Mean M = mean value of phosphorous standards (µg/$\Delta A_{phosphorous}$)

3.2.15 Trypsin Inhibitory Activity

Trypsin inhibitory activity was determined as described by Balandrán-Quintana et al. (1998). The sample of 0.5 g was extracted with 50 mL of distilled water for 30 min with mechanical shaking at a speed of 200 RCF. The sample was destabilised by adding 10 mL of 50mM tris buffer (pH 8.2, containing 10 mM $CaCl_2$). Then the sample was filtered through a Whatman

No.2 paper after being stirred vigorously for 2-3 minutes. The pre-warmed working BAPA (2 mL of benzoyl-DL-arginine-*p*-nitroanilide hydrochloride solution) was mixed with 1 mL of sample. The trypsin working solution (0.5 mL) was added and 0.5 mL of 30% acetic acid was added exactly after 10 minutes to stop the reaction. The absorbance was measured at 410 nm and distilled water was used as the blank.

$Equation\ 13$

$$TUI\ per\ mg\ of\ sample\ = \frac{[(A_{410}^{r}\ - A_{410}^{s})\ X\ 100]/\ mL\ diluted\ sample\ extract}{mg\ of\ sample/\ mL\ diluted\ sample\ extract}$$

3.2.16 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were run using raw and extruded samples from the *in vitro* protein digestion process. Samples were prepared by adding a volume of sample reducing dye (see recipe in Appendix) equivalent to 1/3 of the sample volume (e.g. if the sample volume is 15 µL, then add 5 µL of sample reducing dye), and mixed well before being heating for 5 minutes at 95 °C. Samples were then centrifuged for 30 seconds at 12,000 RCF. All SDS-PAGE analysis was performed using NuPAGE Bis-Tris pre-cast polyacrylamide 10 well mini-gels (Novex by life technologies, Carlsbad, California, USA). Gels, with strip and comb removed, were placed in a BoltTM mini–Gel Tank (Life Technologies TM, Carlsbad, California, USA), and then the anode and cathode chambers were filled with 1XBolt MES SDS running buffer (supplied as 20X, Life TechnologiesTM, Carlsbad, California, USA), ensuring that the wells of the gels were filled with buffer. Samples were loaded into each well; Novex Sharp Pre-stained protein Standard (Novex by life technologies, Carlsbad, California, USA) was used as a molecular weight marker by loading 10 µL into one of the wells of the gel. SDS-PAGE gels were run for 55 min at 165 volts (BioRad PowerPac Basic, BioRad Laboratories, Hercules, California, USA).

Once electrophoresis was complete, gels were removed from the plastic gel cassettes and rinsed with water to remove excess salts and SDS before being stained with Coomassie Blue stain (see recipe in Appendix) on a shaker for 15 min. The stain was discarded, and the gel was rinsed with water to remove the excess stain before being placed in destain solution (see the recipe in Appendix) and left on a shaker until sufficiently destained so that a picture could be taken.

3.2.17 Pasting Properties

The raw formulations (3.4 g from each formulation according to the AACC method 76-21) were weighed into the vessel and transferred to a test canister with 25.1 mL of RO water. The stirrer was placed into the canister and the sample was mixed until clumps cleared. The thermal-visco profiles of the resulting pastes were measured by a Rapid Visco Analyser (Perten Instruments, Hagersten, Sweden). A programmed heating and cooling cycle standard profile 1 was used where the samples were held at 50 °C and heated to 95 °C at 6 °C/min, a holding phase at 95 °C for 1.5 min, a cooling step from 95 to 50 °C at 6 °C/min and holding phase at 50°C for 2 min. The peak viscosity, trough viscosity, final viscosity and breakdown viscosity of the samples were recorded (Ratnaningsih et al., 2020).

3.2.18 Physical analysis

a) Colour measurements

The colour of the extruded snack samples was measured in terms of L* (brightness), a* (redness) and b* (yellowness) by using a tristimulus colour analyser (Minolta Chroma Meter CR 210, Minolta Camera Co., Japan). The instrument was calibrated using a standard white tile (L*=98.03, a*=-0.23, b*=0.25). Total colour change (ΔE) and the browning index (BI) were calculated according to Wani & Kumar (2016c).

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad \text{- } Equation\ 14$$

$$BI = \left(100 \times \frac{(X - 0.31)}{0.17}\right) - Equation\ 15$$

$$Where\ X = (a^* + 1.75)/(5.645L* + a^* - 0.3012b^*)$$

b) Texture analysis of extruded snacks

The instrumental texture of extruded snacks was evaluated using 5-bladed Kramer Shear Cell (HDP/KS5) (Oliveira et al., 2017) Texture Analyser (TA.XT2i; Stable Micro Systems, Godalming, UK) equipped with a 50 kg load cell, adjusted in mode "measure force in compression". The empty shear cell (perspex front forward) was secured in the heavy-duty platform, which was loosely fixed onto the machine base. The blades are attached to the load cell carrier employing the rapid locating adapter and lowered slowly into the sample cell and through the base slots. The heavy-duty platform was then manoeuvred until clearance was visible between the blades and their respective slots. The blades were then raised above the cell to allow for placement of the test sample. The shear cell was filled with a weighed amount by 50% of its capacity. The test was carried out according to the following operation conditions: probe distance of 48 mm, pre-test speed of 1 mm/s, test speed of 2 mm/s and a post-test speed of 10mm/s. Hardness was defined as the peak force (N) of the first compression required for the sample to rupture, while crispiness was the total number of measured force peaks along the curve. Each sample was tested six times.

c) Other Physical Paramerters

Sectional expansion index (SEI): The diameters of twenty pieces of randomly selected extruded snacks from each trial run were measured with a universal craftsman calliper. The SEI was calculated as the ratio between the diameter of the extruded product and the diameter of the die opening (Brennan et al., 2008) as shown in equation (2).

$$SEI = \frac{D_c}{D_m} - Equation\ 16$$

Where Dc is the medium extrudate diameter (mm) and Dm is the die diameter (mm).

The bulk density (g/L) was determined by a volumetric displacement procedure, as described by, (Chiu et al., 2013). Extrudates were weighed (g) and put in a 1 L beaker, and then millet seeds were added to fill up the beaker. The extrudates were taken out, and the volume of millet seeds was measured (L). The bulk density (BD) was calculated according to equation (3).

$$BD\ \left(\frac{g}{L}\right) = \frac{p}{v} \times 100 - Equation\ 17$$

Where p is the extruder sample weight (g) and v is the millet seed volume (L).

Water absorption index (WAI) and water solubility index (WSI): The WAI and WSI of extruded samples were determined according to the method of (Sha et al., 2016) and calculated through the following equations 4 & 5.

$$WAI = \frac{W_{sediment}}{W_{drysolid}} \times 100 - Equation\ 18$$

$$WSI = \frac{W_{dissolved\ solids\ in\ supernatant}}{W_{dry\ solid}} \times 100\ - Equation\ 19$$

3.2.19 Sensory analysis

This research protocol was approved by the Lincoln University Human Ethics Committee (Appendix B, B-1, 2020-30). A total of 70 consumers from the staff and postgraduate students participated in the consumer sessions conducted at Lincoln University in partitioned booths under cool natural lighting. The power analysis was used to determine the number of untrained panalists. the Computerised questionnaires were administered (RedJade® Sensory Solutions, LLC, CA, USA). Each panellist evaluated five pieces of six extruded snack formulations, labelled with three-digit codes, following a counter-balanced presentation order. Before evaluation, panellists signed consent forms for voluntary participation. Unsalted crackers and water were provided for palate cleansing. Appearance

(size and shape, colour), aroma, overall flavour, texture and after taste attributes were scored on nine-point hedonic scales (from 1 = dislike extremely to 9 = like extremely). Consumers completed a CATA question with 20 terms included texture and flavour. The "just-about right" (JAR) scales (from 1 = too little to 3 = too much, with 2 = just-about-right) was used to measure the attributes of crispiness, hardness, and colour.

3.2.20 Statistical analysis

Unless stated otherwise, experiments were performed in triplicate in all sample analysis. Statistical differences were determined by one-way analysis of variance (ANOVA) and Tukey's comparison test ($p<0.05$). Pearson's correlations were also carried out to analyse the significant correlations at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

For the sensory test, CATA (Check-All-That-Apply) data, Penalty analysis (JAR-Just above right), principal component analysis (PCA) and The principal coordinate analysis (PCoA) were performed using XLSTAT statistical software (Addinsoft, NY, USA). Cohran's Q test was used to test the independence of extruded products and their attributes. The principal coordinate analysis (PCoA) was run on the CATA and overall liking data to identify the dissimilarities. PCA is used to identify the relationships between instrumental data and sensory characteristics. Analysis of variance (ANOVA), and Tukey's tests ($p<0.05$) were carried out using Minitab 19.0 (Minitab Inc., State College, PA, USA) to determine whether differences existed among the sensory liking scores.

Chapter 4

Properties of the rice-cowpea and protein concentrate composite flours

Abstract

This study aims to investigate the effects of different levels of cowpea flour and whey protein concentrate (WPC) addition on selected quality characteristics of rice-based raw blends. Rice flour was blended with cowpea flour and WPC at weight ratios of 90:10:0, 80:15:05, 70:20:10, 60:25:15, and 50:30:20 to prepare composite flours. Rice flour was used as a control. The nutritional components, pasting properties, *in vitro* starch and protein digestibility of raw blends were analysed. Results indicated that the peak, breakdown, setback, and final viscosities were significantly lower, and the pasting temperature was significantly higher with cowpea and WPC fortification compared to the control. Higher amounts of cowpea flour and WPC resulted in a reduced amount of reducing sugars being released during *in vitro* starch digestion. An increase in the ratio of cowpea flour and WPC increased the protein and fibre contents and decreased the starch content of raw blends. The increased protein content had an impact on protein digestibility. This provides a new avenue for fortifying gluten-free formulae with high protein and fibre components, this data shows the impact of food processing on quality parameters.

Key words: gluten-free, cowpea, whey protein concentrate, *in vitro* digestibility, pasting properties

4.1 Introduction

Extrusion cooking is one of the most popular processing technique used to develop ready-to-eat snacks and textured products. During extrusion, material is subjected to thermal and shear forces which causes nutritional, physical and chemical transformations within the material and results in variety of end products with different shapes, texture, colour and flavour (Dalbhagat et al., 2019). Many studies have focused on developing nutritious snacks by combining cereal and legume food industry by-products since most of the available snacks are made from refined cereals flours which are high in energy, low in protein, fibre and other bioactive components (Arribas et al., 2019; Ciudad-Mulero et al., 2018; Ramírez-Jiménez et al., 2018). Given the potential to increase protein, fibre and bioactive compounds in gluten-free extruded snacks, this new combination of cowpea, whey protein concentrates, and rice was selected as ingredients.

Cowpea (*V. unguiculata* L. Walp.) is one of the most important protein sources in the diet of the tropical region. It contains 22-30% of protein, 53-66% carbohydrates, 15% TDF and 2.3% fat (Akissoé et al., 2021; Rengadu et al., 2020; Sreerama et al., 2012). Cowpea seeds are a good source of vitamins including B group vitamins, such as folate (vitamin B9) and thiamine (vitamin B1); and minerals rich in potassium, phosphorus, calcium, iron, zinc, sulphur, magnesium, manganese, and copper (Gonçalves et al., 2016; Harmankaya et al., 2016). Processing methods such as dehulling, soaking, fermentation, germination, heat treatments or a combination of these have reducing effects on anti-nutritional factors (Yadav et al., 2019). As legumes have an amino acid composition complementary to that of cereal grains, such legume-cereal combination leads to a high protein mix with good nutritional quality (Jayathilake et al., 2018). White rice (*O. sativa L*.) was selected as the carrier cereal due to its colour, hypo-allergenicity, bland taste and processing characteristics. In this study, WPC was

used as a secondary ingredient to improve the protein content. Whey protein concentrate is a good source of high biological value protein with essential amino acids and has many beneficial effects on human health (Brnčić et al., 2011; Huth et al., 2004).

Extrusion processing has a positive effect on the modification of starch and proteins, enhancing their digestibility, and reducing the content of anti-nutritional components such as trypsin inhibitors, phytic acid, lectins and tannins. The thermal treatment associated with extrusion cooking improves starch digestibility through a starch gelatinisation (Cutillo et al., 2021; Oñate Narciso & Brennan, 2018; Pasqualone et al., 2020)

A decrease in insoluble dietary fibre and an increase in soluble dietary fibre has been observed in extruded bean, pea and lentil-based formulations (Arribas et al., 2019b; da Silva Lindemann et al., 2021; Morales et al., 2015b). The digestibility of proteins has also been shown to increase with extrusion cooking due to the denaturation of proteins; induced by heat and by high friction and shear forces, may improve the accessibility of proteolytic enzymes and the reduction in trypsin inhibitors, which interfere with proteolysis (Ghumman et al., 2016; Patil et al., 2016; Rathod & Annapure, 2016). Arribas et al. (2019b) have reported that extrusion cooking causes an increase in the *in vitro* digestibility of proteins by about 13–18% compared to the bean fortified rice-based raw blends.

Extrusion cooking of cowpea-based mixtures has been reported in recent years (Batista et al., 2010c; Jakkanwar et al., 2018c; Marengo et al., 2017). However, extrusion cooking of a combination of cowpea and WPC in rice-based snack foods has not been studied yet. Before extrusion processing, it is important to analyse the raw blends to detect the effect of extrusion on ready-to-eat snacks developed from these new combinations. Therefore, this chapter shows the experimental data of the raw blends before they were subjected to extrusion processing and was helpful in understanding the effect of extrusion on raw blends.

4.2 Material and Methods

4.2.1 Materials

The materials are described in section 3.1.1.

4.2.2 Moisture content of raw blends

Determination of the moisture content was carried out as described in 3.2.1

4.2.3 Protein content

Determination of the protein content was carried out as described in in 3.2.2

4.2.4 Soluble and total starch contents

Determination of the soluble and total starch content was carried out as described in 3.2.10

4.2.5 Soluble and total fibre contents

Determination of the soluble and total fibre content was carried out as described in 3.2.12

4.2.6 Pasting properties

Determination of the pasting properties was carried out as described in 3.2.17

4.2.7 *In vitro* digestibility of starch

In vitro digestibility of starch was carried out as described in 3.2.4

4.2.8 *In vitro* protein digestibility

In vitro protein digestibility was carried out as described in 3.2.5

4.2.9 Statistical analysis

Statistical analysis was carried out as described in 3.3

4.3 Results and Discussion

4.3.1 Nutritional components in raw blends

Table 4-1 The nutritional composition of raw materials and blends

Samples	Dry Matter g/100 g	Protein g/100 g (Dry basis)	Soluble Starch g/100 g	Total Starch g/100 g	Soluble Dietary Fibre	Total Dietary Fibre
Cowpea	88.74 ± 0.06	21.04 ± 0.08	45.31 ± 1.03	54.50 ± 0.96	0.88 ± 0.14	12.91 ± 0.11
WPC	99.61 ± 0.01	78.47 ± 0.00	-	-	-	-
RF	88.93 ± 0.04	7.38 ± 0.00^{f}	66.91 ± 0.89^{a}	67.11 ± 0.92^{a}	0.04 ± 0.00^{d}	0.06 ± 0.00^{d}
$R_{90}C_{10}$	88.58 ± 0.47	8.59 ± 0.04^{e}	63.05 ± 0.39^{b}	63.39 ± 0.38^{ab}	0.13 ± 0.00^{cd}	0.92 ± 0.01^{c}
$R_{80}C_{15}W_{05}$	89.71 ± 0.38	11.28 ± 0.04^{d}	61.80 ± 1.80^{b}	62.42 ± 1.74^{bc}	0.18 ± 0.01^{c}	1.21 ± 0.04^{bc}
$R_{70}C_{20}W_{10}$	90.04 ± 0.11	17.19 ± 0.00^{c}	60.76 ± 0.14^{bc}	62.05 ± 0.21^{bc}	0.21 ± 0.02^{bc}	1.34 ± 0.08^{bc}
$R_{60}C_{25}W_{15}$	90.68 ± 0.28	18.66 ± 0.04^{b}	57.07 ± 1.07^{c}	58.73 ± 1.10^{c}	0.32 ± 0.00^{ab}	1.70 ± 0.11^{ab}
$R_{50}C_{30}W_{20}$	90.90 ± 0.16	23.44 ± 0.44^{a}	47.80 ± 0.42^{d}	49.51 ± 0.81^{d}	0.35 ± 0.07^{a}	2.00 ± 0.30^{a}

Values are mean ± standard deviation (n=3). Values within a column followed by the same superscript letter are not significantly different from each other ($p<0.05$), according to the Tukey's test. Sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Table 4.1 shows the composition of raw blends where the rice flour was replaced by cowpea flour and WPC. According to the results in Table 4.1, the cowpea sample was shown to be high in protein (21 g 100 g^{-1}) content. Gerrano et al. (2019) reported similar protein contents (23–28 g/100 g) in 22 cowpea varieties from South Africa. Compared to other legumes, the protein content of cowpea flour was higher than that of pea, red bean and lower than that of soybean, lentil, kidney beans and broad beans (Kan et al., 2018). The high protein contents

found in the cowpea varieties can serve as a good source of protein for developing food products with enhanced protein content. An increase in protein content of more than 18% was observed in formulations with 25-30% cowpea flour and 15-20% WPC, fortification of extruded snacks with legumes, (including cowpea) and WPC has previously been shown to increase the protein content and quality in extruded snacks (Jakkanwar et al., 2018; Teba et al., 2017).

The soluble starch content was significantly reduced in all formulations compared to the control. Fortification of 30% cowpea flour + 20% WPC reduced total starch content by 26% compared to the control. The TDF content increased from 0.06% in the control to 0.92-2.0% in the 10-30% cowpea flour and 10-20% WPC fortifications. Soluble dietary fibre content contributed 14-17% of TDF in all cowpeas fortified formulations compared to the control. Carbohydrates were the major component in ready-to-eat snacks made from refined cereal flours (Brennan et al., 2012). Ho et al. (2016) have reported that the bean carbohydrates are rich in non-digestible carbohydrates which are responsible for the low glycaemic index during digestion. It is important to study the relationship between insoluble and soluble fibre due to their different roles in digestion and absorption in the intestine and their effect on physicochemical properties such as expansion ratio (Rodríguez-Vidal et al., 2017), hydration, oil binding and viscosity parameters (Elleuch et al., 2011). The insoluble to soluble fibre ratios ranged from 4.3:1 to 6.0: 1 in cowpea flour fortified formulations compared to the 0.5:1 in the control sample. It has been reported that the higher insoluble fibre ratios decreased the predicted glycaemic index and increased the water absorption and texture of final products (Oh et al., 2014).

4.3.2 Pasting properties of blends

The analysis of pasting properties is an important indicator that predicts how the starch will behave during different food processing conditions. The pasting properties of the raw materials and raw blends, including peak viscosity, trough, breakdown, final viscosities, setback and pasting temperatures, are reported in Table 4.2. These show the variations of the starch viscosity, in a surplus of water, during heating and cooling treatments under controlled conditions. The incorporation of cowpea flour and WPC to rice flour drastically lowered the peak, trough, breakdown, final and setback viscosities. Peak viscosity is a measure of the ability of starches to swell freely before the dissolution of amylose and indicates the strength of the pastes formed during gelatinisation (Asaam et al., 2018). The peak viscosities of the composite flours were found to be in the range 3675– 1045 mPa s compared to the 3920 mPa s in the control sample. The rice flour sample has a higher peak viscosity, which can be explained by the fact that rice flour has a higher starch content compared to rice flour-cowpea/ WPC/ rice composites; this resulted in the higher swelling power, leading to an increase in peak viscosity. Starch and protein interactions restrict the starch swelling and reduce the viscosity (Duta & Culetu, 2015), and high amounts of fibre act as a filler, reducing the starch content and thereby reducing its viscosity (Korus et al., 2017). This observation was supported by the study of Jiao et al.(2020) in which they found a reduction in peak viscosity with an increased proportion of pea starch in rice flour blends. Peak time is the time taken to reach the respective peak viscosity. The greatest peak time recorded was 6.98 min and the shortest 5.98 min with no significant differences ($p<0.05$) existing between them. According to Barak et al. (2013), the flours with high protein content need more time to reach peak viscosity, and peak viscosity shows a negative correlation with protein. Trough viscosity is the minimum viscosity value in the constant temperature phase

Table 4-2 The pasting properties of cowpea flour and WPC blended with rice flour

Samples	Peak Viscosity (Cp)	Trough Viscosity (Cp)	Break down Viscosity (Cp)	Final Viscosity (Cp)	Setback Viscosity (Cp)	Pasting Temperature (°C)	Peak Time (min)
Cowpea	1236.67 ± 4.16	956.67 ± 0.58	279.33 ± 4.51	1497.00 ± 1.00	541.00 ± 0.00	83.09 ± 0.07	5.00 ± 0.01
WPC	151.00 ± 9.54	127.33 ± 7.57	23.67 ± 2.08	380.33 ± 9.29	253.00 ± 2.65	0.00 ± 0.00	6.98 ± 0.04
RF	3920.33 ± 57.5^{a}	2996.67 ± 12.1^{a}	879.67 ± 15.5^{a}	7017.00 ± 1.0^{a}	4119.67 ± 112.5^{a}	81.55 ± 0.0^{c}	5.98 ± 0.1^{b}
$R_{90}C_{10}$	3675.67 ± 33.7^{b}	3110.00 ± 9.2^{b}	563.33 ± 20.8^{b}	6005.33 ± 3.2^{b}	3195.67 ± 16.6^{b}	82.38 ± 0.0^{b}	6.20 ± 0.1^{b}
$R_{80}C_{15}W_{05}$	2478.67 ± 7.5^{c}	2289.00 ± 8.2^{c}	188.00 ± 1.0^{c}	5004.00 ± 1.0^{c}	2713.33 ± 7.8^{c}	82.65 ± 0.4^{b}	6.00 ± 0.0^{b}
$R_{70}C_{20}W_{10}$	1708.67 ± 13.6^{d}	1686.33 ± 10.7^{d}	29.67 ± 3.1^{d}	3647.00 ± 7.5^{d}	1895.33 ± 12.2^{d}	84.42 ± 0.4^{a}	6.20 ± 0.1^{b}
$R_{60}C_{25}W_{15}$	1278.00 ± 2.0^{e}	1253.00 ± 3.0^{e}	25.00 ± 1.0^{d}	2555.33 ± 3.1^{e}	1301.67 ± 6.0^{e}	84.64 ± 0.1^{a}	6.98 ± 0.0^{a}
$R_{50}C_{30}W_{20}$	1045.67 ± 8.7^{f}	997.67 ± 6.1^{f}	47.33 ± 2.5^{d}	1947.00 ± 18.5^{f}	934.67 ± 2.5^{f}	84.80 ± 0.0^{a}	6.98 ± 0.0^{a}

Values are mean ± standard deviation (n=3). Values within a column related to the raw blends followed by the same superscript letter are not significantly different from each other ($p<0.05$), according to the Tukey's test. sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

of the RVA profile measuring the ability of paste to withstand breakdown during cooling (Asaam et al., 2018). Trough viscosity results for all formulations ranged from 3110 to 997 mPa s and significantly decreased with an increasing cowpea flour and WPC substitution.

The breakdown viscosity measures how easily the granular structure of the starch breaks after maximum swelling at peak viscosity (Oñate Narciso & Brennan, 2018). This rise could be due to protein denaturation. The breakdown viscosity of the flours ranged from 879 to 47 mPa s with formulations 30% cowpea flour + 20% WPC and control recording the lowest and highest values respectively. Significant differences *($p<0.05$)* existed between all the formulations. Smaller breakdown viscosities suggest that pastes are more stable under hot conditions resulting from lower concentrations of starch in a sample. The final viscosity of the formulations ranged from 7017 to 1947 mPa s and there is a significant reduction *($p<0.05$)* in the final viscosities with the increased proportion of cowpea and WPC. The reduction in the final viscosities of blends may be attributed to the loss of the ability of the amylose chain to retrograde during cooling, possibly due to the presence of the incorporated proteins and the proteins may act as plasticisers, and prevent molecular rearrangement of amylose in starch gels, leading to lower final viscosities (Oñate Narciso & Brennan, 2018). Setback viscosity measures the retrogradation tendency of a paste prepared from a starchy food (Kaushal et al., 2012). The control formulation had the highest setback, 4119 mPa s. As cowpea and WPC fortification increased, setback viscosity decreased significantly. The pasting temperature indicates the minimum temperature required to cook or gelatinise flour. The results showed that pasting temperatures of the composite flours ranged from 81–85 °C with significant differences *($p<0.05$)* among the formulations. The highest gelatinisation temperature was found for 30% cowpea + 20% WPC blend (84.80 °C) and the lowest for rice control (81.55 °C). The gelatinisation temperatures of blends with 20-30%

cowpea and 10-15% WPC were significantly higher than that of blends with 10-15% cowpea and 5% WPC. This could be due to the physical competition for water between protein gelation and starch gelatinisation of blends containing high protein and starch. This observation of the changes in viscosity of the blends was supported by the study of (Iwe et al., 2016) with cowpea fortified rice formulations.

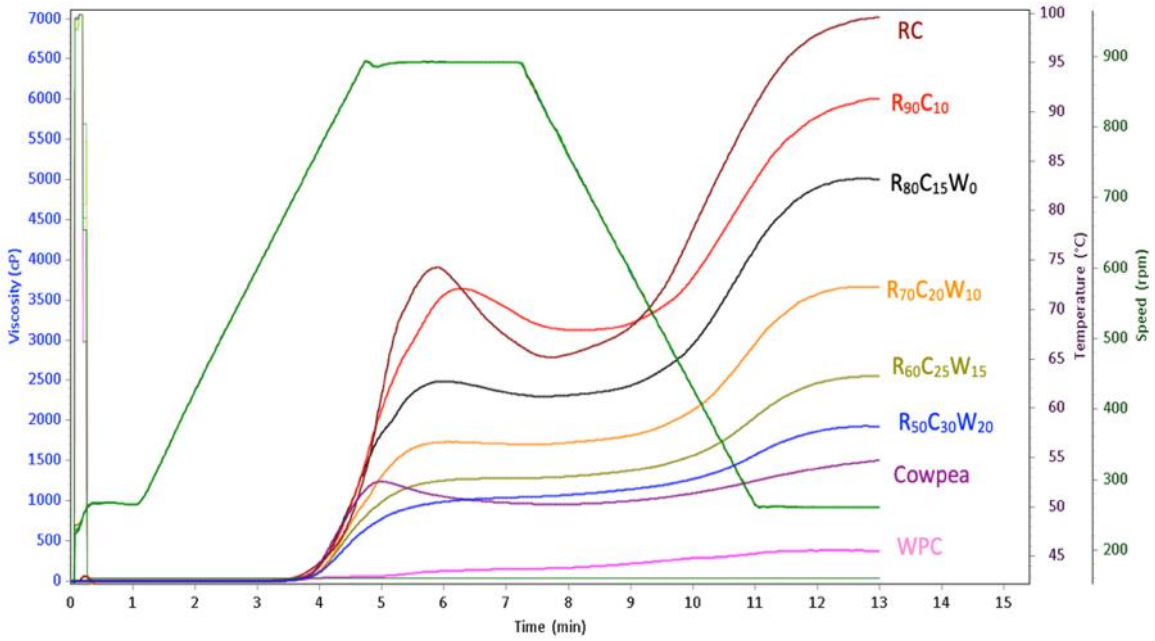

Figure 4-1 Effect of cowpea flour and WPC on rice flour pasting profile

sample codes: RC (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

4.3.3 *In vitro* digestibility of protein and starch of raw blends

Determining the *in vitro* digestibility of the raw formulation provides information about the effect of extrusion on these parameters. The protein *in vitro* digestibility of raw blends

increased significantly with the addition of 15-30 % cowpea flour and 5-20% WPC compared to the control sample for both pepsin and pancreatin digestion. This rise could be due to the protein denaturation during extrusion, which would expose more polypeptide bonds to proteolytic enzymes and the inactivation of thermo-labile anti-nutritional factors (protease inhibitors, phytic acid) of legumes that impair protein digestibility (Arribas, Cabellos, et al., 2019b).

Table 4-3 The *in vitro* digestibility of protein in the raw blends of cowpea, WPC and rice flour

Samples	1h Pepsin	2 h Pancreatin
RC	26.70 ± 1.40^{c}	41.69 ± 0.58^{c}
$R_{90}C_{10}$	29.50 ± 6.19^{bc}	42.03 ± 2.79^{bc}
$R_{80}C_{15}W_{05}$	33.80 ± 2.92^{abc}	49.82 ± 5.11^{ab}
$R_{70}C_{20}W_{10}$	35.67 ± 1.26^{ab}	49.92 ± 0.96^{ab}
$R_{60}C_{25}W_{15}$	37.52 ± 0.39^{a}	53.57 ± 0.80^{a}
$R_{50}C_{30}W_{20}$	38.22 ± 0.67^{a}	57.63 ± 4.00^{a}

Values are mean ± standard deviation (n=3). Values within a column followed by the same superscript letter are not significantly different from each other ($p<0.05$), according to the Tukey's test. Sample codes: RC (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

The mg of glucose released per g of sample from *in vitro* starch digestion of rice flour (control) and raw blends of rice, cowpea and WPC was obtained for each time point (0, 20, 60, 120 min) (Figure 4.2). It was noticeable that there was a significant difference between the control sample and the cowpea and WPC fortified samples. In all samples, the values of

reducing sugars increased dramatically in the first 20 min and the peak values were reached between 20 -120 min. There were significantly more reducing sugars released from the control blends than from all the cowpea and WPC fortified blends.

The control showed the highest rate of hydrolysis, and 30% cowpea flour + 20% WPC formulation had the lowest rate of hydrolysis over 120 min period. The starch hydrolysis values reduced as the ratio of cowpea+WPC/rice increased compared to the control sample. Fortification with 10-30% cowpea flour and 5-20% WPC in the rice-based raw mixtures resulted in a reduction in reducing sugar released after 2 h in the range of 20-47% compared to the control. Higher inclusion rates of CP and WPC led to greater reductions in reducing sugar released at 0, 20, 60 and 120 min compared to the control. This could be explained by the nutritional components; dietary fibre (12-14%), resistant starch (9-14%) and protein (16-31%) present in the cowpea flour (Jayathilake et al., 2018; Rengadu et al., 2020a). The results for reducing sugar are expected due to the amount of total carbohydrates was not similar in all formulations since rice flour has replaced with cowpea and WPC.

Starch can be classified into different fractions which are total starch, rapidly digested starch, slowly digested starch and resistant starch. Rapidly digested starch can be hydrolysed to reducing sugars within 20 min, while slowly digested starch is hydrolysed to reducing sugars in 120 min when subjected to *in vitro* assay. The starch that remains after 120 of hydrolysis is known as the resistant starch (Englyst, H.N., Kingman, S. M., & Cummings, 1992; Rengadu et al., 2020b). The proteins in the cowpea flour and the WPC may encapsulate the starch reducing enzyme hydrolysis, while the dietary fibre in the cowpea flour may reduce the starch digestibility due to its high-water absorbing capacity (Foschia et al., 2015). Lower digestibility of starch has been related to many health benefits; favourable postprandial glycaemia and reduced oxidative stress through a controlled delivery of glucose to the body involving different mechanisms. Starch can also escape digestion as an effect of dietary fibre

reducing the transit time of the bolus in the small intestine (Harris et al., 2000). There is recent research evidence showing that this reduced ratio of carbohydrates to protein is helpful in weight loss and specifically it has been shown that leucine, a branched-chain amino acid present in whey proteins contributes more to weight loss diets (Ferrando, A. A., Williams, B. D., Stuart, C. A., Lane, H. W., & Wolfe, 1995; Meroni et al., 2020; Onwulata et al., 2010). These results agreed with the findings of (da Silva Lindemann et al., 2021), starch hydrolysis values reduced as the ratio of beans/rice increased, regardless the bean source used.

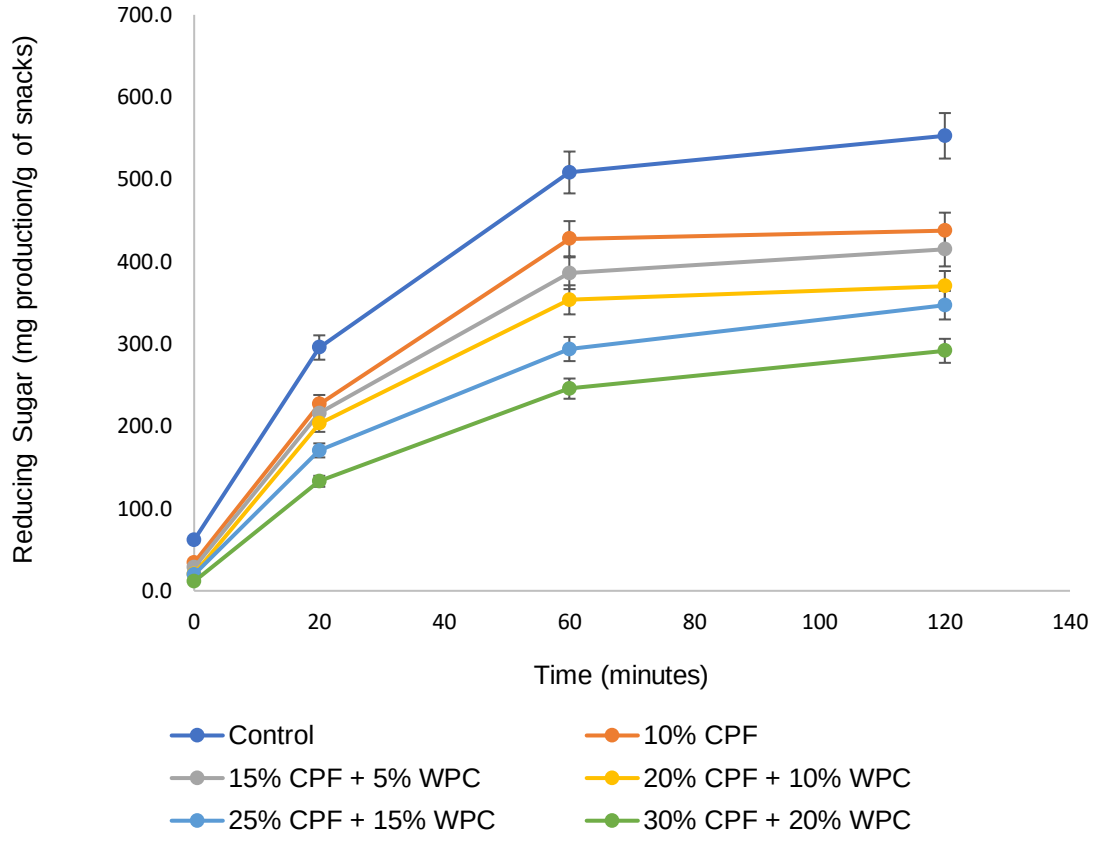

Figure 4-2 The amount of glucose released (mg/ g of sample) from control and cowpea flour and WPC fortified rice-based raw blends

Values are mean ± standard deviation (n=3). sample codes: RC (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

4.4 Conclusion

These experiments confirmed that the fortification with cowpea and WPC in rice-based blends improved the protein and fibre contents. The *in vitro* protein digestibility of raw blends increased significantly with the addition of more than 20% cowpea flour and 5% WPC compared to the control sample. Incorporation of a higher proportion of cowpea and WPC led to decreases in peak, trough, breakdown, final and setback viscosities and increases in pasting temperature and peak time in rice-based blends. According to the results obtained in this study, the reduction in starch hydrolysis rate when greater amounts of cowpea and WPC are added to rice is a result of (1) lower starch content of the blend, (2) greater content of resistant starch and fibre components, (3) the phenomenon of interactions of cowpea, WPC and rice constituents. These raw blends can be considered as a good alternative for developing ready-to-eat extruded snacks with more protein, fibre, and low carbohydrate content. Production of novel snacks offers a new pathway to increase legume consumption and provide new food for consumers who have gluten-sensitivity.

Chapter 5

The Effect of Cowpea and Whey Protein Concentrate on Nutritional, Physicochemical and Textural Properties of Ready-to-eat Extruded Rice Snacks

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Abstract

An attempt was undertaken in this study to develop rice-based extruded snacks with high protein and fibre with cowpea flour and whey protein concentrate (WPC). Nutritional, physicochemical, and textural properties of extrudates were evaluated, at five levels of cowpea and WPC (10:0, 15:05, 20:10, 25:15, 30:20) fortifications, rice flour was used as a control. The protein and fibre content in the extrudates significantly increased ($p<0.05$) with cowpea (10-30%) and WPC (5-20%) fortification compared to the control. Study results showed that increasing cowpea and WPC content in extrudates made their colour lightness decrease, and their mechanical properties increase. The number of peaks during compression increased with the higher incorporations of cowpea and WPC. Correlation analysis revealed that the protein, fibre contents were significantly related to hardness, crispiness, bulk density, water absorption index and colour properties of the extrudates. The essential and non-essential amino acid profiles increased in the extrudates, proportionally to the cowpea and WPC fortification.

Keywords: Extrusion processing, cowpea, whey protein concentrate, Nutritional composition, texture

5.1 Introduction

Extrusion processing has become an important manufacturing process for the development of a wide range of cereal-based products such as breakfast cereals, ready-to-eat snacks, cereal-based baby food, and textural proteins (Alam et al., 2016). During extrusion, the food material is subject to strong shear force with controlled elements – for example, temperature or moisture. The extrusion of starchy foods leads to the degradation and gelatinisation of starch particles, the denaturation of proteins and the formation of starch-protein or starch-lipid complexes, with changes in their physical, chemical, and microscopic properties of extrudates (Dalbhagat et al., 2019; Xu et al., 2016). Ready-to-eat snacks are widely consumed by the world's population due its comfort and satiety. Starch is the main constituent of the extruded snacks, and these snacks are dense in energy but are nutritionally poor in terms of protein, fibre and micronutrients (Brennan et al., 2013a). There are many studies on enhancing protein, fibre and other micronutrients in extruded snacks by combining cereals and legumes (Ciudad-Mulero et al., 2020; Martin et al., 2020c; Neder-Suárez et al., 2021b; Yağcı et al., 2020b).

Cereals are the main sources of dietary carbohydrates for human nutrition. Wheat, corn, and rice are the three most important cereal crops used for human consumption. Rice (*O. sativa L.*); a starchy cereal is the staple food for over half of the global population, and it is further utilised as raw material for developing value-added extruded products such as snacks, breakfasts due to its characteristics of flavour carrying capability, hypo-allergenicity, bland taste or good processing performance (Henry, 2019; Kadan et al., 2003; Xu et al., 2016).

Pulse crops offer an important and affordable source of protein, dietary fibre, micronutrients such as vitamin B, minerals (as iron and zinc) and a variety of antioxidants in human diets (FAO, 2016). Cowpea (*V. unguiculata* L. *Walp*) is a food legume, contains 24.2% of protein are rich in lysine, glutamine, phenylalanine, and tyrosine, but they lack cysteine and methionine, thus being complementary to those of cereal grains. It has been recognised as a source of dietary fibre (14.4%) and resistant starch, and its slowly digestible starch results in limited and slow increases in plasma glucose and insulin upon ingestion, which positively affects human health (Gonçalves et al., 2016b). Cowpea seeds are a good source of health-promoting bioactive compounds, mainly phenolic acids derivatives (148–1176 μg/g), flavonol glycosides (27–1060 μg/g) and peptides. These polyphenols and peptides have a significant anti-inflammatory effect, and benefits against cancer, diabetes, and cardiovascular disease (Awika & Duodu, 2017). Apart from the macronutrients, cowpea provides micronutrients, including vitamins (vitamin B-complex, C, carotenoids, E) and minerals (potassium, phosphorus, calcium, sulphur, magnesium, iron, zinc, manganese, and copper) (Gonçalves et al., 2016c). However, the presence of antinutrients such as phytic acid, trypsin inhibitors and alpha-galactooligosaccharides has limited the food consumption of cowpea seeds (Sharma et al., 2021a; Sreerama et al., 2012b).

Extruded snacks can be fortified with whey proteins; a dairy industry by-product widely available in the health food market as flavoured shakes, flavoured protein bars and dietary supplements, to enhance the nutrient density by increasing protein content. Whey protein concentrate is rich in essential amino acids; lysine (9.5%), leucine (11.8%), methionine (3.0%), valine (4.7%), threonine (4.6%), and phenylalanine (3.0%) (Etzel, 2004; Yadav et al., 2014).

The consumer acceptance of direct-puffed snacks depends on unique quality attributes such as texture, appearance, taste, flavour, and colour; texture is the most important attribute in a second-generation snack. Refined cereal flours are used intensively as raw materials for extruded products developments due to their high expansion capacity and appreciated textural properties. It is beneficial to incorporate the locally available legumes in the cereal-based formula to improve the nutritious and healthy fractions, protein and fibre in extruded products. The textural and mechanical properties of puffed products should be intensively studied when they completely or partially replace the common cereals in the extruded formula. Comparative studies on the effect of extrusion on different combinations of rice, cowpea and WPC are scarce, especially at the same processing conditions. Previous studies showed that the extrusion significantly affects nutritional and expansion properties of cowpea fortified extruded snacks and breakfast cereals (Batista et al., 2010c; Marengo et al., 2017; Nahemiah et al., 2015). The aim of this chapter is to investigate the performance of combinations of rice, cowpea and WPC on extrusion in comparison with common refined rice flour. Within this frame, all flours were extruded at the same condition. Their nutritional, physicochemical and mechanical properties were assessed.

5.2 Materials and Methods

5.2.1 Materials

Raw materials were used as mentioned in 3.1.1.

5.2.2 Preparation of extrudates

Extrusion trials were carried out as outlined in 3.1.2.

5.2.3 Moisture content

Moisture content was determined as described in 3.2.1.

5.2.4 Protein content

Protein content was measured as mentioned in 3.2.2.

5.2.5 Soluble and total dietary fibre contents

Soluble and total fibre contents were determined as outlined in 3.2.12.

5.2.6 Soluble and total starch contents

Soluble and total starch contents were determined as described in 3.2.10.

5.2.7 Determination of total amino acid profile

Total amino acid profile was determined as described in 3.2.6.

5.2.8 Physical analysis

a) Colour analysis was performed as mentioned in 3.2.18 (a).

b) Texture analysis was performed as described in 3.2.18 (b).

c) Sectional expansion index, bulk density, water absorption index (WAI) and water solubility index (WSI) were determined as described in 3.2.18 (C).

5.2.9 Statistical analysis

Statistical analysis was carried out as mentioned in 3.3.

5.3 Results and Discussion

A co-rotating twin-screw extruder was used to obtain rice-based extrudates (Figure 5. 1) containing cowpea and WPC in five different combinations and used rice as control in triplicates. Process conditions were screw speed at 252 $\pm$ 0.75 rpm, feed rate at 7.98 $\pm$ 0.00 kg h^{-1}, water rate at 90.67 $\pm$ 0.52 kg h^{-1}, die temperature 111.5 ^{0}C $\pm$ 3.00 and die diameter was 3 mm (Table 5.1).

Table 5.2 shows the proximate composition of the extruded snack products fortified with cowpea and whey protein concentrate. Total starch and protein contents were the major components of extrudates representing > 90% of the dry matter. The highest protein content was found for 30% cowpea flour and 20% WPC fortification (27.22%), and it was about 3-fold compared to the control. In practical terms, a serving of 100 g of the 30% cowpea flour and

20% WPC fortified extruded products would provide 50% of the recommended dietary allowance of protein for an adult (recommended daily allowance of protein 53 g). The total starch content decreased in the extrudates from 3.6% (10% cowpea flour) to 33% (30% cowpea flour +20% WPC) in comparison to the control sample; the total starch content was greater than 50% in all formulations. These relationships are due to the replacement of rice flour with legume flour and WPC, which proportionately reduce the starch content in formulations. Less than 50% of dietary fibre was soluble in the formula fortified with 15-30% of cowpea and 5-20% WPC. In this study, TDF contents of cowpea enriched snacks were significantly higher than control and proportional to replacement levels of cowpea flour *($P<0.05$).*

The extrusion cooking process induces modifications in the dietary fibre fractions. The high pressure in the machine barrel and screw speed easily ruptures large molecules, which increases the degree of decomposition of fibre material molecules, thus converting them into smaller molecules that contain many water solubility parts (Jing & Chi, 2013). The high temperatures used in extrusion breaks down the glycosidic bonds in the polysaccharides, thus increasing the amount of soluble dietary fibre by converting the insoluble dietary fibre to soluble dietary fibre in extrudates. The moisture content of products was 7–9 g per 100 g on a dry weight basis, which is characteristic of extruded cereal-based snacks (Renoldi et al., 2021). These results are also in accordance with other previous studies (Cappa et al., 2020; Natabirwa et al., 2020a).

Table 5-1 Ingredient's formulations for extruded snacks and the processing parameters of extrusion cooking.

Sample ID	Rice flour (%)	Cowpea flour (%)	WPC (%)	Screw speed (rpm)	Feed rate (kg/h)	Water rate (kg/h)	Torque (Nm)	Die temperature °C	Power KW
RF	100	-	-	253	7.99	91	78	115	2
$R_{90}C_{10}$	90	10	-	253	7.99	91	80	115	2.01
$R_{80}C_{15}W_{05}$	80	15	05	252	7.99	90	72	111	1.74
$R_{70}C_{20}W_{10}$	70	20	10	251	7.98	90	68	111	1.82
$R_{60}C_{25}W_{15}$	60	25	15	252	7.99	91	72	109	2.1
$R_{50}C_{30}W_{20}$	50	30	20	252	7.97	91	77	108	2.14

Abbreviations: RF – rice flour, CPF- cowpea flour, WPC- whey protein concentrate

Sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Table 5-2 Nutritional properties of the extruded ready-to-eat snacks

	Dry Matter g/100 g	Protein g/100 g (Dry basis)	Soluble Starch g/100 g	Total Starch g/100 g	Soluble Dietary Fibre g/100 g	Total Dietary Fibre g/100 g
RF	93.4±1.20^{a}	7.56 ± 0.00^{f}	81.10 ± 1.11^{a}	82.07 ± 1.09^{a}	0.09 ± 0.00^{c}	0.11±0.00^{d}
$R_{90}C_{10}$	92.10±0.45^{a}	9.94 ± 0.00^{e}	77.66 ± 1.66^{ab}	79.09 ± 1.64^{bc}	0.45 ± 0.07^{bc}	1.17±0.24^{c}
$R_{80}C_{15}W_{05}$	91.48±1.36^{a}	14.63 ± 0.09^{d}	72.91 ± 0.07^{b}	75.24 ± 0.09^{b}	0.67 ± 0.07^{ab}	1.64±0.04^{bc}
$R_{70}C_{20}W_{10}$	92.44±0.42^{a}	19.06 ± 0.09^{c}	64.93 ± 0.35^{c}	67.46 ± 0.25^{c}	0.90 ± 0.25^{ab}	1.99±0.28^{ab}
$R_{60}C_{25}W_{15}$	91.54±1.69^{a}	20.81 ± 0.00^{b}	57.68 ± 3.57^{d}	60.78 ± 3.54^{d}	0.97 ± 0.18^{ab}	2.27±0.15^{ab}
$R_{50}C_{30}W_{20}$	92.00±0.51^{a}	27.22 ± 0.04^{a}	50.58 ± 3.16^{e}	54.90 ± 3.40^{d}	1.07 ± 0.09^{a}	2.59±0.06^{a}

Values = Mean ± standard deviation (n=3). Values within a vertical column followed by the same letter are not significantly different from each other (*p<0.05*) . Abbreviations: RF – rice flour , CPF- cowpea flour, WPC- whey protein concentrate. Sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Table 5-3 Properties of extruded ready-to-eat snacks

Sample ID	Colour Properties					Expansion Ratio	Bulk Density (g/ml^3)	WAI ($g.g\ gel^{-1}$)	WSI (%)	Hardness (N)	Crispiness (N /mm)
	L*	a*	b*	ΔE	BI						
RF	75.92 ± 0.93^{a}	-0.45 ± 0.02^{e}	12.24 ± 0.21^{d}	-	-	4.12 ± 0.16^{a}	13.48 ± 0.99^{d}	5.13 ± 0.16^{a}	24.39 ± 0.62^{b}	223.35 ± 10.67^{c}	45.4 ± 6.23^{a}
$R_{90}C_{10}$	68.30 ± 0.98^{b}	0.32 ± 0.05^{d}	12.77 ± 0.46^{d}	7.69	20.56	4.07 ± 0.18^{a}	14.07 ± 0.56^{d}	4.86 ± 0.08^{ab}	23.06 ± 0.41^{bc}	243.32 ± 12.88^{c}	39.8 ± 4.32^{a}
$R_{80}C_{15}W_{05}$	68.07 ± 0.65^{b}	2.26 ± 0.08^{c}	18.90 ± 0.79^{c}	10.65	34.29	3.62 ± 0.15^{b}	16.54 ± 0.24^{c}	4.82 ± 0.06^{ab}	21.60 ± 2.24^{bc}	308.87 ± 32.30^{b}	37.8 ± 2.17^{a}
$R_{70}C_{20}W_{10}$	67.14 ± 0.72^{bc}	3.06 ± 0.13^{a}	21.62 ± 0.06^{a}	13.32	41.38	4.09 ± 0.66^{a}	19.33 ± 0.59^{b}	4.76 ± 0.14^{b}	20.79 ± 0.26^{c}	326.23 ± 22.61^{b}	40.4 ± 1.14^{a}
$R_{60}C_{25}W_{15}$	65.93 ± 0.97^{c}	2.34 ± 0.09^{c}	20.07 ± 0.14^{b}	13.00	38.13	3.94 ± 0.19^{a}	20.76 ± 0.87^{ab}	4.66 ± 0.10^{b}	24.38 ± 1.20^{b}	339.11 ± 20.18^{b}	38.8 ± 1.30^{a}
$R_{50}C_{30}W_{20}$	65.75 ± 0.33^{c}	2.69 ± 0.12^{b}	21.83 ± 0.47^{a}	14.33	42.47	3.66 ± 0.14^{b}	21.18 ± 1.03^{a}	4.12 ± 0.13^{c}	29.66 ± 1.21^{a}	446.11 ± 15.46^{a}	23.8 ± 6.80^{b}

Values = Mean ± standard deviation (n=3). Values within a vertical column followed by the same letter are not significantly different from each other. (*p<0.05*). Abbreviations: RF – rice flour, CPF- cowpea flour, WPC- whey protein concentrate, WAI=Water absorption index, WSI = Water solubility index, L - lightness, a* - redness, b*- yellowness, ΔE – colour change, BI – Browning index; sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

The colour of products is an important quality attribute, which contributes to consumer acceptability. The colour, which was measured by L*, a*, and b*values, are summarised in Table 5.2. L*values represent the lightness of the extrudate by a level of 0–100. The redness of the extrudate is indicated by the a* values. The b* values measure the yellowness of the extrudate. Regarding the instrumental colour measurements, the addition of cowpea and WPC led to an increase in a* values (redness) and a reduction in L*values (lightness), indicating a significant (*P<0.05*) darkening of the extrudate samples compared to the control after processing. In this study, the screw speed and temperature conditions remained constant (Table 5.2), so the proportion of cowpea and WPC present in the extrudates was an important factor in the colour of the samples. The colour was influenced by varying the levels of cowpea and WPC, which was mainly attributed to the reactions between the residues of sugar by the degradation of starch and the amino acids in WPC. Reducing sugars and amino acids react and promote nonenzymatic browning, also known as Maillard reaction, which darkens the final product. The decreased *L values could be due to the increased fibre content in formulations enhancing the moisture retention and reducing expansion and porosity in the extrudates (Lu et al., 2020). This finding is confirmed by the significant (*p<0.05*) negative correlation (Table 5.4) between the lightness and fibre values observed in the study.

The total colour change (ΔE) ranged from 7.69 to 14.33 for extruded formulations with 10-30% cowpea and 5-20% WPC compared to the control (extruded rice). Browning is the development of a brown colour due to the non-enzymatic reactions through Maillard reactions or caramelisation. The browning index was calculated based on the colorimeter results and was in the range of 20.56 - 42.47, showing that an increase in cowpea and WPC content resulted in an increase in the browning index. The results are similar to those

reported by Wani & Kumar (2016a) and Yu et al. (2017) with high protein blends of pea flour and WPC in extruded snacks.

Figure 5-1 Coloured images of developed ready-to-eat extruded snacks

Rice flour (RF), cowpea flour (CPF) and whey protein concentrate (WPC); A: 100% RF, B: 90% RF + 10%CPF, C: 80% RF + 15% CPF + 05% WPC, D: 70% RF + 20% CPF + 10% WPC, E: 60% RF + 25% CPF + 15% WPC and F: 50% RF + 30% CPF + 20% WPC

A high value for expansion index is a key physical indicator of quality for extruded snacks. The addition of 15% and 30% of cowpea and 5% and 20% WPC led to a significant reduction in the expansion index compared to the control. The expansion index decreased with increasing cowpea and WPC fortification. With the increasing fortification, the starch content decreased, and the fibre content increased. At high levels of fortification, the starch matrix cannot maintain its integrity and thus collapses, resulting in a lower expansion in extrudates (Wang et al., 2019). Dietary fibres bind water more strongly than the starch granules resulting reduced the moisture loss at the die (Bisharat et al., 2013; Lu et al., 2020). The

increasing WPC content contributes more proteins to the matrix. Hence this affects expansion due to its molecular structure and conformation of protein has effect on the distribution of water in the matrix, as the stretching properties during extrusion cooking are affected (Moraru & Kokini, 2003).

The bulk density of the extrudates significantly increased with the incorporation of cowpea and WPC at higher levels (20–30%). The bulk density value of the extrudates ranged from 13.48 – 21.18 g/mL^3. The addition of 15% cowpea flour and 5 % WPC or more significantly reduced (*p<0.05*) the bulk density compared to the control. The bulk density of extrudates has a significant positive correlation with the protein content (r = 0.966), *p<0.01*) and fibre content (r=0.932, *p<0.01*) and a negative correlation with starch content (r=-0.972, *p<0.01)*. Bulk density is inversely related to expansion ratio because the decrease in expansion leads to an increase in bulk density and *vice versa*. Previous studies by other researchers also found that that the density increased with the supplementation of materials containing high fibre and protein to the starch-based extruded products (Bisharat et al., 2013; Stojceska et al., 2008; Wang & Ryu, 2013). Dietary fibre applies a concentration-dependent effect on extrudate expansion regardless of the nature of the source. The presence of excess fibre may rupture the bubble cell walls and prevents the expansion of air bubbles to their maximum level, which is reflected in bulk density (Arivalagan et al., 2018)

Water plays a key role including starch gelatinisation, degradation, and melting during extrusion processing (Wang et al., 2019). The WSI is related to the level of molecular degradation of the material and can be used as an indicator for shear-induced starch fragmentation (Arivalagan et al., 2018). In general, extrusion processing promotes the mechanical breakdown of starch granules and polymers which increases WSI (Singha et al., 2018). The WAI measures the capacity to absorb water or as an index of the degree of starch gelatinization, and WSI is an indicator of the amount of degradation of soluble starch

components. Extrudate interaction with water or water-based liquids can be measured using WAI and WSI. Extrudates with higher values of WSI could be used in ready-to-eat snacks for small children as they dissolve easily, and extrudates with low values of WAI are suitable for use as cereals or as ingredients for energy bars because they are not prone to absorb the water and remain crispy for an extended period of time (Kowalski et al., 2015). The WAI and WSI of extrudates were in the range of 5.13 - 4.12 g/g.gel and 24.39 - 29.66%, respectively, WAI and WSI showed an inverse relationship. The WAI was not significantly affected with 10-25% cowpea flour and 5-15% WPC fortifications. This could be due to the proportion of starch damage as well as the interactions with proteins and other non-starch components in the matrix. Table 5.4 illustrates the negative correlation between WAI and the protein content (r = -0.928, $p<0.01$), fibre content (r= -0.859, $p<0.05$); positive correlation with starch content (r=0.904, $p<0.05$). The changes in WSI did not show a consistent pattern in relation to increasing proportions of cowpea flour and WPC in the formulations. Initially WSI values were reduced from 24.39 to 20.79% in 20% cowpea flour + 10% WPC and after that slight increase was observed up to 29.66% with 30% cowpea flour and 20% WPC fortifications. The initial decrease could be attributed to the decreased starch content, increasing levels of fibre due to cowpea flour fortification and the increase in WPC, which lowers the WSI. The variations in WSI values may be attributed to protein/flour interactions during extrusion. These variations have previously been reported in extrusion processing of legume and/or WPC fortified rice-based snacks (Altaf et al., 2021; Onwulata et al., 2001; Sharma et al., 2017c).

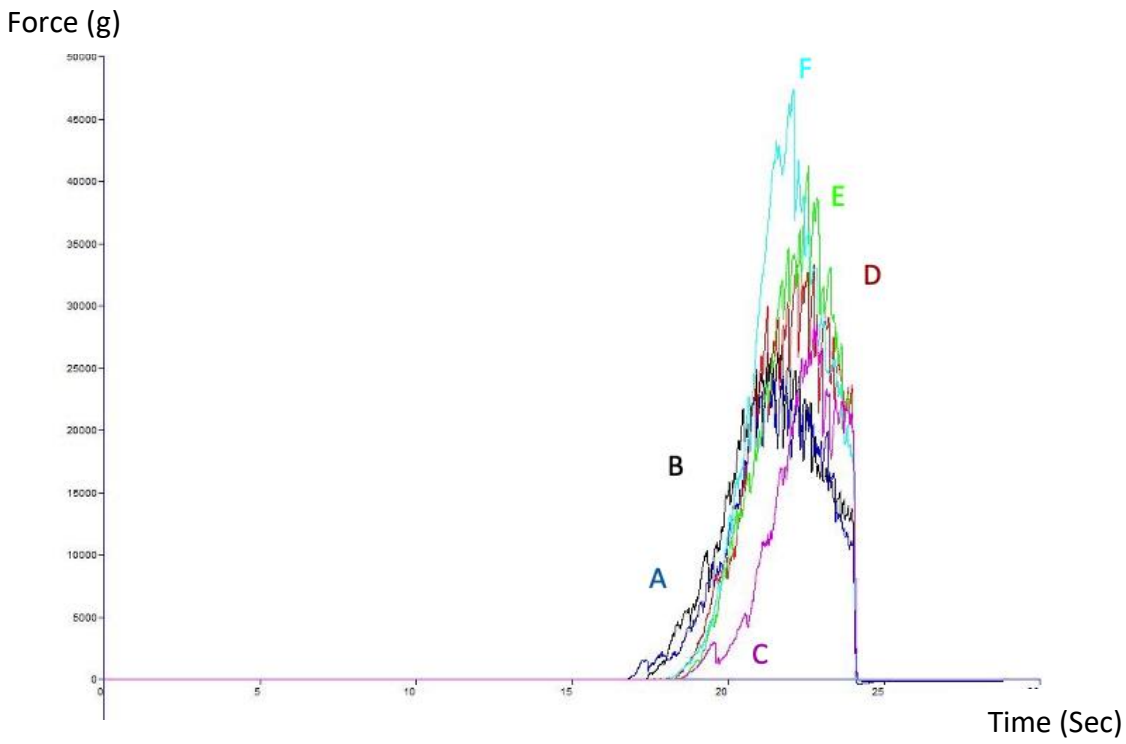

Figure 5-2 Typical compression curve of extruded snacks

Rice flour (RF), cowpea flour (CPF) and whey protein concentrate (WPC); A: 100% RF, B: 90% RF + 10%CPF, C: 80% RF + 15% CPF + 05% WPC, D: 70% RF + 20% CPF + 10% WPC, E: 60% RF + 25% CPF + 15% WPC and F: 50% RF + 30% CPF + 20% WPC

The hardness of the extruded product is an important index and is used to measure the textural property of expansion using peak force. Hardness is a result of the interactions between the components in the matrix and it is affected by the microstructure of the expanded products, such as the bubble size, the distribution of bubbles, and the bubble cell wall (Yu, Liu, Tang, & Liu, 2017). Table 5.3 and Figure 5.2 show the results obtained for the mechanical texture of the extrudates. The overall trend of hardness was that the peak force value increased with the incorporation of cowpea from 10% to 30% and WPC from 5% to 20%. There were no significant differences observed in the addition of cowpea and WPC in the range of 15-25% and 5-15%, respectively. These observations are in accordance with previous findings, stating that high protein extrudates are harder compared to samples with

lower protein contents. Extrudates rich in native proteins (such as beans, peas, lentils) resulted in harder extrudates, due to the protein unfolding and cross-linking with other molecules, such as starch and lipids. This protein aggregation is caused by the heat and shear of extrusion processing. Table 5.4 demonstrates the positive correlation between extrudates hardness with protein content (r = 0.980, $p<0.01$) and fibre content (r =0.897, $p<0.05$) and negative correlation with starch content (r=-0.945, $p<0.01$). Hood-Niefer & Tyler, 2010 stated that the observation of extrudates hardness with increased pea flour content.

Crispness measures the presence of fragile porous structures through the number of force peaks recorded during the compression test (Dogan & Kokini, 2007). The attribute of crispiness in extrudates results in adjoining fractures, which can be seen in a typical compression curve (Figure 5.2). A high crispness and low hardness in extruded snacks is an indication of high-quality that denotes consumer acceptance. According to Figure 5.2, a linear increase of the applied external force continued until the first fracture occurs. This fracture stops and the applied force drops due to the air-filled bubbles. An increase in force is required for the next fracture to further compress the samples (Dogan & Kokini, 2007).. The crispiness and hardness of 30% cowpea flour and 20% WPC were significantly different $p<0.05$ to the other extrudates. No significant differences ($P=0.05$) in crispness were observed between products containing cowpea 10-25% and WPC 5-15% enriched samples compared to control. Changes that may be due to fortification with fibre are reduced elasticity and weakening of bubble cell structure when starch-fibre interactions take place, leading to harder structures (Renoldi et al., 2021).

Table 5-4 Pearson's correlation coefficient of physicochemical and nutritional properties of extruded ready-to-eat snacks

	DM	PC	SS	TS	SDF	TDF	L*	a*	b*	ER	BD	WAI	WSI	H
PC	-0.508													
SS	0.492	-0.988**												
TS	0.483	-0.985**	1.000**											
SDF	-0.682	0.947**	-0.928**	-0.926**										
TDF	-0.734	0.938**	-0.919**	-0.915	0.994**									
L*	0.837*	-0.785	0.765	0.761	-0.922**	-0.947**								
a*	-0.626	0.850*	-0.791	-0.786	0.933**	0.909*	-0.826*							
b*	-0.546	0.909*	-0.861*	-0.857*	0.937**	0.906*	-0.770	0.983**						
ER	0.659	-0.549	0.481	0.461	-0.502	-0.554	0.478	-0.516	-0.528					
BD	-0.525	0.966**	-0.971**	-0.972**	0.958**	0.932**	-0.785	0.884*	0.934**	-0.429				
WAI	0.487	-0.928**	0.912*	0.904*	-0.834*	-0.859*	0.745	-0.670	-0.728	0.627	-0.809			
WSI	0.012	0.535	-0.581	-0.578	0.279	0.310	-0.136	0.027	0.167	-0.386	0.383	-0.714		
H	-0.493	0.980**	-0.953**	-0.945**	0.894*	0.897*	-0.735	0.804	0.869	-0.665	0.903*	-0.961**	0.611	
C	0.453	-0.828*	0.794	0.782	-0.707*	-0.749	0.643	-0.556	-0.613	0.728	-0.661	0.969**	-0.753	-0.907*

DM, Dry matter; PC, Protein content; SS, Soluble starch; TS, Total starch; SDF, Soluble dietary fibre; TDF, Total dietary fibre; L*, Lightness; a*, Redness; b*,Yellowness; ER, Expansion ratio; BD, Bulk density; WAI, Water absorption index; WSI, Water-soluble index; H, hardness; C, crunciness. * Significant at *p<0.05*. **Significant at *p<0.01.*

The Maillard reaction is a chemical reaction involving amino groups and carbonyl groups, and results in browning and flavour production. It also leads to a reduction in the availability of amino acids. Lysine is the limiting amino acid in cereals and appeared to be the most reactive amino acid with two available amino acids (Singh et al., 2007). Inactivation of anti-nutritional factors during extrusion improved enzyme hydrolysis of the protein. Therefore, further studying of the composition of amino acids profile after extrusion and during the *in vitro* digestion process would be worthwhile. The albumin (water-soluble), globulin (salt-soluble), prolamin (alcohol-soluble) and glutelin (alkaline-soluble) fractions are the main storage proteins present in plant-based sources (Osborne et al., 1924). Teka et al. (2020) found that the globulins and albumins are the main seed proteins of cowpea seed; Leucine was the most abundant followed by lysine, valine, tryptophan and histidine, whereas methionine was the least abundant essential amino acid; glutamic acid and asparagine were the most abundant non-essential amino acids. Cowpea cultivars are frequently reported to be deficient in sulphur-containing amino acids such as methionine and cysteine (Baptista et al., 2017). WPC contained 33.3% protein, which is composed of essential amino acids, glutamic acid was the most abundant followed by leucine, lysine, threonine, isoleucine and valine and aspartic acid, proline and alanine as the non-essential amino acids (Barone et al., 2020. A combination of cowpea and WPC in rice-based extruded snacks is rich in proteins. A 100 g serving of the 30% cowpea and 20% WPC extruded product will supply 79% of the recommended daily allowance (RDA) of protein (RDA of 34 g/day) for children up to 12 years old. Table 5.5a and 5.5b show the changes in amino acids composition during the *in vitro* protein digestion process. The content of essential and non-essential amino acids increases after 3 h of digestion. Valine was the most abundant essential amino acid amount followed

Table 5-5 Essential amino acid composition (μM/mL) of extruded snacks during protein digestion at 0, 1 hr and 3 hr

		Phenylalanine	Methionine	Isoleucine	Lysine	Leucine	Histidine	Tryptophan	Valine	Threonine	Taurine
RF	0	5.16 ± 0.0	-	4.29 ± 0.0	6.72 ± 0.0	4.61 ± 0.1	-	1.19 ± 0.0	6.50 ± 0.0	10.6 ± 0.0	-
	1 hr	5.55 ± 0.0	-	4.53 ± 0.0	6.92 ± 0.5	5.01 ± 0.0	13.59 ± 0.3	1.36 ± 0.0	7.31 ± 0.0	11.63 ± 0.6	-
	2 hr	806.77 ± 40.1	450.20 ± 17.2	619.99 ± 32.1	932.52 ± 51.2	1071.59 ± 51.0	428.14 ± 8.2	468.70 ± 24.5	1190.98 ± 61.1	705.39 ± 22.8	5.28 ± 0.0
$R_{90}C_{10}$	0	6.30 ± 0.0	-	4.51 ± 0.0	6.98± 0.0	4.88 ± 0.3	16.28 ± 0.1	2.35 ± 0.0	7.96 ± 0.1	11.98 ± 0.0	-
	1 hr	7.07 ± 0.4	5.88 ± 0.0	4.74 ± 0.0	7.33 ± 0.0	5.52 ± 0.0	17.85 ± 0.0	2.77 ± 0.0	8.17 ± 0.0	12.71 ± 0.0	-
	2 hr	1058.06 ± 22.7	540.42 ± 11.3	806.33 ± 17.7	1291.48 ± 42.1	1387.96 ± 38.9	496.39 ± 8.1	622.20 ± 16.5	1497.83 ±5 .1	870.75 ± 18.9	6.80 ± 0.0
$R_{80}C_{15}W_{05}$	0	6.15 ± 0.2	5.92 ± 0.0	4.60 ± 0.0	7.13 ± 0.1	5.68 ± 0.0	17.96± 0.1	2.56 ± 0.1	8.02 ± 0.0	12.7 ± 1.01	-
	1 hr	7.38 ± 0.1	5.96 ± 0.03	5.29 ± 0.0	8.05 ± 0.1	5.96 ± 0.0	19.28 ± 0.1	3.00 ± 0.1	8.60 ± 0.2	13.33 ± 0.1	0.02 ± 0.0
	2 hr	1299.23 ± 53.9	675.39 ± 27.1	1101.93 ± 50.2	1758.22 ± 71.0	1868.24 ± 66.6	577.24 ± 9.6	840.31 ± 43.5	1975.91 ± 90.0	1232.22 ± 11.6	7.08 ± 0.0
$R_{70}C_{20}W_{10}$	0	6.66 ± 0.1	5.96 ± 0.0	4.87 ± 0.0	7.24 ± 0.7	5.08 ± 0.4	19.53 ± 0.0	3.0 ± 0.0	8.39 ± 0.1	13.93 ± 0.0	-
	1 hr	7.94 ± 0.1	6.05 ± 0.0	5.80 ± 0.2	10.05 ± 0.2	5.76 ± 0.0	21.21 ± 0.1	4.14 ± 00	9.25 ± 0.0	14.35 ± 0.2	5.85 ± 0.0
	2 hr	1534.18 ± 111.1	800.62 ± 53.34	1380.07 ± 102.4	2185.04 ± 176.4	2266.09 ± 176.4	637.08 ± 39.3	1018.91 ± 63.8	2316.0 ± 146.8	1494.81 ± 45.4	7.6 ± 0.1
$R_{60}C_{25}W_{15}$	0	7.47 ± 0.2	6.01 ± 0.0	5.06 ± 0.0	8.13 ± 0.0	5.38 ± 0.5	21.51 ± 0.0	3.56 ± 0.3	9.01 ± 0.0	15.06 ± 0.0	-
	1 hr	8.79 ± 0.0	6.14 ± 0.0	6.00 ± 0.0	10.89 ± 0.1	5.60 ± 0.1	24.13 ± 0.1	5.35 ± 0.1	9.32 ± 0.0	15.94 ± 0.0	7.47 ± 0.0
	2 hr	1572.45 ± 97.1	791.67 ± 41.0	1384.25 ± 84.3	2205.59 ± 139.8	2315.59 ± 151.4	637.16 ± 23.2	1023.25 ± 77.5	2413.62 ± 154.8	1431.65 ± 80.8	8.81 ± 0.0
$R_{50}C_{30}W_{20}$	0	7.07 ± 0.6	5.88 ± 0.0	5.12 ± 0.0	7.72 ± 1.1	5.69 ± 0.2	19.38 ± 0.4	3.14 ± 0.3	7.79 ± 0.5	14.61 ± 0.3	-
	1 hr	8.15 ± 0.1	6.11 ± 0.0	5.79 ± 0.6	9.21 ± 3.0	6.22 ± 3.1	22.73 ± 3.1	4.57 ± 0.2	8.24 ± 0.0	16.92 ± 3.0	6.96 ± 0.1
	2 hr	1586.59 ± 148.3	860.34 ± 88.0	1541.81 ± 138.2	2684.69 ± 98.9	2598.34 ± 103.03	710.06 ± 38.04	1178.61 ± 42.50	2497.54 ± 6.27	1833.92 ±184.9	8.83 ± 1.83

Results in the table represent the mean ± standard deviation. CPF-Cowpea flour, WPC-Whey protein concentrate sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Table 5-6 Non-essential amino acid composition (µM/mL) of extruded snacks during protein digestion at 0, 1 hr and 3 hr

		Arginine	Alanine	Glutamic acid	Glycine	Proline	Serine	Aspartic acid	Cysteine	Asparagine	Glutamine	Tyrosine
RF	0	12.24 ± 0.0	14.71 ± 0.1	17.92 ± 0.3	8.42 ± 0.0	13.41 ± 0.6	14.77 ± 0.0	2.72 ± 0.1	-	48.20 ± 1.0	2.89 ± 0.0	5.24 ± 0.0
	1 hr	13.34 ± 0.4	17.11 ± 0.7	19.52 ± 0.6	10.53 ± 1.1	13.52 ± 0.2	15.28 ± 0.3	2.84 ± 0.1	-	60.62 ± 3.8	4.20 ± 0.1	6.05 ± 0.0
	2 hr	1818.03 ± 101.7	1464.31 ± 82.5	866.31 ± 55.4	466.59 ± 17.9	383.75 ± 18.6	662.02 ± 20.7	311.16 ± 34.7	566.48 ± 14.5	1841.24 ± 93.1	1018.42 ± 51.3	1395.93 ± 69.8
$R_{90}C_{10}$	0	34.18 ± 0.2	20.25 ± 0.2	24.62 ± 0.4	11.76 ± 0.0	15.25 ± 1.5	30.60 ± 0.0	9.73 ± 0.8	22.81 ± 0.1	101.99 ± 0.8	2.91 ± 0.0	6.57± 0.0
	1 hr	38.05 ± 0.0	23.45 ± 0.1	28.18 ± 1.6	13.90 ± 0.1	16.40 ± 0.0	35.43 ± 0.1	10.99 ± 0.2	26.35 ± 0.0	125.38 ± 2.4	3.10 ± 0.0	6.90 ± 0.0
	2 hr	2332.00 ± 58.5	1812.38 ± 43.9	1137.09 ± 37.4	560.09 ± 12.5	414.25 ± 3.7	785.17 ± 17.9	394.77 ±14.6	699.65 ± 20.3	2346.04 ± 54.7	1233.05 ± 29.7	1756.16 ± 44.4
$R_{80}C_{15}W_{05}$	0	42.79 ± 0.3	22.73 ± 0.2	31.68 ± 0.2	12.58 ± 0.0	15.93 ± 0.7	37.85 ± 0.1	12.72 ± 0.3	28.42 ± 0.5	120.19 ± 0.9	2.97 ± 0.0	7.16 ± 0.0
	1 hr	45.90 ± 0.8	25.71 ± 0.2	33.84 ± 0.0	14.34 ± 0.2	16.71 ± 0.1	44.28 ± 0.5	14.80 ± 0.4	31.13 ± 0.1	141.29 ± 2.7	3.05 ± 0.0	7.18 ± 0.0
	2 hr	2809.90 ± 138.8	2315.36 ± 70.8	1545.61 ± 82.3	696.18 ± 8.7	496.32 ± 1.3	1061.30 ± 42.8	550.75 ± 31.6	954.75 ±56.7	2889.63 ± 161.8	1523.73 ± 86.3	2226.72 ± 102.7
$R_{70}C_{20}W_{10}$	0	58.94 ± 0.4	27.13 ± 0.2	42.61 ± 0.2	13.87 ± 0.0	17.40 ± 0.2	62.98 ± 0.1	15.83 ± 0.3	46.99 ± 0.0	166.03 ± 0.3	3.16 ± 0.0	7.10 ± 0.0
	1 hr	64.09 ± 0.9	31.12 ± 0.3	45.36 ± 0.4	15.87 ± 0.4	17.96 ± 0.0	78.85 ± 1.3	21.04 ± 1.6	54.83 ± 0.5	204.91 ± 3.6	3.23 ± 0.0	7.75 ± 0.0
	2 hr	3258.72 ± 123.2	2600.52 ± 66.34	1865.94 ± 157.5	753.51 ± 56.8	531.47 ± 22.8	1174.62 ± 115.5	673.19 ± 74.3	1196.74 ± 31.0	3265.10 ± 95.8	1809.65 ± 153.0	2580.69 ± 133.8
$R_{60}C_{25}W_{15}$	0	80.95 ± 0.4	31.23 ± 0.2	54.51 ± 0.2	15.64 ± 0.0	18.62 ± 1.3	95.10 ± 0.4	18.51 ± 0.4	69.72 ± 1.1	223.68 ± 0.7	3.42 ± 0.0	8.12 ± 0.0
	1 hr	85.89 ± 0.0	36.60 ± 0.2	60.95 ± 2.8	17.64 ± 0.3	19.16 ± 1.3	121.47 ± 1.8	28.5 ± 0.3	82.67 ± 0.1	277.15 ± 4.2	3.51 ± 0.0	8.07 ± 0.0
	2 hr	3205.95 ± 66.8	2815.18 ± 44.0	1909.93 ± 123.4	737.31 ± 36.7	523.34 ± 31.9	1160.90 ± 65.9	679.65 ± 54.8	1151.69 ± 61.2	3573.17 ± 135.2	1796.33 ± 101.9	2648.06 ± 166.3
$R_{50}C_{30}W_{20}$	0	69.25 ± 2.2	28.75 ± 0.6	51.99 ± 0.7	14.82 ± 0.6	17.41 ± 0.9	81.86 ± 2.5	14.47 ± 0.9	58.66 ± 3.1	193.58 ± 10.9	3.34± 0.1	7.50 ± 0.4
	1 hr	73.85 ± 9.3	33.05 ± 3.2	65.68 ± 20.7	18.07 ± 4.6	17.78 ± 0.0	108.80 ± 18.7	23.11 ± 0.3	77.35 ± 15.2	287.47 ± 6.4	3.43 ± 0.1	7.39 ± 0.1
	2 hr	3307.78 ± 150.7	3038.79 ± 172.0	2046.68 ±139.0	896.16 ± 97.0	533.96 ± 8.5	1212.46 ± 58.7	936.17 ± 68.4	1189.17 ± 36.7	3863.47 ± 107.0	1835.49 ± 111.0	2872.03 ± 158.5

Results in the table represent the mean ± standard deviation. CPF-Cowpea flour, WPC-Whey protein concentrate; sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

by leucine, lysine, phenylalanine, isoleucine, tryptophan, and histidine up to 25% cowpea and 15% WPC fortifications. Asparagine was the most abundant non-essential amino acid followed by arginine, alanine, tyrosine, glutamine or glutamic acid, serine, cysteine, glycine, aspartic acid, and proline. The total essential amino acid content was found to be 15.5 ± 0.84 mmol /mL and the total non-essential amino acid content was found to be 21.73 ± 1.09 mmol /mL in digested extracts after 3 h in 30% cowpea and 20% WPC fortification. It has been reported that the extrusion process reduces the essential amino acids profile in extruded snacks made from common beans and maize (Félix-Medina et al., 2021b; Simons et al., 2015). The results of this study found that fortification of extruded rice snacks with cowpea and WPC improved the amino acid profiles in both essential and non-essential amino acids.

5.4 Conclusion

Blending cowpea flour and WPC with rice to produce ready-to-eat extruded snacks resulted in a product that was higher in fibre and protein and with a better amino acid profile than rice only extruded snacks. The cowpea and WPC fortified snacks had low levels of hardness and were crispy and have the potential to replace traditional snack foods which are low in nutrients. The protein, starch, and fibre contents were all significantly correlated to crispiness and hardness. Extrusion processing has many advantages over conventional food processing methods such as flexibility, reduced cost benefits, high production rate, and quality products. These new and novel extruded products could increase legume and protein consumption.

Chapter 6

Effects of Extrusion Processing on the Bioactive Constituents, *in Vitro* Digestibility, Amino Acid Composition, and Antioxidant Potential of Novel Gluten-free Extruded Snacks Fortified with Cowpea and Whey Protein Concentrate

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Abstract

The bioactive compounds (sucrose-galactosyl oligosaccharides, insoluble dietary fibre, resistant starch, phytic acid, trypsin inhibitory activity), protein digestibility, amino acid composition, total phenolic content and antioxidant properties of different rice-cowpea-WPC blends were evaluated in non-extruded and extruded formulations. Rice flour (100%) was used as a control. Extrusion increased the oligosaccharides (2-3-fold) and resistant starch (1-3 fold), whereas the insoluble fibre content was not significantly affected. Extrusion increased protein digestibility ($p<0.05$) and amino acid composition in snacks. Extruded and raw samples enriched with cowpea and WPC had an increase in total phenolic content and antioxidant activity. Extrusion significantly reduced the TPC, and antioxidant properties of extruded snacks compared to their raw counterparts. The results obtained in this study facilitate the growing interest of the food industry to cater to consumer demand for healthy novel gluten-free expanded snacks with bioactive compounds.

Keywords: Ready-to-eat snacks, protein, fibre, bioactive phytochemicals, glycaemic response

6.1 Introduction

The principle of extrusion cooking is that raw materials are fed into the extruder barrel and the screws convey the food along with it. The use of high temperature, increased pressure and shearing causes changes in structural, nutritional, and functional properties such as the gelatinisation of starches, changes in dietary fibre content and lipid oxidation, Maillard browning, inactivation of anti-nutritional compounds, changes in bioactive compounds, changes in antioxidant properties, and the improvement of sensory attributes (Arribas, Pereira, et al., 2019a).

The extrusion process modified starch, protein, fibre, and other components in the food matrix. Starch digestibility is affected by extrusion conditions and so too is the composition of the formulation, for instance research has shown that starch digestibility increases with severe treatment at low moisture level or decreases with the formation of starch-protein complex, starch-lipid complex, or presence of resistant starch (Cappa et al., 2020; Oñate Narciso & Brennan, 2018; Renoldi et al., 2021). Protein digestibility was shown to increase after extrusion due to the denaturation of proteins, inactivation of anti-nutritional factors such as trypsin inhibitors, phytic acid and tannins (Yağcı et al., 2020b; B. Zhang et al., 2017). Bioactive compounds such as α-galactosides (raffinose, stachyose, verbascose and ajugose), phytic acid, trypsin inhibitors and lectins have been traditionally considered as anti-nutritional factors that are affected during the extrusion process (Arribas et al., 2019c; Ciudad-Mulero et al., 2020). Polyphenols have been reported to have high sensitivity to thermal stress as many other nutrients such as vitamins, minerals. Recent studies documented that polyphenol content and antioxidant activities of extrudates reduced after extrusion processing (Félix-Medina et al., 2021a; Shevkani et al., 2019a).

This chapter reports on the effects of extrusion on bioactive compounds (indigestible fibre, α-galactosides, resistant starch, trypsin inhibitory activity and phytic acid), starch and protein digestibility, amino acids, total polyphenols and antioxidant activities of gluten-free rice-based formulations with cowpea and WPC. Raw mixtures of the formulations were compared with the extruded snacks. Commercial white rice flour and extruded white rice were used as controls.

6.2 Materials and Methods

6.2.1 Raw materials

Raw materials were combined as mentioned in 3.1.1.

6.2.2 Extrusion processing

Extrusion processing was performed as described in 3.1.2

6.2.3 Raffinose-serious oligosaccharides

Raffinose-serious oligosaccharides were determined as mentioned in 3.2.13

6.2.4 Trypsin inhibitory activity

Trypsin inhibitory activity was measured as mentioned in 3.2.15

6.2.5 Phytic acid content

Phytic acid content was determined as described in 3.2.14

6.2.6 Resistant starch

Resistant starch content was determined as described in 3.2.11

6.2.7 Insoluble dietary fibre

Insoluble fibre content was determined as mentioned in 3.2.12

6.2.8 *In vitro* starch digestibility

In vitro starch digestibility was meseaured as mentioned in 3.2.4

6.2.9 *In vitro* protein digestibility

In vitro protein digestibility was meseaured as described in 3.2.5

6.2.10 Amino acid determination

Amino acid profile was determined as mentioned in 3.2.6

6.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was determined as described in 3.2.16

6.2.12 Total phenolic contents and antioxidant activities

Total phenoli contents and antioxidant activities were determined as described in 3.2.9

6.3 Results and Discussion

6.3.1 Bioactive compounds

From a physiological point of view, the most important group of carbohydrates in legumes are the raffinose family oligosaccharides (RFOs) which are indigestible α-galactosides of sucrose. They include raffinose, stachyose and verbascose, which have prebiotic potential and confer health benefits and are also known as flatulence factors because the human mucosa of the lower intestinal tract lacks the α-1,6- galactosidase hydrolytic enzyme. These oligosaccharides are fermented by the gut microflora and produce gases such as carbon dioxide, hydrogen, and methane (Rackis & Regional, 1974).

The cowpea used in this study had 7.92±1.7 mmol/100g of RFOs (Table 6.1) which was determined as a group of oligosaccharides (Data not shown). Sreerama et al., (2012b) studied the content of raffinose, stachyose and verbascose in cowpea flour and reported values of 10.3, 17.8 and 3.6 mg/g, respectively. The addition of 10 - 30% (w/w) cowpea significantly increased the RFO content (1.0 - 2.3 mmol/100g) in the raw formulations ($p<0.05$). After extrusion, the RFO content significantly ($p<0.05$) increased two to three folds in all the formulations compared to their non-extruded counterparts. This increase could be

associated with improved extraction of RSOs from the extrudate matrix due to the disruption of the cell wall during the extrusion cooking or could be due to the extrusion process itself, altering the structure of other carbohydrates. These prebiotic galacto-oligosaccharides facilitate the growth of intestinal bifidobacteria, conferring health benefits to the human gut (Arribas et al., 2017).

As the cowpea flour proportion increased in the formulations, the trypsin inhibitory units (TIU) content increased significantly ($p<0.05$) to reach 23.83 TIU/mg (at 30% cowpea) in the raw formulations (Table 6.1). Cowpea flour contained 32.51 $\pm$ 0.45 TIU/mg of trypsin inhibitory units, as the main source of TIU in raw formulations. The control extruded samples had the lowest TIU values of all formulations. The same trend was observed in the non-extruded formulations as occurred in the extruded formulations - increasing cowpea flour increased the TIUs. The effect of extrusion on trypsin inhibitors was determined by comparing the extrudate with the respective raw formulation. There was a significant reduction ($p<0.05$) in TIUs from non-extruded formulations to extruded formulations (ranging from 57% to 81% of reduction). The highest reduction of trypsin inhibitors (16.43 TIU/mg) was observed in the samples containing 30% cowpea. Extrusion cooking reduced the trypsin inhibitors, which confirmed the thermo-liable nature of protease inhibitors. Furthermore, in most recent years, extrusion cooking has been considered as the effective thermal processing for TIUs inactivation. Yadav et al., (2019) and Batista et al., (2010a) have reported a reduction of TIUs through extrusion cooking of cowpea. The presence of a thermo-liable anti-nutritional factor such as trypsin inhibitors reduces the bioavailability of protein and micro minerals of legumes by forming complexes with useful protein and minerals, However, there is evidence that protease inhibitors of legume seeds are effective at preventing or suppressing carcinogen-induced transformation (Faris et al., 2020).

Table 6-1 Bioactive compounds in raw formulations and extruded snacks fortified with cowpea and WPC

Sample	Galactosyl-sucrose Oligosaccharides millimoles/100 grams		Insoluble dietary fibre (g/100 g)		Resistant Starch (g/100 g)		Trypsin inhibitor activity (TIU/mg)		Phytic acid (mg/100g)	
	Raw	Extruded	Raw	Extruded	Raw	Extruded	Raw	Extruded	Raw	Extruded
RF	$0.01 \pm 0.02^{d,B}$	$0.92 \pm 0.06^{d,A}$	$0.02 \pm 0.03^{d,ns}$	$0.02 \pm 0.01^{e,ns}$	$0.22 \pm 0.03^{c,B}$	$0.97 \pm 0.04^{e,A}$	$1.20 \pm 0.94^{d,A}$	$0.23 \pm 0.07^{e,B}$	$22.21 \pm 3.65^{b,ns}$	$11.34 \pm 1.79^{,ns}$
$R_{90}C_{10}$	$1.05 \pm 0.26^{c,B}$	$3.34 \pm 0.14^{c,A}$	$0.79 \pm 0.13^{c,ns}$	$0.72 \pm 0.17^{d,ns}$	$0.30 \pm 0.08^{c,B}$	$1.43 \pm 0.04^{d,A}$	$10.22 \pm 2.47^{c,A}$	$4.44 \pm 0.18^{d,B}$	$84.67 \pm 8.13^{b,A}$	$13.40 \pm 3.22^{bc,B}$
$R_{80}C_{15}W_{05}$	$0.90 \pm 0.60^{c,B}$	$3.56 \pm 0.14^{c,A}$	$1.02 \pm 0.17^{b,ns}$	$0.97 \pm 0.02^{cd,ns}$	$0.59 \pm 0.06^{c,B}$	$2.33 \pm 0.04^{c,A}$	$13.06 \pm 3.21^{c,A}$	$5.25 \pm 0.18^{c,B}$	$123.53 \pm 6.33^{ab,A}$	$18.04 \pm 3.22^{abc,B}$
$R_{70}C_{20}W_{10}$	$1.50 \pm 0.60^{a,B}$	$4.18 \pm 0.07^{b,A}$	$1.13 \pm 0.13^{bc,ns}$	$1.09 \pm 0.03^{bc,ns}$	$1.27 \pm 0.06^{b,B}$	$2.53 \pm 0.11^{c,A}$	$16.59 \pm 2.87^{bc,A}$	$6.56 \pm 0.19^{b,B}$	$240.13 \pm 11.43^{ab,A}$	$25.78 \pm 2.36^{abc,B}$
$R_{60}C_{25}W_{15}$	$2.15 \pm 0.26^{bc,B}$	$4.54 \pm 0.08^{a,A}$	$1.38 \pm 0.31^{ab,ns}$	$1.30 \pm 0.03^{ab,ns}$	$1.60 \pm 0.12^{ab,B}$	$3.10 \pm 0.13^{b,A}$	$21.77 \pm 2.73^{ab,A}$	$7.04 \pm 0.04^{a,B}$	$280.38 \pm 10.03^{ab,A}$	$26.29 \pm 2.68^{ab,B}$
$R_{50}C_{30}W_{20}$	$2.30 \pm 0.03^{a,B}$	$4.71 \pm 0.24^{a,A}$	$1.65 \pm 0.39^{a,ns}$	$1.52 \pm 0.03^{a,ns}$	$1.79 \pm 0.31^{a,B}$	$4.32 \pm 0.25^{a,A}$	$23.83 \pm 2.70^{a,A}$	$7.40 \pm 0.08^{a,B}$	$365.05 \pm 12.46^{a,A}$	$33.23 \pm 7.39^{a,B}$

Values are mean ± standard deviation (n=3). In each column, the different lowercase letters mean statistically (*P<0.05*) differences in between the treatments, whereas superscript capital letters mean the significant difference between the same raw formulation and extruded snacks, "ns": non-significant. Abbreviations: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

The phytic acid content (Table 6.1) ranged from 22.21 to 365.05 mg/100 g and 11.34 to 33.23 mg/100g in non-extruded and extruded formulations, respectively. The results showed that the content of phytic acid increased significantly ($p<0.05$) with increased cowpea flour in both non-extruded and extruded formulations. Phytic acid content decreased significantly ($p<0.05$) after processing in cowpea fortified samples. This reduction ranged between 84 % and 90 % from the initial concentration of phytic acid in raw formulations. Phytic acid is a polyphosphate compound, which lowers the bioavailability of minerals, and inhibits the digestibility of proteins through its chelating properties. Phytic acid is involved in the ''hard-to-cook'' phenomenon of legumes. However, phytic acid has anti-inflammatory, antioxidant, and anti-cancer properties (Ciro et al., 2020).

In this study, the resistant starch content of the non-extruded formulations ranged from 0.3 to 1.52 g/100g (Table 6.1), compared to 0.22 g/100g in rice flour. When the cowpea concentration in the non-extruded formulations increased from 20 to 30%, there was a significant ($p<0.05$) increase in the resistant starch content. The extrusion process also produced a significant ($p<0.05$) increase in resistant starch (0.75 - 2.8 g/100 g) for all formulations compared to their respective non-extruded flour counterparts. The cowpea (30%) formulation had the highest resistant starch contents of 1.79 g/100 g and 4.32 g/100 g for both the non-extruded and extruded products, respectively. Formulations that included cowpea flour had a significant increase in the resistant starch content for both raw and extruded products compared to the control ($p<0.05$). Legume starch contains 60–70% amylopectin and 30–40% amylose, whereas other starchy food contains 70–75% amylopectin and 25–30% amylose. Themeier et al., (2005) reported that the high amylose starch granules were more resistant to amylase attack than the amylopectin-rich starch granules. The formation of resistant starch in this study, could be due to the use of mild extrusion conditions (<130 °C) with the presence of dietary fibre retarding the starch

gelatinisation and as a result of full gelatinisation may not happen during extrusion and the formation of resistant starch may also occur (Arribas et al., 2017).

When starch is digested into sugars it creates a glycaemic impact and the body produces a glycaemic response to absorb the sugars from the bloodstream (Englyst, H.N., Kingman, S. M., & Cummings, 1992; Sajilata, M. G., Singhal, R. S., & Kulkarni, 1996). Starch quality can be defined by the rate at which it is digested as rapidly digestible - starch digested within 20 min, slowly digestible starch - starch which takes up to 2 hours to digest, and resistant starch – starch that generally remains undigested by the small intestine. When resistant starch reaches the large intestine, it is fermented by the microbiota and produces short-chain fatty acids. Interest in resistant starch is growing due to its potential role as a protective factor in colon health by helping protect against colorectal cancer. The consumption of resistant starch rich food products can reduce the rate of glucose release into the bloodstream, and they can be considered as low glycaemic impact foods (Englyst, H.N., Kingman, S. M., & Cummings, 1992; Sajilata, M. G., Singhal, R. S., & Kulkarni, 1996).

The IDF (Table 6.1) content of the raw formulations varied from 0.02% to 1.65%, and this amount was proportionate to the cowpea fortification; the higher the proportion of cowpea flour, the higher the IDF content. After extrusion, IDF contents were not affected significantly. This could be related to the low-temperature extrusion conditions used in the study. Arribas et al. (2019b) reported that the redistribution of insoluble dietary fibre to soluble dietary fibre depends on the extrusion conditions (140°C - 180°C, 25 – 30 % moisture).

Dietary fibre plays an important role in human health by reducing constipation, decreasing the risk of non-communicable diseases such as cardiovascular diseases, colon cancer, and type 2 diabetes. In addition, dietary fibre acts as a functional food and it is used in weight control diets due to its low caloric value. Therefore, the incorporation of fibre rich legumes

such as cowpea in rice-based extruded snacks improves the nutritional and functional value of the food products.

6.3.2 *in vitro* starch digestibility

The *in vitro* digestion process mimics gastric and intestinal digestion and provides a predictive glycaemic response to the food analysed. Figure 6.1 represents an interpretation of the amount of reducing sugars released over 120 min of *in vitro* digestion between the raw and extruded samples, reflecting the contribution of raw materials.

It was noticeable that there was a decrease of iAUC values observed with increased inclusion levels of cowpea and WPC in both raw and extruded samples. For instance, with 30% CP and 20% WPC in raw formulations and extruded snacks a 51% and 34% reduction of reducing sugar release, respectively, was observed compared to the rice control. Substitution of rice flour with 10-30% cowpea and 5-20% WPC decreased the reducing sugar released by 19-65% and 13-34% in the raw and extruded snacks, respectively. This could be due to the higher amount of protein associated with an increased proportion of cowpea and WPC possibly creating a stronger protein network, hence reducing the starch availability to enzyme attack; dietary fibre fractions from legume also contributed to this reduction through slow and/or uneven hydration of polysaccharide matrix, which delays/hinders encapsulation of the protein-starch matrix until the later stage of digestion (Foschia et al., 2015). Occurrence/formation of resistant starch in a starchy material with the presence of moisture can act as a plasticiser which facilitates the retrogradation could also contribute to lower the glycaemic properties (Beigh et al., 2020).

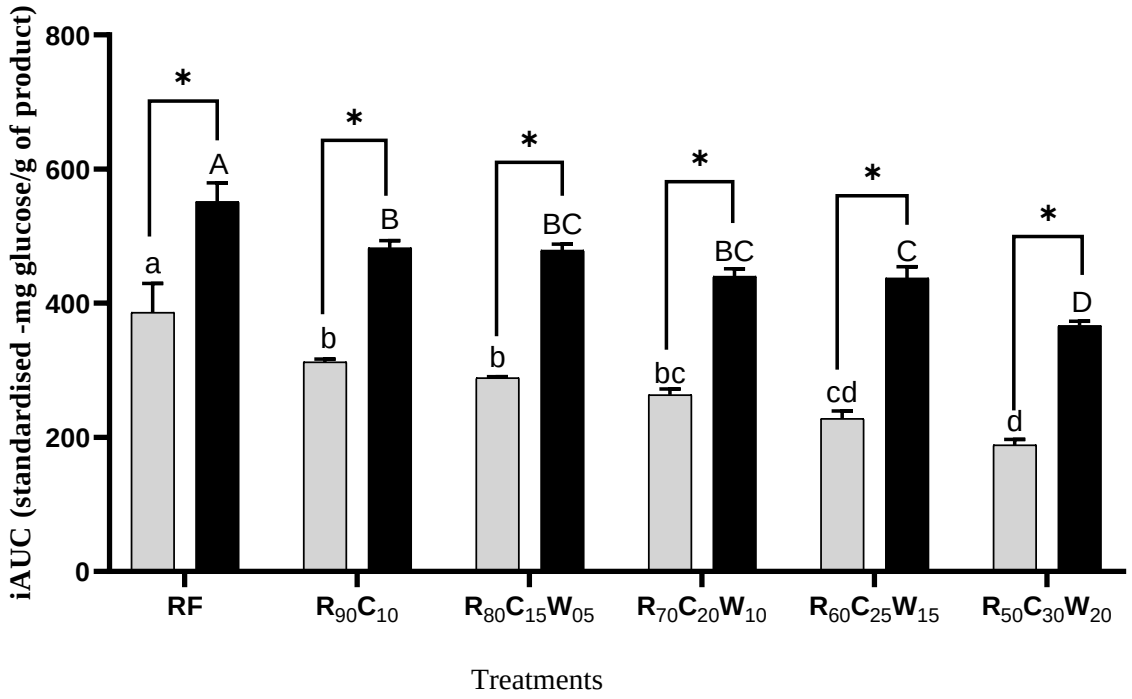


Figure 6-1 The effect of extrusion processing on the average incremental area under the curve values (iAUC) for cowpea and WPC fortified raw blends and extrudates

The column values = mean (n=3). Error bars indicate standard deviation. Comparison among raw formulations is expressed by different lowercase letters, and within extruded snacks is expressed by different upper-case letters, while before and after extrusion is indicated by the asterisks *, and different letters indicate the samples that are significantly different from each other(*P<0.05*). Abbreviations: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

A significant increase in reducing sugar released was observed as an effect of extrusion in all extruded samples compared to their raw counterparts. The iAUC values of 10-30% CP and 5-20% WPC fortifications in extruded snacks increased by 1.5-2 folds compared to their raw formulations. This increased *in vitro* digestibility of starch after extrusion is due to the loss of structural integrity of the starch granule due to increased shearing action develops heat through dissipation of mechanical energy ultimately increasing the starch susceptibility towards enzymatic reactions (Altan et al., 2009).

6.3.3 Protein digestibility and amino acid composition

The *in vitro* protein digestibility results are presented in Table 6.2. It showed a significant (*P<0.05*) increase in the protein digestibility for all the blends when compared to their corresponding raw counterparts, reaching values from 84 to 89 %.

Table 6-2 The effect of extrusion on the *in vitro* protein digestibility of cowpea and WPC formulations and extrudates

Treatment	***In vitro* protein digestibility (%)**	
	Raw formulations	Extruded snacks
RF	$41.77 \pm 0.72^{e, B}$	$84.36 \pm 0.46^{c, A}$
$R_{90}C_{10}$	$43.76 \pm 0.36^{de, B}$	$85.57 \pm 0.65^{bc, A}$
$R_{80}C_{15}W_{05}$	$46.53 \pm 0.75^{cd, B}$	$86.23 \pm 1.67^{abc, A}$
$R_{70}C_{20}W_{10}$	$49.19 \pm 0.36^{c, B}$	$87.14 \pm 1.38^{ab, A}$
$R_{60}C_{25}W_{15}$	$53.83 \pm 1.03^{b, B}$	$87.98 \pm 0.75^{ab, A}$
$R_{50}C_{30}W_{20}$	$57.52 \pm 2.09^{a, B}$	$88.89 \pm 0.38^{a, A}$

values = means ± standard deviation (n=3); Mean values in the same column followed by a different lowercase superscript letters and mean values in the same raw followed by the different upper-case superscript letters are significantly different (*P<0.05*). sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Arribas et al. (2017) also observed an increase in the *in vitro* protein digestibility of rice, when pea and carob flour were added to the blends. This could be explained by heat and shear forces used in the extrusion process that can thermally unfold the proteins' structure and increase the surface area for (1) enzymatic reactions, (2) inactivation of the anti-nutritional factors such as phytates, and trypsin inhibitors, and (3) increases in the protein digestibility (Arribas et al., 2017).

This study aimed to create ready-to-eat extruded rice snacks with an increased protein content by enriching the rice with cowpea and WPC in different proportions. The cereal-legume combinations provide a high-quality protein profile with an adequate balance of the amino acids in the formula (Table 6.3). Cowpea contains 80.04 mg/g DM of essential amino acids and 19.78 mg/g DM of non-essential amino acids, respectively and WPC contains 307.74 mg/g DM of essential amino acids and 63.47 mg/g DM of NESAA as raw formulations. The first limiting amino acid in the raw rice control sample is methionine and it's followed by histidine, tyrosine, threonine, isoleucine. The combination of rice with cowpea and WPC increased these limiting amino acids significantly ($P<0.05$) in both raw and extruded samples except tyrosine, histidine, and lysine in the 10%, 15% and 30% cowpea and 5% and 20% WPC substituted formulations. Glutamic acid is the most abundant non-essential amino acid followed by aspartic acid, arginine and alanine in both raw and extruded formulations (Table 6.3). In the raw formulations, the addition of 15-30% cowpea and 5-20% WPC increased significantly the amounts of essential amino acids (1.4 to 2.8 fold) and non-essential amino acids (1.0 to 2.0 fold) compared to those of the raw rice control samples. On the other hand, the extrusion process significantly increased the amounts of essential amino acids (2.3 to 4.0 fold) and non-essential amino acids (2.0 to 3.3 fold) compared to the raw formulations.

Table 6-3 The effect of extrusion processing on the essential and non-essential amino acid composition of raw formulations and extruded snacks fortified with cowpea flour and whey protein concentrate

	Cowpea	WPC	RF		$R_{90}C_{10}$		$R_{80}C_{15}W_{05}$		$R_{70}C_{20}W_{10}$		$R_{60}C_{25}W_{15}$		$R_{50}C_{30}W_{20}$	
	Raw	Raw	Raw	Extruded	Raw	Extruded	Raw	Extruded	Raw	Extruded	Raw	Extruded	Raw	Extruded
Essential AA														
Phenylalanine	10.66 ± 0.43	17.33 ± 0.17	2.89 ± 0.02^{d}	2.86 ± 0.03^{c,ns}	2.28 ± 0.09^{e}	3.90 ± 0.46^{bc}	3.18 ± 0.08^{d}	5.27 ± 0.34^{b}	3.84 ± 0.00^{c}	7.75 ± 0.15^{a}	4.54 ± 0.11^{b}	7.94 ± 0.72^{a}	5.42 ± 0.14^{a}	7.65 ± 0.24^{a}
Tyrosine	5.09 ± 0.12	16.23 ± 0.19	2.04 ± 0.03^{de}	2.00 ± 0.17^{d}	1.69 ± 0.12^{e}	2.01 ± 0.17^{d,ns}	2.37 ± 0.15^{cd}	3.18 ± 0.02^{c}	2.79 ± 0.1b^{c}	4.85 ± 0.17^{a}	3.26 ± 0.04^{b}	4.56 ± 0.24^{ab}	3.95 ± 0.17^{a}	4.15 ± 0.01^{b,ns}
Isoleucine	7.55 ± 0.50	36.89 ± 0.92	2.21 ± 0.02^{e}	2.19 ± 0.01^{c}	1.69 ± 0.06^{f}	2.86 ± 0.13^{c}	3.11 ± 0.17^{d}	5.41 ± 0.26^{b}	4.22 ± 0.09^{c}	8.89 ± 0.15^{a}	5.32 ± 0.08^{b}	8.81 ± 0.72^{a}	6.85 ± 0.14^{a}	10.13 ± 0.42^{a}
Lysine	16.93 ± 0.33	68.51 ± 3.97	2.89 ± 0.01^{de}	2.64 ± 0.10^{d}	2.70 ± 0.11^{e}	4.55 ± 0.85^{d,ns}	5.85 ± 0.43^{cd}	8.72 ± 0.90^{c,ns}	8.22 ± 0.47^{bc}	14.92 ± 0.26^{b}	10.25 ± 0.22^{ab}	16.73 ± 0.22^{ab}	11.95 ± 1.73^{a}	17.85 ± 0.09^{a}
Methionine	2.32 ± 0.06	12.07 ± 0.26	1.25 ± 0.10^{e}	1.30 ± 0.06^{c}	1.17 ± 0.02^{e}	1.49 ± 0.08^{c}	1.68 ± 0.10^{d}	2.26 ± 0.13^{b}	2.06 ± 0.04^{c}	3.42 ± 0.06^{a}	2.49 ± 0.03^{b}	3.33 ± 0.11^{a}	3.01 ± 0.09^{a}	3.41 ± 0.03^{a}
Threonine	7.45 ± 0.00	48.79 ± 3.05	2.08 ± 0.08^{e}	2.15 ± 0.01^{d}	1.56 ± 0.03^{e}	2.85 ± 0.00^{d}	3.03 ± 0.25^{d}	5.72 ± 0.11^{c}	4.21 ± 0.00^{c}	9.63 ± 0.39^{b}	5.43 ± 0.16^{b}	9.98 ± 0.25^{b}	6.90 ± 0.18^{a}	11.45 ± 0.23^{a}
Valine	8.39 ± 0.58	32.61 ± 1.03	3.01 ± 0.04^{e}	3.04 ± 0.02^{c}	2.15 ± 0.02^{f}	3.95 ± 0.38^{c}	3.47 ± 0.18^{d}	5.95 ± 0.16^{b}	4.42 ± 0.08^{c}	9.25 ± 0.09^{a}	5.36 ± 0.08^{b}	9.11 ± 0.50^{a}	6.70 ± 0.14^{a}	10.21 ± 0.41^{a}
Leucine	14.50 ± 0.41	64.82 ± 1.87	4.51 ± 0.07^{f}	4.61 ± 0.11^{e}	3.48 ± 0.05^{e}	6.26 ± 0.23^{d}	6.24 ± 0.23^{d}	10.50 ± 0.24^{c}	8.36 ± 0.21^{c}	16.56 ± 0.31^{b}	10.87 ± 0.27^{b}	16.76 ± 0.77^{b}	14.18 ± 0.22^{a}	19.38 ± 0.24^{a}
Histidine	7.15± 0.96	10.49 ± 0.30	1.41 ± 0.13^{e}	1.31 ± 0.13^{c}	1.81 ± 0.01^{d}	2.35 ± 0.39^{bc,ns}	2.43 ± 0.13^{c}	3.23 ± 0.33^{ab,ns}	2.79 ± 0.08^{c}	4.71 ± 0.24^{a}	3.25 ± 0.06^{b}	4.61 ± 0.63^{a}	3.84 ± 0.12^{a}	4.46 ± 0.33^{a,ns}
Non-Essential AA														
Arginine	15.69 ± 1.01	17.63 ± 1.87	5.23 ± 0.03^{c}	5.16 ± 0.21^{c}	3.85 ± 0.09^{e}	6.52 ± 0.38^{bc}	4.79 ± 0.09^{d}	7.49 ± 0.44^{b}	5.25 ± 0.05^{c}	10.34 ± 0.21^{a}	5.84 ± 0.11^{b}	10.49 ± 0.06^{a}	6.62 ± 0.20^{a}	10.05 ± 0.25^{a}
Alanine	8.67 ± 0.20	32.80 ± 1.06	3.40 ± 0.04^{d}	3.45 ± 0.07^{e}	2.48 ± 0.03^{e}	4.36 ± 0.17^{d}	3.86 ± 0.15^{d}	6.45 ± 0.13^{c}	4.86 ± 0.03^{c}	9.70 ± 0.20^{b}	5.97 ± 0.13^{b}	9.99 ± 0.00^{b}	7.29 ± 0.19^{a}	10.91 ± 0.12^{a}
Glutamic acid	31.99 ± 2.25	110.36 ± 2.37	9.73 ± 0.19^{e}	9.96 ± 0.55^{c}	7.27 ± 0.08^{f}	14.42 ± 1.09^{c}	11.97 ± 0.36^{d}	21.02 ± 0.47^{b}	15.10 ± 0.19^{c}	32.02 ± 0.23^{a}	18.55 ± 0.07^{b}	32.62 ± 1.19^{a}	22.30 ± 0.44^{a}	36.54 ± 3.05^{a}
Glycine	7.47 ± 0.02	10.77 ± 0.50	2.65 ± 0.08^{d}	2.68 ± 0.06^{c}	2.00 ± 0.04^{e}	3.25 ± 0.01^{bc}	2.60 ± 0.03^{d}	4.01 ± 0.04^{b}	2.96 ± 0.03^{c}	5.57 ± 0.32^{a}	3.41 ± 0.07^{b}	6.07 ± 0.34^{a}	3.93 ± 0.12^{a}	5.81 ± 0.25^{a}
Proline	8.05 ± 0.26	37.31 ± 1.63	2.67 ± 0.11^{c}	2.64 ± 0.09^{c}	1.77 ± 0.45^{d}	3.61 ± 0.17^{c}	2.43 ± 0.23^{cd}	6.04 ± 0.72^{b}	3.07 ± 0.02^{bc}	9.52 ± 0.27^{a}	3.65 ± 0.07^{b}	9.49 ± 0.13^{a}	4.77 ± 0.07^{a}	10.02 ± 0.72^{a}
Serine	8.60 ± 0.18	33.06 ± 1.05	2.12 ± 0.10^{e}	2.09 ± 0.04^{d}	1.74 ± 0.01^{f}	2.70 ± 0.10^{d}	2.95 ± 0.15^{d}	4.55 ± 0.01^{c}	3.82 ± 0.05^{c}	7.28 ± 0.35^{b}	4.80 ± 0.24^{b}	8.04 ± 0.39^{ab}	5.79 ± 0.21^{a}	8.44 ± 0.36^{a}
Aspartic acid	19.78 ± 1.14	63.47 ± 0.73	4.82 ± 0.02^{e}	4.97 ± 0.19^{d}	3.82 ± 0.06^{f}	7.33 ± 0.32^{c}	6.27 ± 0.22^{d}	11.38 ± 0.10^{b}	7.94 ± 0.16^{c}	17.92 ± 0.06^{a}	9.54 ± 0.11^{b}	18.20 ± 0.75^{a}	11.28 ± 0.46^{a}	19.94 ± 1.16^{a}

values = mean ± standard deviation (n=3). In each column, the different letters mean statistically (*P<0.05*) differences in between the treatments, whereas "ns" mean the non-significant difference due to extrusion processing between the same raw formulation and extruded snacks. sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

The main purpose of fortifying with cowpea and WPC was to improve the protein, fibre and bioactive compound content in the rice-based formula. Therefore, it is important to study the effect of extrusion on soluble and insoluble protein profiles formulations. The molecular weight distribution profiles for protein hydrolysis were evaluated by SDS-PAGE for all raw formulations and extruded snacks and is presented in Figure 6.2 and 6.3. The total proteins extracted covered a range of molecular weights on SDS-PAGE, within the region 10–250 kDa. The cowpea has a unique protein profile with four major fractions, namely 43-55% of globulins (at least 16 protein bands), 20-35% of albumins (at least 20 protein bands), 18.4-32.21% of glutelins (21 protein bands) and 0.73-2.70% of prolamins (one protein band) (Tchiagam et al., 2011 and Santos et al., 2012). The globulin fraction represents the four main polypeptides with molecular masses of 50, 56, 60, 65 (kDa) and minor peptides distributed over a range of 42-28 KDa. Subunits of vicilin proteins which are the predominant cowpea globulin are reported to range from 49 -63 kDa (Chan & Phillips, 1994). The most dominant albumin peptides can be seen in the range of 23-99 kDa. Glutelins were found in the range of 44-62 kDa and prolamin polypeptides were reported with the molecular masses of 54, 59, 62 and 105 kDa (Chan & Phillips, 1994). The β-lactoglobulin (18.4 kDa), α-lactalbumin (14.4 kDa), bovine serum albumin (66.5 kDa) and immunoglobulins are the major protein fractions present in whey protein concentrates.

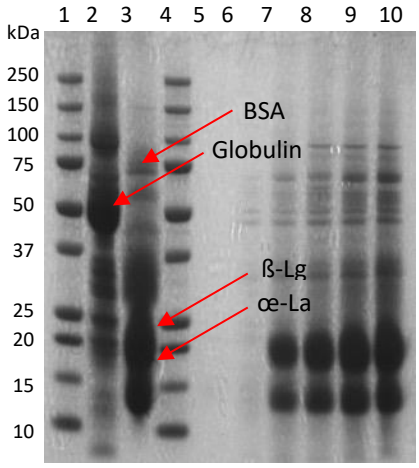
The protein band profiles of raw materials and raw blends showed considerable similarities concerning the number of protein bands and their band intensities. Before digestion (Figure 6.2 (a), cowpea has highly intense bands around the 50 kDa in soluble and insoluble electrophoretic patterns, which represents the globulin fraction with molecular weights of 47 to 65 kDa. The globulin fraction is further divided into legumin (11S) and vicilin/ß-vignina (7S) (Chan & Phillips, 1994). Alghamdi et al. (2019) have reported that the albumin and

glutelin fractions were separated into polypeptide bands with molecular weights ranging between 15 to 110 kDa and 15 to 130 kDa, respectively. According to the SDS PAGE results, higher proportions of these proteins present in raw formulations are soluble in water than the insoluble portion, and a comparison of protein bands revealed that higher proportions of cowpea in raw blends increased the protein concentration than the control sample. The proteins present in the extruded snacks were generally not soluble in water and there were more insoluble proteins (Figure 6.3b) at the beginning of the digestion process. This could be a result of the denaturation of globular protein due to the use of high temperatures during the extrusion process. The extruded snacks with a higher proportion of cowpea and WPC contained higher levels of low molecular weight protein components than those from the control sample. There are clear bands that can be seen around 20 kDa band. This could be a combination of 19-23 kDa from basic glutelin precursor from rice flour and β-lactoglobulin from WPC. The α-lactalbumin can be seen in between 10-15 kDa. The band thickness increased as the concentration of the protein fractions increased in line with the increases in WPC compared to the control.

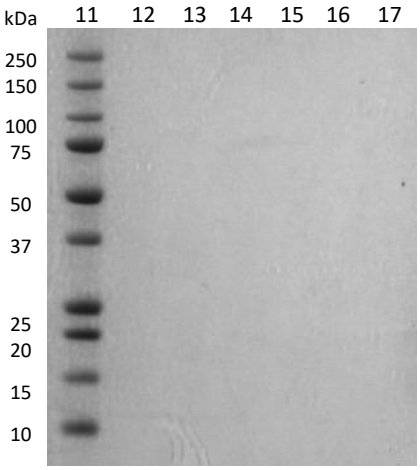
After digestion with pepsin for 1 h, aggregates between 37-150 kDa in the stacking gel degraded and disappeared. The aggregates on the top of the separating gel also degraded with the appearance of high intense bands with lower molecular fraction. The globulin was mostly digested, with the total disappearance of the acid subunits of legumin and significant degradation of the basic subunits of legumin. This suggests that acid subunits were more vulnerable to pepsin digestion. After 2 h of pancreatin digestion, aggregates between 10-25 kDa of the separating gel were almost digested. According to the SDS-PAGE electrophoretic pattern of the WPC, bands corresponding to α-lactalbumin (14.4 kDa), β-lactoglobulin (18.4 kDa), and bovine serum albumin (66.5 kDa) were visible before digestion. Similar patterns

were observed by Miralles et al. (2018). This is noted particularly where the high intense bands indicated the presence of *β*-lactoglobulin and bovine serum albumin in the insoluble form in water. After 1h of pepsin digestion, bovine serum albumin was no longer visible in all digests of raw and blends. The *β*-lactoglobulin band remained after 1 hr but reduced after the 2 hr pancreatin digestion in all digests.

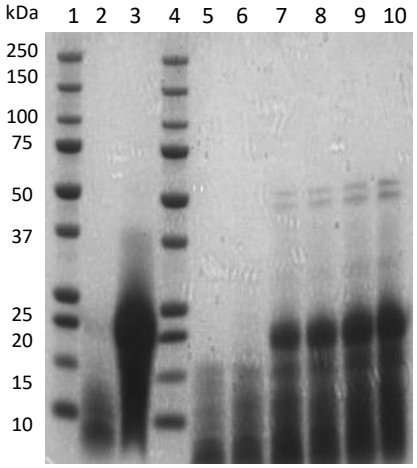
Sousa et al. (2020) have reported that the β-lactoglobulin was resistant to pepsin in the gastric phase and immediately hydrolysed at the beginning of the intestinal phase. Soluble protein fractions are increasing with the digestion process while insoluble proteins are decreasing. The other bands that are still visible at the end of the digestion could be corresponding to the mixture of rice albumin (15–56 kDa), rice globulin (11-12 kDa), rice glutelin (30-40 for acidic or 19-23 for basic), vicilin and legumin A from cowpea (Sousa et al., 2020). Based on the analysis of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), the globulin fractions of cowpea and WPC snacks contained much more low molecular weight protein components (21–14 kDa) than the control sample, and the disappearance of some bands in the high molecular weight region was observed. The findings in this study agree with those previously reported (Alonso et al., 2000; Fang et al., 2013). It has been reported that extrusion processing resulted in major changes in the band patterns of the albumin and globulin fractions (Alonso et al., 2000; Li & Lee, 1996). This information will be useful in studying the molecular mechanism of protein interactions, changes in molecular weight distribution and quantity of soluble and insoluble fractions after extrusion processing.



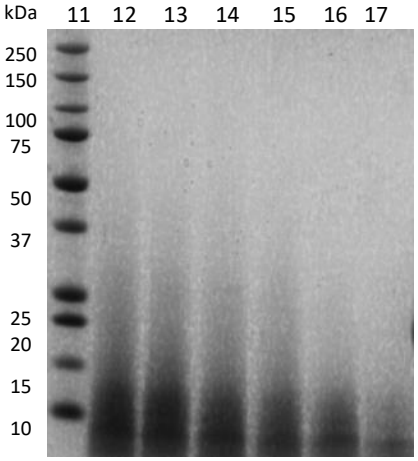
(a) 0 min – Raw



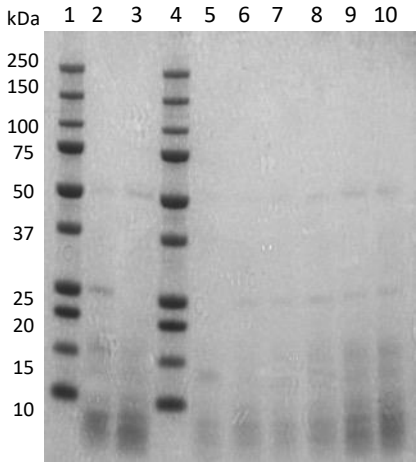
(b) 0 min - Extruded



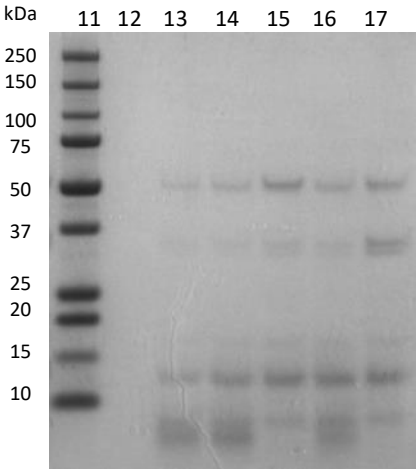
(c) 1 hr – Raw



(d) 1 hr - Extruded



(e) 2 hr – Raw



(f) 2 hr - Extruded

Figure 6-2 SDS-PAGE showing profiles for soluble proteins

Lane 1: protein ladder, lane 2: whole seed cowpea, lane 3: WPC, lane 4: protein ladder, lane 5: RC (rice flour), lane 6: $R_{90}C_{10}$, lane 7: $R_{80}C_{15}W_{05}$, lane 8: $R_{70}C_{20}W_{10}$, lane 9: $R_{60}C_{25}W_{15}$, and lane 10 $R_{50}C_{30}W_{20}$. BSA, Bovine serum albumin; ß-lg, ß-lactoglobulin; α-La- α-lactalbumin

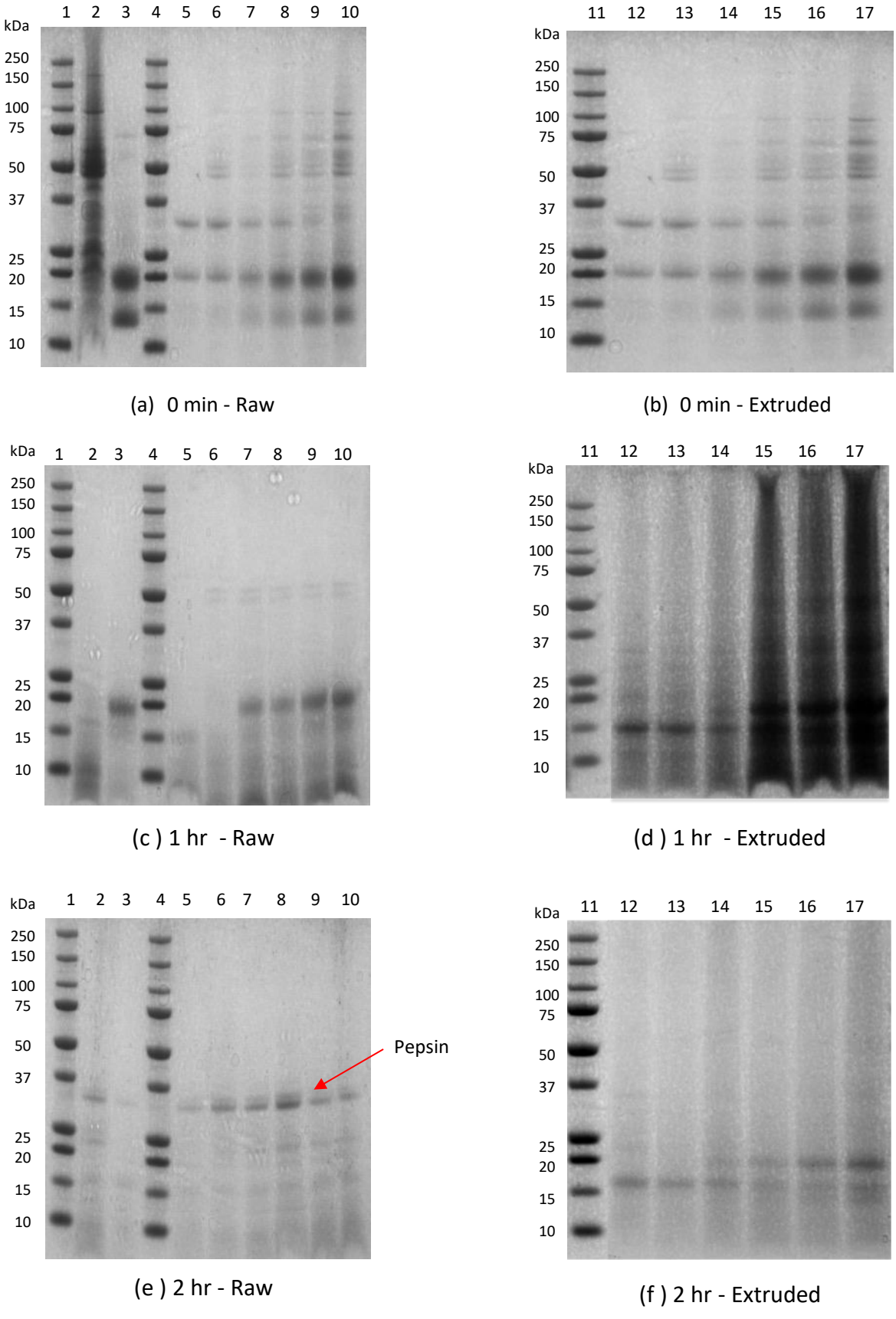


Figure 6-3 SDS- PAGE showing profiles for insoluble proteins

Lane 1: protein ladder, lane 2: whole seed cowpea, lane 3: WPC, lane 4: protein ladder, lane 5: RC (rice flour), lane 6: $R_{90}C_{10}$, lane 7: $R_{80}C_{15}W_{05}$, lane 8: $R_{70}C_{20}W_{10}$, lane 9: $R_{60}C_{25}W_{15}$, and lane 10 $R_{50}C_{30}W_{20}$. BSA, Bovine serum albumin; ß-lg, ß-lactoglobulin; α-La- α-lactalbumin

6.3.4 TPC and antioxidant properties (DPPH, ABTS and FRAP)

The TPC and antioxidant properties including DPPH, ABTS, and FRAP of the non-extruded formulation and extrudates are shown in Figure 6.4. The TPC of the non-extruded formulations substituted with cowpea (10-30%) and WPC (5-20%) ranged from 1.02 to 2.95 mg GAE/g. As expected, the TPC increased significantly ($p<0.05$) with increasing legume content in the non-extruded formulations. The addition of 15-30% cowpea and 5-20% WPC increased significantly the TPC by 2-3 fold compared to the control sample (100% rice). However, the TCP of the 10% CP substitution was not significantly different compared to the control sample. In general, higher DPPH, ABTS and FRAP radical scavenging activities were detected in the non-extruded formulations with higher amounts of cowpea flour and WPC. The sample containing 30% cowpea and 20% WPC had the highest antioxidant activity and the control had the lowest antioxidant activity.

Extrusion resulted in a significant ($P<0.05$) decrease in TPC and DPPH, ABTS and FRAP radical scavenging activities of all the samples compared to their corresponding non-extruded formulations. However, the fortification of rice flour with cowpea and WPC increased the antioxidant properties significantly ($P< 0.05$) compared to that of the extruded rice control sample. Increases and decreases in both the phenolic content and in the antioxidant activity of the different legumes have been reported depending on the extrusion conditions. Reduction of TPC and the antioxidant properties could be due to the decomposition or alteration in the molecular structure of phenolic compounds during the extrusion process at temperatures above 80 °C (Zadernowski et al., 1999). The reduced reactivity and a decrease in phenolics extractability due to the formation of insoluble protein–polyphenol complexes/polymerisation might be the reasons for the reduction of TPC in extrudates in this study.

In contrast, some studies have reported increases in TPC and antioxidant activity with an extrusion that could be due to the lack of leaching water-soluble phenols such as soaking, the release of some non-available phenols bound to the cell walls, the presence of higher molecular weight MRPs and the improved extractability (Stojceska et al., 2008). The use of low temperatures (<140 °C) and relatively low moisture (<14%) can retain higher contents of phenolics and increase the antioxidant activity (Arribas et al., 2019c).

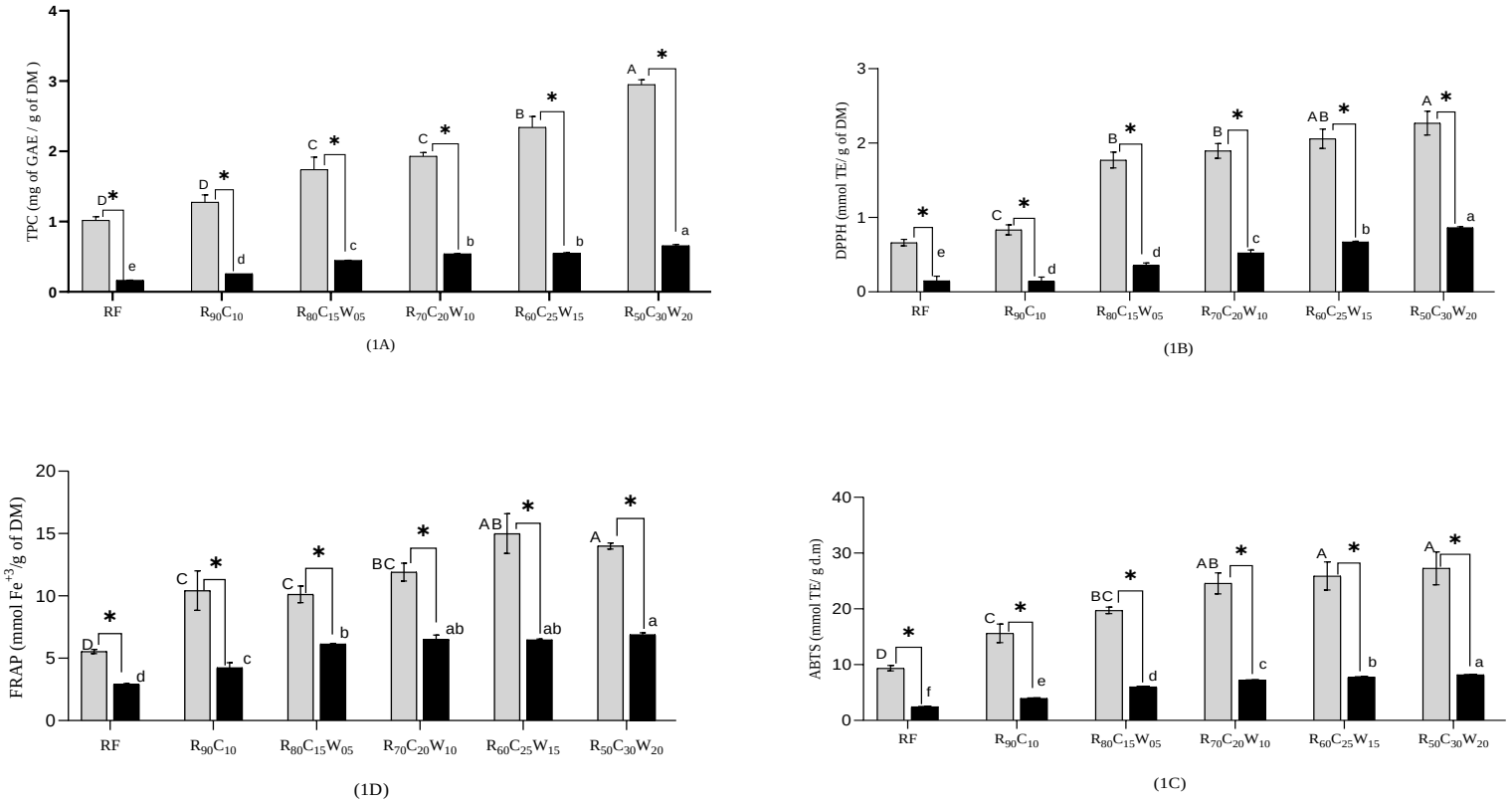


Figure 6-4 (1A) The effect of extrusion processing on the total phenolic content (TPC) and (1B) antioxidant capacity; DPPH assay, (1C) ABTS assay and (1D) FRAP assay of non-extruded formulations and extruded snacks.

Value = means (n=3). Error bars indicate standard deviation. Comparison among non-extruded formulations is expressed by different capital letters, and within extruded snacks is expressed by different lowercase letters, while before and after extrusion processing. sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

6.4 Conclusion

From the findings of this study it can be concluded that the Extrusion processing significantly increased the resistant starch and raffinose series oligosaccharides in the extruded snacks compared to the non-extruded formulation and trypsin inhibitory activity, phytic acid content, phenolic content and antioxidant activity all decreased significantly for all extruded snacks. Extrusion processing increased protein and starch digestibility and amino acids profile in extrudates as compared to the non-extruded blends. The snacks that had been fortified with cowpea and whey protein had increased dietary fibre, antioxidant properties, and amino acid profile. *In vitro* digestion highlighted that the fortification of rice flour with cowpea and WPC reduced the glycaemic response of extruded snacks. Based on the analysis of SDS-PAGE, the extruded snacks with increased proportion of cowpea and WPC observed with more low molecular weight protein components (21–14 kDa) than the control sample. The development of this novel gluten-free ready-to-eat product with increased fibre and protein can be considered as healthy functional snacks which cater for the consumer demand for nutritious snacks. At the same time, this would be a good way to increase pulse consumption and healthy food for those suffering from gluten sensitivity.

Chapter 7

Gluten-free Extruded Snacks Enriched with Cowpea and Whey Protein Concentrate: *In Vitro* Starch Digestibility, Phenolic Contents, and Antioxidant Properties

Abstract

Low glycaemic foods can be considered as a non-drug therapy for improving postprandial hyperglycaemia. Rice flour-based extruded snacks were fortified with cowpea and WPC at different levels to investigate the effect of fortification on the *in vitro* starch and protein digestion, total phenolics and antioxidant properties. Incorporation of 10-30% cowpea flour and 5-20% of whey protein concentrate in rice-based extruded snacks significantly decreased the reducing sugar released in the range of 14-41%, compared to the control sample. After the *in vitro* digestion process, the antioxidant activity of the cowpea and WPC fortified snacks was more than 2-fold higher compared to their extruded counterparts. These results show the potential beneficial use of cowpea and WPC, as functional ingredients to improve the nutritional profile and reduce the glycaemic index of rice-based extruded snacks.

Keywords: extruded snacks, gluten-free, glycaemic, antioxidant

7.1 Introduction

The application of low moisture extrusion technology has been taken up in the manufacture of ready-to-eat snack products. The use of high temperature and shear forces during extrusion causes structural, chemical, and nutritional transformations such as starch gelatinisation, protein denaturation, lipid oxidation, Maillard browning, inactivation of anti-

nutritional compounds, changes in bioactive compounds and antioxidant properties (Arribas et al., 2019b).

Most ready-to-eat snack products are made from refined cereal flours and are generally high in calories and low in protein and fibre (Brennan et al., 2012). As consumer awareness of the link between diet and disease increases, the snack industry has advanced to develop healthy snacks with more protein, fibre, and other phytochemicals. In recent years, there have been many studies focused on combining different food sources to develop healthy snack formulations such as the use of legumes and pulses, namely lentil, faba bean, lupin, pea, chickpea, cowpea (Ciudad-Mulero et al., 2020; Martin et al., 2020a; Philipp et al., 2017; Yadav et al., 2019); fruits such as orange and apple pomace (Huang & Ma, 2016; Leyva-Corral et al., 2016); and dehydrated vegetables (Bisharat et al., 2013). Most of these studies focused on understanding the effect on the nutritional and physicochemical characteristics including bioactive compounds and antioxidant properties. Based on the literature reviewed, there is limited information available on the behaviour of these components after the *in vitro* digestion process.

White rice (*O. sativa L*.) has been used in making gluten-free cereal-based snacks due to its colour, flavour, hypo-allergenicity, bland taste and better processing characteristics. The fortification of rice-based products with legumes improves the nutritional composition in terms of protein, fibre and bioactive compounds (Dalbhagat et al., 2019). Cowpea (*V. unguiculata L. Walp*) is one of the most important food legumes of the tropical region. Cowpea seeds contain 24.2% protein, 63.5% carbohydrates, 14.4% dietary fibre and 2.5% of fat on a dry basis. Besides the nutritional benefits, cowpea is an important source of bioactive compounds phenolic acids and flavonoids (Ojwang et al., 2012). The presence of anti-nutritional factors, such as phytic acid, trypsin inhibitory activity and α-galactosides (raffinose, stachyose and verbascose) lowers the bioavailability of minerals, inhibits protein

digestibility, and causes flatulence in humans. However, there is research that shows these factors also have a positive role as by exhibiting antioxidant and anticancer effects and that they favour the growth of beneficial microflora (Sreerama et al., 2012b; Turner et al., 2002). Cowpea seeds contain a high amount of resistant starch and dietary fibre, which reduces calories and improves glucose regulation in diabetic patients (Oboh & Agu, 2010).

This study used, whey protein concentrate (WPC) as a secondary ingredient to improve the proteins and minerals content in extrudates, as it is a rich source of essential amino acids; leucine (11.8%) lysine (9.5%), valine (4.7%), threonine (4.6%), methionine (3.1%) and phenylalanine (3.0%) (Brnčić et al., 2011; Huth et al., 2004). Afizah & Rizvi, 2014 reported that whey proteins have the potential to use as a functional ingredient due to their solubility, gel and form forming ability and emulsions with greater viscosity and stability during the extrusion process.

Therefore, the aim of this chapter is to analyse the predictive glycaemic response, total phenolics and antioxidant properties of ready-to-eat extruded snacks formulated by combining rice flour, cowpea flour and WPC.

7.2 Materials and Methods

7.2.1 Raw materials

Raw materials were used as described in 3.1.1

7.2.2 Extrusion processing

Extrusion processing was carried out as described in 3.1.2

7.2.3 *In vitro* starch digestion

In vitro starch digestion was carried out as described in 3.2.4

7.2.4 Total phenolic content and Antioxidant Properties (DPPH, ABTS and FRAP activities)

TPC, DPPH, ABTS, and FRAP determinations were carried out as described in 3.2.9

7.2.5 Statistical analysis

Statistical analysis was carried as described in 3.3

7.3 Results and Discussion

The *in vitro* enzymatic digestion was performed to mimic gastric and pancreatic digestion. These *in vitro* studies on starch digestibility of foods provide important data in the initial screening of the predictive glycaemic response of foods. The starch fractions are defined as rapidly digested starch, slowly digestible starch, and resistant starch (Englyst et al, 1992). In an *in vivo* study, rapidly available glucose was found to be an excellent predictor of glycaemic response and explained approximately 61% of the glycaemic response (Englyst et al, 1999). The glycaemic index quantifies the increase in blood glucose after the consumption of a set amount of carbohydrates. The rapidly available glucose is highly correlated to the glycaemic index in human studies (Englyst et al., 2003). The *in vitro* method used in this study, predicts the glycaemic response, and has the advantage of not requiring human subjects. Several studies have reported high correlations of *in vitro* starch hydrolysis to the glycaemic index and glycaemic response of foods (Goni, I., Garcia-Alonso, a., 1997; Onipe et al., 2020; Seal et al., 2003; Woolnough et al., 2010). Figure 7.1 represent an interpretation of the amount of reducing sugars released over 120 min *in vitro* digestion between samples, reflecting the contribution of raw materials.

It was noticeable that there were more reducing sugars released from the control extruded snacks compared to the CP and WPC enriched extruded snacks samples. This trend was maintained over the 2 h of digestion. In all samples, the release of reducing sugars increased dramatically in the first 20 min. In particular, the amount of reducing sugars in CP and WPC

fortified samples was lower at 20, 60 and 120 min of time digestion compared to the control. However, there was no significant difference in released glucose amount in 15% CP with 5% WPC and 20% CP and 10% WPC fortification levels after 2 h. The observed total reduction after 2 h for the extruded samples was in the range of 14-41% at the inclusion levels of 10-30% CP and 5-20% of WPC, respectively. Higher inclusion rates of CP and WPC led to greater reductions in glucose released in 0, 20, 60 and 120 min compared to the control. This could be explained by the addition of CP and WPC alters the starch degradation or enzyme hydrolysis, reducing sugar release compared to the control.

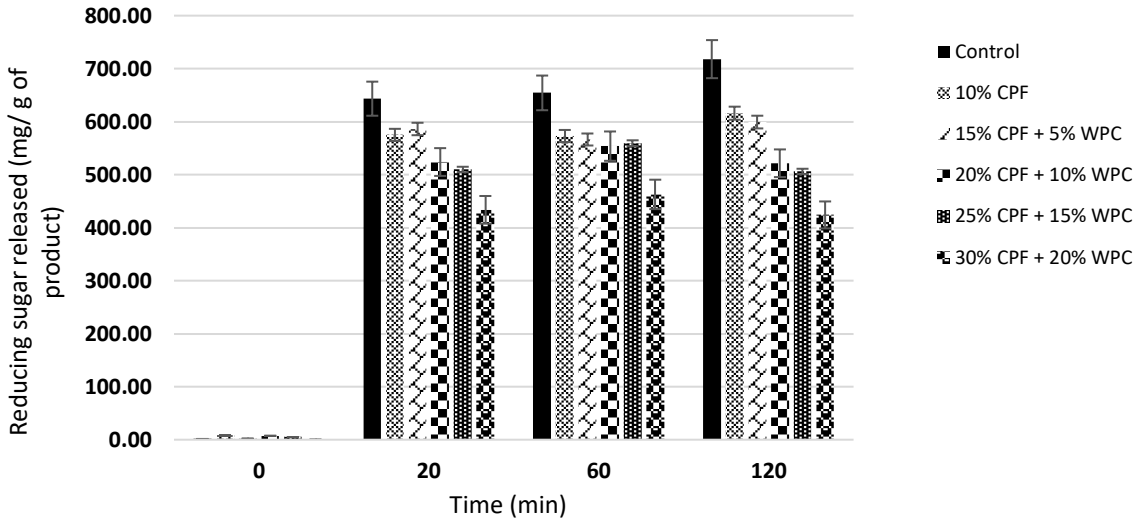


Figure 7.2 The amount of glucose released (mg/g of sample) from control (100% rice flour) and cowpea flour (CPF) and whey protein concentrate (WPC) fortified samples in rice-based blends. Values are means n=3, error bars show the standard deviation

Value = means (n=3). Error bars indicate standard deviation. Comparison among raw formulations is expressed by different capital letters, and within extruded snacks is expressed by different lowercase letters, while before and after extrusion processing. sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

The TDF content of cowpea ranges from 12 to 14g $100g^{-1}$ (Martín-Cabrejas, 2019). Majority of dietary fibre composed of insoluble fibre (13.1g $100g^{-1}$) fraction in cowpea flour (Sajilata et al., 2006; Sreerama et al., 2012b). Dietary fibre content increased with the increasing proportion of cowpea flour in formulae. Food rich in fibre protect the carbohydrates from

digestive enzymes. The high-water binding capacity of dietary fibres also reduce the glucose releasing rate into the system by making water not available for starch swelling and forming gel-like substances which slow gastric emptying and control the absorption of nutrients (Lu et al., 2018).

The cowpea starch fraction was composed of 38-53 g $100g^{-1}$ of rapidly digested starch, 1-10 g $100g^{-1}$ of slowly digested starch, and 9-12 g $100g^{-1}$ of resistant starch (Rengadu et al., 2020b). Resistant starch resists enzymatic and acidic hydrolysis and absorption within the upper gastrointestinal tract and undergoes fermentation as a prebiotic by the microbiota present in the colon. Resistant starch has no calorific value to the human body, does not increase blood sugar and has comparable physiological effects to dietary fibre (Sajilata et al., 2006). Previous research has indicated that the addition of legume flours, lentil flours, and mushroom powder into extruded snacks reduced the amount of readily digestible carbohydrates (Wani et al., 2021; Hardacre et al., 2005; Lu et al., 2018).

Cowpea, a food legume, and whey protein concentrate were combined with cereal in this study to improve the nutrient profile. Cowpea and WPC contribute more protein to the formulations. With the increased proportions of cowpea and WPC, the ratio of carbohydrates to protein is in the formulations is reduced compared to the control. Recent research evidence suggests that the reduced ratio of carbohydrates to protein is helpful in weight loss and leucine, a branched-chain amino acid present in whey proteins, help in body weight control diets (Ferrando et al, 1995; Onwulata et al., 2010; Meroni et al., 2020).

Therefore, the reduction in the *in vitro* starch hydrolysis observed in treatments that have higher amounts of cowpea and WPC could be a result of (1) lower starch content, (2) greater amount of dietary fibre and resistant starch (3) the interactions of protein/carbohydrates low-GI diets contribute to weight control because they promote satiety through reducing the gastric emptying time, minimise postprandial insulin secretion, maintain insulin sensitivity,

and lower the risks for various conditions associated with hyperinsulinemia, such as diabetes mellitus and cardiovascular disease (Meroni et al., 2020).

The whole grains are a good source of phenolic compounds which are well known for their natural scavenging capabilities and reduce lipid oxidation in food systems. However traditional processing conditions including polishing, soaking, cooking reduce their content by removing or leaching into the cooking water (Rocchetti et al., 2017). Some studies have shown that, extrusion processing which is carried out without effluent increase the TPC in comparison to their non-extruded counterparts (Arribas et al., 2019c; Morales et al., 2015a). The decreases of TPC were reported by Ciudad-Mulero et al. (2018) and Arribas et al. (2019b) with extruded legumes. Polyphenols present in extruded formulations are affected by two counter-acting mechanisms. The use of high temperature and pressure used during extrusion causes decomposition of the thermo-labile phenolic compounds and may also lead to polymerisation of phenolic compounds which could reduce their extractability (Morales et al., 2015a). The mechanical shear applied during extrusion enhances the liberation of bound phenolics through the mechanical breaking of conjugated moieties (Acosta-Estrada et al., 2014).

Table 7.1 compares the TPC and the antioxidant capacity of the extruded snacks, before and after *in vitro* digestion. All the cowpea and WPC fortified extruded snacks have a significantly higher TPC than control extruded snacks. The extrudate fortified with 30% cowpea flour and 20% WPC had the greatest TPC (0.66 mg/g dm) and the rice only extrudate had the lowest TPC (0.16 mg/g dm). The greater the amount of cowpea in the formula the greater the content of phenolic compounds in the extruded snacks. In general, higher DPPH, ABTS and FRAP radical scavenging activities were detected in the extruded snacks with higher amounts of cowpea flour and WPC. The sample containing 30% cowpea and 20% WPC had the highest antioxidant activity and the control had the lowest antioxidant activity.

A similar trend was observed in digested samples. After *in vitro* digestion, the cowpea and WPC fortified digesta showed a significant increase ($p<0.05$) in TPC content in comparison to their control counterpart. The TPC values after *in vitro* digestion increased by up to three-fold in cowpea fortified samples. These observations can be extended to what was observed in the levels of DPPH, ABTS and FRAP antioxidant activities after *in vitro* starch hydrolysis of rice-cowpea-WPC extruded snacks, that is, they increased significantly compared to the control sample after digestion. This could be due to the enzymatic hydrolysis of the starch and protein which breaks down the bonds between phenolic compounds and sugars, cell wall polysaccharides, or amines (Han et al., 2019) Phenolic compounds may undergo degradation and oxidation upon thermal processing and may bind to other components such as proteins that could reduce their extractability. Conversely, changes to cell wall components at high temperature processing or hydrolysis of insoluble-bound phenolic compounds can occur leading to increased extractability (Hachibamba et al., 2013; Wang et al., 2020b).

Table 7-1 Total phenolic content and antioxidant activities in extruded snacks fortified with cowpea and WPC

Sample	Total polyphenols (mg/g d.m)		DPPH (mmol TE/ g d.m)		ABTS (mmol TE/ g d.m)		FRAP (mmol Fe^{+3}/g d.m)	
	Extruded	After digestion	Extruded	After digestion	Extruded	After digestion	Extruded	After digestion
RF	0.16 ± 0.01^{e}	0.83 ± 0.00^{F}	0.15 ± 0.06^{e}	$0.07 \pm 0.04^{C,ns}$	2.45 ± 0.03^{d}	8.06 ± 0.30^{F}	2.93 ± 0.03^{f}	4.44 ± 0.12^{E}
$R_{90}C_{10}$	0.25 ± 0.01^{d}	0.97 ± 0.01^{E}	0.36 ± 0.02^{d}	0.23 ± 0.04^{D}	3.96 ± 0.09^{c}	9.93 ± 0.09^{E}	4.24 ± 0.40^{e}	5.42 ± 0.07^{D}
$R_{80}C_{15}W_{05}$	0.45 ± 0.01^{c}	1.31 ± 0.03^{D}	0.37 ± 0.02^{d}	0.51 ± 0.04^{C}	6.03 ± 0.03^{b}	13.24 ± 0.14^{D}	6.14 ± 0.05^{d}	7.51 ± 0.04^{C}
$R_{70}C_{20}W_{10}$	0.54 ± 0.00^{b}	1.53 ± 0.01^{c}	0.52 ± 0.04^{c}	0.90 ± 0.05^{B}	7.22 ± 0.06^{ab}	14.73 ± 0.13^{C}	6.51 ± 0.35^{c}	10.11 ± 0.08^{B}
$R_{60}C_{25}W_{15}$	0.55 ± 0.01^{b}	1.61 ± 0.02^{B}	0.67 ± 0.01^{b}	1.00 ± 0.08^{B}	7.77 ± 0.07^{ab}	16.19 ± 0.17^{B}	6.47 ± 0.07^{b}	10.87 ± 0.19^{A}
$R_{50}C_{30}W_{20}$	0.66 ± 0.01^{a}	1.91 ± 0.02^{A}	0.86 ± 0.01^{a}	1.19 ± 0.04^{A}	8.17 ± 0.08^{a}	17.86 ± 0.12^{A}	6.88 ± 0.16^{a}	10.90 ± 0.13^{A}

Values are mean ± standard deviation (n=3). In each column, the different upper-case letters mean statistically (*P< 0.05*) differences in between the treatments, whereas “ns” mean the non-significant difference between extruded and digested snack samples. sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

7.4 Conclusion

The addition of cowpea and WPC to ready-to-eat extruded rice snacks lowered the predicted glycaemic response and increased the TPC and antioxidant capacity. The greater reduction in starch hydrolysis rate when greater amounts of cowpea and WPC are added to rice could be a result of lower starch content of the formula, greater content of constituents such as fibre, resistant starch, and the occurrence of interactions of cowpea/rice constituents. After *in vitro* digestion, the digesta of extruded snacks showed an increase in TPC and antioxidant abilities compared to the extruded samples.

Chapter 8

Understanding Consumer Perception of Ready-to-eat Gluten-free Snacks Containing Cowpea Flour and Whey Protein Concentrate

Published Conference paper

- H.N., Nadeesha Dilrukshi, Damir D. Torrico, Margaret A. Brennan, and Charles S. Brennan. 2021. "Instrumental and Sensory Properties of Cowpea and Whey Protein Concentrate-Fortified Extruded Rice Snacks" *Proceedings* 70(1): 95. https://doi.org/10.3390/foods_2020-07704

Abstract

The effect on sensory properties of cowpea and whey protein concentrate (WPC) fortification of rice-based ready-to-eat extruded was investigated. Six samples of extruded snacks were evaluated for liking scores with a 9-point hedonic scale, sensory properties using the check-all-that-apply (CATA) method and just-about-right (JAR) scale by the 70 consumers. The cowpea and WPC fortified samples had higher scores for overall liking than the control sample made with 100% rice flour. The 15-25% cowpea and 5-15% WPC fortified samples had the highest JAR scores for penalty analysis for colour and texture attributes. The panellists perceived and identified texture attributes; soft and crunchy terms were significant for extrudates. Overall, the analysis of all sensory attributes demonstrated that the fortification with 15% cowpea and 5% WPC had higher acceptance by consumers involved in this study. A principal component analysis (PCA) revealed the positive associations between the groups, crispiness and L* value, and hardness b* and a* values, respectively. The fortification of rice-based snacks with cowpea and WPC could be a valuable

alternative with high protein and a sustainable source of fibre in gluten-free extrudates for consumers.

Keywords: extruded snacks, gluten-free, cowpea, consumer, sensory analysis

8.1 Introduction

The combination of novel ingredients in developing extruded snacks has an important effect on sensory attributes, which can create acceptance or rejection by consumers. The approach of health benefits can link with sensory attributes based on previous experiences and existing expectations (Tepsongkroh et al., 2020). The addition of fibre rich sources to a food matrix results in changes in texture and rheological properties (Han et al., 2018; Martin et al., 2020a). Previous findings have highlighted that the addition of fibre rich sources such as faba beans, lentils, peas and mushrooms to extruded products alters the physical, textural and sensorial properties (Lu et al., 2020; Martin et al., 2020a; Saldanha do Carmo et al., 2019). As a high-quality protein source, whey protein concentrate can be used as an ingredient in extrusion processing due to its rheological and functional properties; high solubility and high gel and foam-forming ability (Gong et al., 2021). Many of the existing ready-to-eat snacks on the market are based on wheat or rice with high energy and low nutrients contents. Therefore, the development of healthy snacks with consumer acceptance is a timely requirement for the food industry.

Therefore, the objective of this study was to evaluate sensorial characteristics of cowpea - WPC rice-based extrudates as affected by raw material characteristics (cowpea, WPC to rice ratio).

8.2 Materials and Methods

8.2.1 Raw materials

Raw materials were used as described in 3.1.1.

8.2.2 Extrusion processing

Extrusion was carried out as described in 3.1.2.

8.2.3 Sensory analysis

Sensory analysis was carried out as described in 3.2.21.

8.2.4 Statistical analysis

Statistical analysis was carried out as described in 3.3.

8.3 Results and Discussion

8.3.1 Sensory liking scores

Sensory characteristics of foods are widely considered to be key determinants of the acceptability of food among consumers. Therefore, these characteristics can be used to determine the degree of liking or dislike for a new product. Based on the hedonic rating of 1–9, mean scores for sensory attributes >5 (Table 8.1) was obtained indicating that appearance, colour, aroma, overall flavour, texture, after taste and overall acceptability attributes were moderately liked by panellists. Overall, cowpea and WPC addition improved the sensory liking scores of the extrudates compared to the control. The results show that there were no significant differences ($p<0.05$) in appearance, colour, aroma and overall flavour of all extrudates. In terms of sensory liking scores, 15% CPF and 5% WPC incorporation resulted in a more favourable appearance (5.97), colour (6.19), aroma (5.73) and overall flavour (5.89) than the other combinations and the control. Overall flavour scores were indicative that consumers did not detect the beany flavours or that nutty smells could be desirable to consumers. An increased beany flavour has been reported in legume fortified extruded snacks due to the lower degree of macromolecular degradation at higher values of

feed moisture contents (Lazou & Krokida, 2010). This nutty smell is due to browning reactions during the extrusion process and removal of beany flavour from legumes by extrusion (Cueto et al., 2018). The panellists scored texture more favourably than the control in this study. The 10-25% cowpea flour and 5-15% WPC fortified extrudates received liking scores between 6.04 - 6.36, which were significantly higher than the control. Previous studies have reported that the addition of fibre rich material increases the hardness of extrudates due to increased water retention and changes in the protein-starch interactions or the starch-fibre interactions (Brennan et al., 2013b; Robin et al., 2015; Yu, Liu, Tang, Shen, et al., 2017). As observed (Table 8.1) high cowpea and WPC contents had a less significant effect on the sensory characteristics evaluated in the study. All the formulations and control samples scored more than 5, a score considered the lower limit of acceptability for any sensory attribute (Nyombaire et al., 2011). Overall, 15% cowpea and 5% WPC fortification in rice-based extruded snacks had the highest liking scores for all sensory attributes. In general, the moderate sensory scores from this study were indicative of the acceptability of cowpea and WPC fortified snack products to consumers. These findings agreed with Natabirwa et al., 2020a and Shadan et al., 2017 who observed moderate liking scores for bean and cowpea based composite snacks.

Figure 8-1 Sensory analysis for extruded snacks

8.3.2 Contingency table for sensory attributes

Tables 8.3 and 8.4 are contingency tables showing the selected proportions of individual terms listed on the CATA question by panellists. A higher proportion (closer to 100) indicates the term was more frequently selected by panellists. Few of the sensory attributes used to describe the extruded snacks in the CATA questions were statistically different ($P<0.05$). According to Cochran's Q test, panellists perceived and identified texture attributes; soft and crunchy terms were significant among the other sensory attributes. A biplot of correspondence analysis (Fig 2), explaining 51.5% of the total variance, explain the association between the cowpea and WPC fortification levels and sensory attributes. More specifically, less than 20% cowpea and 10 % WPC fortifications were associated with "crunchy", "crispy", "satisfied", "balanced", "happy", "relaxed" and "energetic" attributes. Fortification with 15% cowpea and 5% WPC had the highest frequencies for crunchy and crispy attributes (Table 4). These results gave a view of how variations in the level of fortification with cowpea and WPC affected the sensory attributes of the extruded snacks

Table 8-1 Mean scores (and standard deviations) of consumer responses based on a 9-point hedonic scale for sensory attributes

Sample	Appearance	Colour	Aroma	Overall Flavour	Texture	After Taste	Overall Liking
RF	5.70 ± 1.77 [a]	5.65 ± 1.77 [a]	5.44 ± 1.30 [a]	5.17 ± 1.50 [a]	5.29 ± 1.71 [b]	5.04 ± 1.77 [b]	5.11 ± 1.65 [b]
$R_{90}C_{10}$	5.69 ± 1.55 [a]	5.79 ± 1.74 [a]	5.63 ± 1.38 [a]	5.80 ± 1.55 [a]	6.16 ± 1.49 [a]	5.67 ± 1.56 [ab]	5.77 ± 1.44 [ab]
$R_{80}C_{15}W_{05}$	5.97 ± 1.52 [a]	6.19 ± 1.31 [a]	5.73 ± 1.34 [a]	5.89 ± 1.85 [a]	6.36 ± 1.51 [a]	5.99 ± 1.80 [a]	6.07 ± 1.53 [a]
$R_{70}C_{20}W_{10}$	5.61 ± 1.48 [a]	5.73 ± 1.58 [a]	5.56 ± 1.40 [a]	5.24 ± 1.61 [a]	6.04 ± 1.50 [a]	5.41 ± 1.58 [a,b]	5.46 ± 1.58 [ab]
$R_{60}C_{25}W_{15}$	5.53 ± 1.50 [a]	5.73 ± 1.56 [a]	5.46 ± 1.44 [a]	5.21 ± 1.70 [a]	6.04 ± 1.65 [a]	5.01 ± 1.71 [b]	5.31 ± 1.7 [ab]
$R_{50}C_{30}W_{20}$	5.64 ± 1.60 [a]	5.69 ± 1.48 [a]	5.35 ± 1.36 [a]	5.2 ± 1.63 [a]	5.99 ± 1.55 [ab]	5.06 ± 1.74 [b]	5.20 ± 1.64 [b]

Values are mean ± standard deviation (n=80). Values in the same row followed by different letters are significantly different ($p < 0.05$). Sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Based on the set of CATA selected by untrained panellists, cowpea and WPC fortified extruded snack samples could be classified into three groups by F1 dimension. In other words, the sensory attributes of the extruded snack samples could be perceived differently in three composition ranges: 1) 100% rice flour, 2) 25 to 30% cowpea and 15 to 20% WPC fortification, and 3) 10 to 20% cowpea and 5 to 10% WPC fortification. As shown in Table 1 and Figure 2, sensory attributes were found to be affected by the fortification levels of cowpea and WPC in rice-based extruded snacks. This result indicates that consumers may experience, detect and identify different sensory attributes depending on the fortification levels. This study demonstrated that 10-20% cowpea and 5-10% WPC were more frequently characterised as having “soft”, “crunchy” and “crispy” attributes. The results presented in this paper agreed with the previous finding of the development of extruded snacks with high protein and fibre (Proserpio et al., 2020; Shah et al., 2017).

Table 8-2 Frequency counts (%) of check-all-that-apply (CATA) emotional terms used to describe the extruded snack products and results of Cochran's Q test for comparison among the samples

Emotional term	Samples						*P* value
	$R_{50}C_{30}W_{20}$	$R_{60}C_{25}W_{15}$	$R_{70}C_{20}W_{10}$	$R_{80}C_{15}W_{05}$	$R_{90}C_{10}$	RF	
Satisfied	17.1 ^{ab}	20.0 ^{ab}	12.9 ^{a}	30.0 ^{b}	21.4 ^{ab}	14.3 ^{ab}	0.046
Relaxed	24.3 ^{ab}	22.9 ^{ab}	25.7 ^{ab}	30.0 ^{ab}	34.3 ^{b}	14.3 ^{a}	0.075
Sharing	14.3 ^{a}	17.1 ^{a}	21.4 ^{a}	21.4 ^{a}	11.4 ^{a}	17.1 ^{a}	0.263
Joyful	12.9 ^{a}	18.6 ^{a}	12.9 ^{a}	17.1 ^{a}	17.1 ^{a}	8.6 ^{a}	0.392
Exciting	8.6 ^{a}	5.7 ^{a}	10.0 ^{a}	11.4 ^{a}	7.1 ^{a}	5.7 ^{a}	0.673
Balanced	18.6 ^{a}	28.6 ^{ab}	30.0 ^{ab}	28.6 ^{ab}	38.6 ^{b}	25.7 ^{ab}	0.087
Energetic	8.6 ^{a}	5.7 ^{a}	11.4 ^{a}	12.9 ^{a}	1.4 ^{a}	2.9 ^{a}	0.018
Bored	34.3 ^{a}	38.6 ^{a}	24.3 ^{a}	24.3 ^{a}	30.0 ^{a}	35.7 ^{a}	0.169
Guilty	4.3 ^{a}	2.9 ^{a}	4.3 ^{a}	1.4 ^{a}	2.9 ^{a}	0 ^{a}	0.523
Happy	14.3 ^{ab}	18.6 ^{ab}	11.4 ^{ab}	21.4 ^{ab}	25.7 ^{b}	8.6 ^{a}	0.012
Sad	12.9 ^{a}	5.7 ^{a}	4.3 ^{a}	2.9 ^{a}	4.3 ^{a}	10.0 ^{a}	0.080
Calm	27.1 ^{a}	32.9 ^{a}	32.9 ^{a}	32.9 ^{a}	31.4 ^{a}	28.6 ^{a}	0.912
Annoyed	10.0 ^{a}	11.4 ^{a}	5.7 ^{a}	7.1 ^{a}	2.9 ^{a}	15.7 ^{a}	0.076
Angry	0 ^{a}	2.9 ^{a}	4.3 ^{a}	2.9 ^{a}	1.4 ^{a}	4.3 ^{a}	0.547
Surprised	11.4 ^{a}	10.0 ^{a}	8.6 ^{a}	8.6 ^{a}	4.3 ^{a}	4.3 ^{a}	0.416
Peaceful	20.0 ^{a}	15.7 ^{a}	21.4 ^{a}	20.0 ^{a}	22.9 ^{a}	18.6 ^{a}	0.819
Contempt	8.6 ^{a}	4.3 ^{a}	11.4 ^{a}	7.1 ^{a}	10.0 ^{a}	8.6 ^{a}	0.606
Disgusted	14.3 ^{a}	11.4 ^{a}	2.9 ^{a}	2.9 ^{a}	5.7 ^{a}	14.3 ^{a}	0.009

*The same letter in superscript within same raw indicates homogeneous groups established by ANOVA ($p< 0.05$). sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

Table 8-3 Frequency counts (%) of check-all-that-apply (CATA) attributes used to describe the extruded snack products and results of Cochran's Q test for comparison among the samples

Sensory Attribute	Samples						*P* value
	$R_{50}C_{30}W_{20}$	$R_{60}C_{25}W_{15}$	$R_{70}C_{20}W_{10}$	$R_{80}C_{15}W_{05}$	$R_{90}C_{10}$	RF	
Sweet	11.4 [a]	5.7 [a]	10.0 [a]	8.6 [a]	7.1 [a]	5.7 [a]	0.673
Soft	5.7 [a]	7.1 [a]	7.1 [a]	11.4 [a]	15.7 [a]	18.6 [a]	0.017
Burnt	15.7 [a]	11.4 [a]	12.9 [a]	11.4 [a]	8.6 [a]	8.6 [a]	0.724
Sour	1.4 [a]	1.4 [a]	2.9 [a]	1.4 [a]	1.4 [a]	1.4 [a]	0.973
Crunchy	50.0 [ab]	42.9 [ab]	51.4 [ab]	57.1 [b]	48.6 [ab]	38.6 [a]	0.050
Chewy	10.0 [a]	7.1 [a]	7.1 [a]	14.3 [a]	15.7 [a]	20.0 [a]	0.081
Brittle	12.9 [a]	15.7 [a]	11.4 [a]	8.6 [a]	15.7 [a]	14.3 [a]	0.594
Bitter	12.9 [a]	12.9 [a]	7.1 [a]	8.6 [a]	5.7 [a]	4.3 [a]	0.201
Crispy	62.9 [ab]	64.3 [ab]	68.6 [ab]	75.7 [b]	62.9 [ab]	57.1 [a]	0.068
Hard	11.4 [a]	11.4 [a]	5.7 [a]	4.3 [a]	2.9 [a]	11.4 [a]	0.063
Sticky	12.9 [ab]	15.7 [ab]	5.7 [ab]	7.1 [a]	25.7 [bc]	34.3 [c]	<0.0001
Firm	14.3 [a]	11.4 [a]	12.9 [a]	11.4 [a]	11.4 [a]	12.9 [a]	0.988
Bland	28.6 [a]	30.0 [a]	30.0 [a]	30.0 [a]	31.4 [a]	25.7 [a]	0.956
Dry	47.1 [a]	52.9 [a]	50.0 [a]	47.1 [a]	50.0 [a]	47.1 [a]	0.944
Off-flavour	21.4 [a]	17.1 [a]	18.6 [a]	14.3 [a]	12.9 [a]	20.0 [a]	0.525
Mealy	7.1 [a]	10.0 [a]	8.6 [a]	8.6 [a]	14.3 [a]	11.4 [a]	0.520
Dairy	4.3 [a]	1.4 [a]	5.7 [a]	7.1 [a]	4.3 [a]	4.3 [a]	0.291
Salty	7.1 [a]	8.6 [a]	2.9 [a]	2.9 [a]	4.3 [a]	1.4 [a]	0.222
Umami	5.7 [a]	7.1 [a]	8.6 [a]	2.9 [a]	1.4 [a]	1.4 [a]	0.143

*The same letter in superscript within same raw indicates homogeneous groups established by ANOVA ($p< 0.05$). sample codes: RF (100% rice flour); $R_{90}C_{10}$ (90% rice flour + 10% cowpea flour); $R_{80}C_{15}W_{05}$ (80% rice flour + 15% cowpea flour + 5% WPC); $R_{70}C_{20}W_{10}$ (70% rice flour + 20% cowpea flour + 10% WPC); $R_{60}C_{25}W_{15}$ (60% rice flour + 25% cowpea flour + 15% WPC); $R_{50}C_{30}W_{20}$ (60% rice flour + 25% cowpea flour + 15% WPC)

8.3.3 Attitudinal and JAR (Just-About-Right) analysis

In this case, to find out the degree of correlation between instrumental and sensory attributes, Pearson correlation coefficients were determined (Table 8.4). There were no significant correlations between instrumental colour and sensory colour (r = 0.68 and 0.87, respectively) and crispiness and hardness with sensory texture (r = 0.48 and 0.49, respectively). Significant correlations were found between hardness and b* value (r = 0.86, $p<0.05$) and crispiness and L* value (r =0.64, $p<0.05$), respectively. The texture of a product is a critical sensory attribute of extruded snacks. Some studies have shown that instrumental texture analysis correlates with sensory results of extruded snacks (Paula & Conti-Silva, 2014). This outcome may be due to the variation between selected instrumental techniques and the way consumers perceive food characteristics; because mouth feel, texture and colour perception are all highly variable among individuals and those factors are strongly influenced by the environment (Paula & Conti-Silva, 2014). The PCA (Figure 8.2a) allowed discrimination between the formulations and instrumental/sensory variables. The fortification of the snacks with cowpea flour (20%-30%) and WPC (10%-20%) lead to an increase in hardness, and a* and b* colour parameters. The snacks fortified with 10%-15% cowpea flour and 5% WPC showed the highest liked texture and hedonic scores for most of the sensory attributes.

A bi-plot of correspondence analysis (Figure 8.2b), explaining 69.83% of the total variance, illustrates the associations between samples and the emotional attributes. More specifically 10-15% cowpea and 5% WPC incorporated snacks were associated with “happy”, “balanced”, “joyful”, “relaxed”, “satisfied”, “peaceful”, “calm” and “exciting”. Based on the CATA emotional terms selected by panellists, extruded snacks samples could be classified into two groups, i.e., 1)

10-20% cowpea and 5-10% WPC, and 2) 25-30% cowpea and 15-20% WPC, mainly by the X-axis (F1) that explains 48.30% of the total variance. According to Principal Coordinate Analysis (PCoA; Figure 2), overall liking was associated with the attributes firm, crispy, crunchy and sticky.

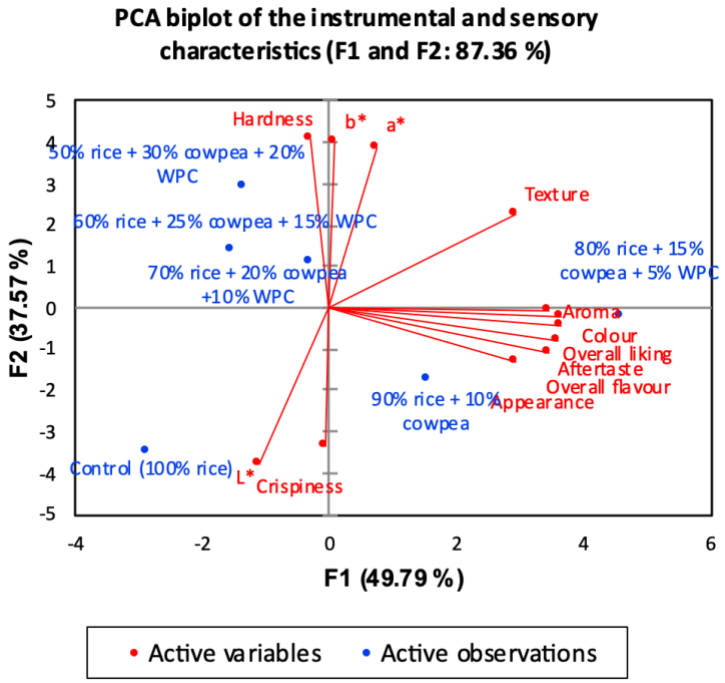


Figure 8-2 Principal component analysis (PCA) for the levels of substitution of rice flour by cowpea flour and WPC (5%, 10%, 15%, 20%, 25% and 30% w/w) in relation to the Instrumental colour, texture and sensory scores

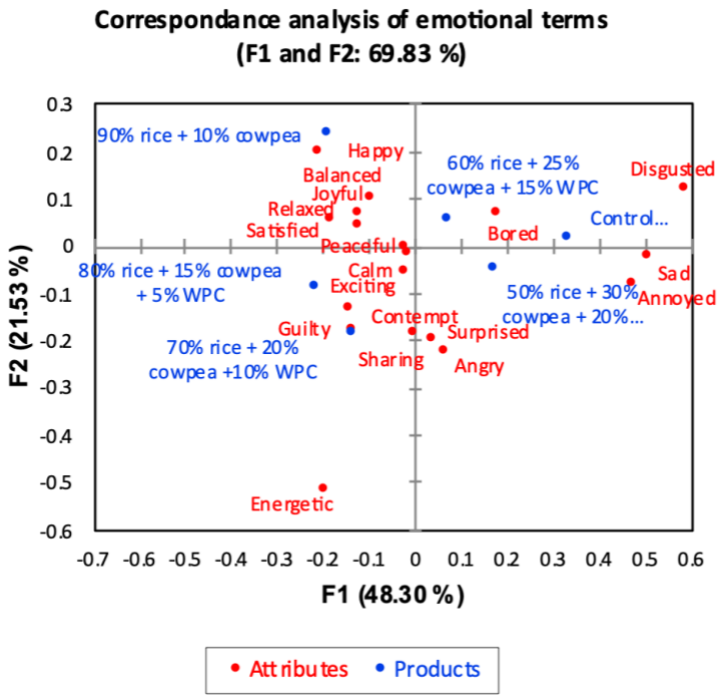


Figure 8-3 A bi-plot drawn by the correspondence analysis for CATA (Check-All-That-Apply) emotions

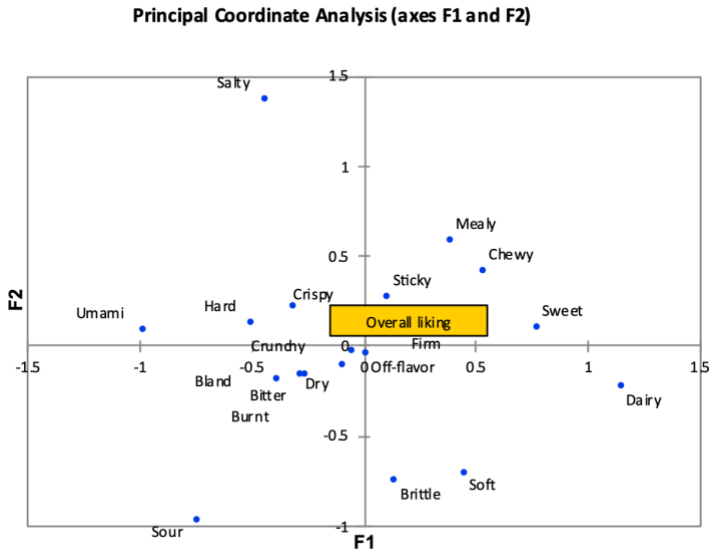


Figure 8-4 Principal coordinates analysis (PCoA) for overall liking and CATA attributes

Figure 8.4 contains the JAR (Just-About-Right) results measured for the following product attributes: colour, crispiness and hardness. JAR scale values of 1 of 1–3 categorised them as “Too Little,” “Just-About-Right” and “Too Much,” respectively. The JAR scale quantifies the optimum intensity of sensory attributes using bipolar scales. The percentage of panellists that rated the attributes as just-about-right increased with 10-25% cowpea and 5 -15% WPC for colour and hardness attributes and 15-25% cowpea and 5-15% WPC for crispiness. A large improvement in JAR values was observed in 15% cowpea flour and 5% WPC addition for colour, crispiness and hardness attributes 83%, 89% and 94% respectively.

Table 8-4 Correlation values between instrumental and sensory attributes

Variables	Appearance	Colour	Aroma	Overall flavour	Texture	Aftertaste	Overall liking	Crispiness	Hardness	L*	a*	b*
Appearance	**1**	**0.870**	0.791	0.732	0.294	0.780	0.731	0.071	-0.222	0.216	-0.104	-0.217
Colour	**0.870**	**1**	**0.875**	0.801	0.656	**0.857**	**0.894**	0.034	-0.069	-0.181	0.219	0.089
Aroma	0.791	**0.875**	**1**	**0.930**	0.738	**0.985**	**0.971**	-0.019	-0.128	-0.242	0.142	-0.049
Overall flavour	0.732	0.801	**0.930**	**1**	0.644	**0.932**	**0.951**	0.105	-0.329	-0.119	-0.139	-0.310
Texture	0.294	0.656	0.738	0.644	**1**	0.671	0.768	-0.360	0.359	**-0.818**	0.618	0.480
Aftertaste	0.780	**0.857**	**0.985**	**0.932**	0.671	**1**	**0.974**	0.149	-0.277	-0.139	0.054	-0.142
Overall liking	0.731	**0.894**	**0.971**	**0.951**	0.768	**0.974**	**1**	0.108	-0.217	-0.260	0.114	-0.069
Crispiness	0.071	0.034	-0.019	0.105	-0.360	0.149	0.108	**1**	**-0.907**	0.643	-0.556	-0.589
Hardness	-0.222	-0.069	-0.128	-0.329	0.359	-0.277	-0.217	**-0.907**	**1**	-0.735	0.804	**0.860**
L*	0.216	-0.181	-0.242	-0.119	**-0.818**	-0.139	-0.260	0.643	-0.735	**1**	**-0.826**	-0.781
a*	-0.104	0.219	0.142	-0.139	0.618	0.054	0.114	-0.556	0.804	**-0.826**	**1**	**0.977**
b*	-0.217	0.089	-0.049	-0.310	0.480	-0.142	-0.069	-0.589	**0.860**	-0.781	**0.977**	**1**

L*, Lightness; a*, Redness; b*,Yellowness

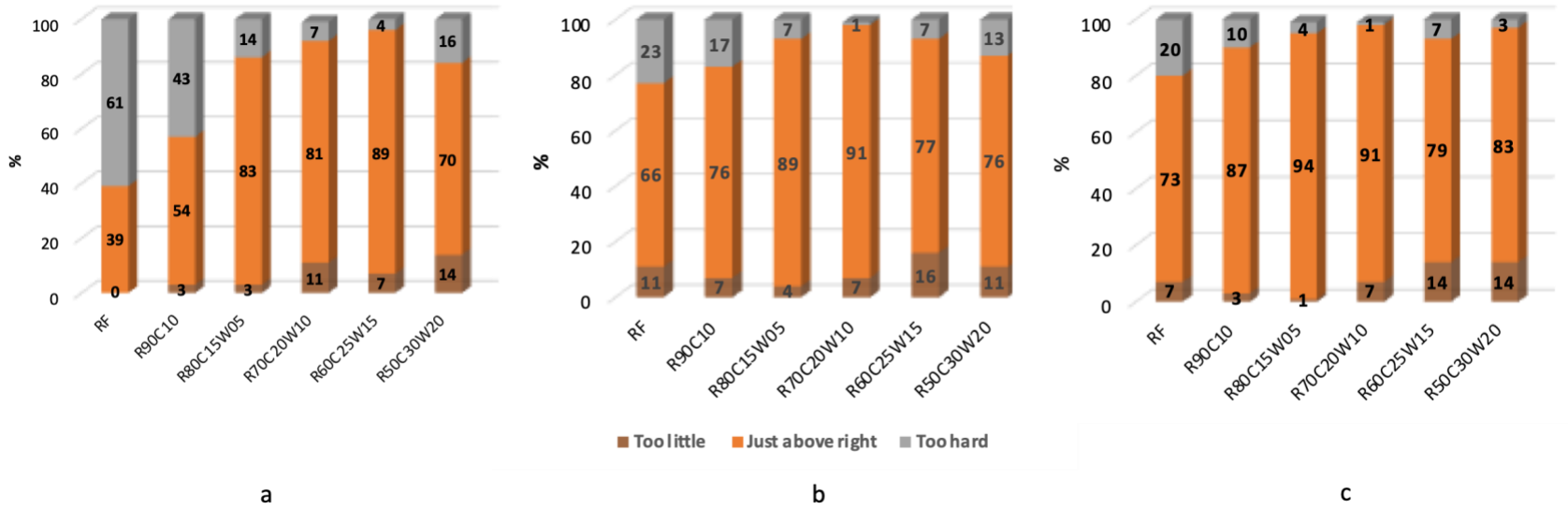


Figure 8-5 JAR results from consumer acceptance test for extrudates with (a) Colour; (b) Crispiness; (c) Hardness

8.4 Conclusions

Fortification, of rice based extruded snacks, with cowpea and WPC did not significantly affect the sensory attributes of the extrudates. Overall flavour scores were indicative that consumers did not detect beany flavours or that nutty smells could be desirable to consumers. Cowpea–WPC fortified gluten-free extruded snack containing 10–15% cowpea flour and 5% WPC was found to exhibit the most desirable consumer acceptability. The study demonstrated that extrusion cooking could be used to develop acceptable and nutritious composite snacks from protein-rich legumes. Thus, ingredient complementation can be strategically applied in the development of gluten-free extruded with cowpea and WPC to boost nutritional quality and sensory acceptability.

Chapter 9

The Influence of Light Emitting Diodes on the Nutritional, Phenolic and Antioxidant Properties in Cowpea Sprouts

Abstract

The effects of various light emitting diodes (LEDs), such as red, blue, and white fluorescent lamps and darkness, on the nutritional components, mineral content, levels of phenolic compounds and antioxidant activities in cowpea sprouts were evaluated. The protein and total dietary fibre concentrations of the cowpea sprouts significantly increased with blue and red LED lights compared to the dark conditions. The phytic acid, trypsin inhibitory activity, raffinose series oligosaccharides and starch fractions of cowpea sprouts germinated under the light conditions were not significantly different from those germinated under light conditions. The total phenolic and antioxidant properties of the cowpea sprout under the white and red LED light were significantly higher when compared to the blue and dark environment. The red LED grown cowpea sprouts had the highest content of mineral followed by white, blue, and dark conditions, respectively. Taken together, the application of red LED light or the white fluorescent light source is a good practice for the improvement of nutritional, total phenolics and antioxidant properties of cowpea sprouts.

Key words: cowpea sprouts, light emitting diode (LED), nutritional components, total phenolics, mineral

9.1 Introduction

Legumes are important source of food in the human diet as they provide essential macro and micro nutrients for nutrition and health. The current recommended daily allowance (RDA) for protein is may be higher, more than 1.1-1.2 g protein/kg bodyweight for adults of all ages, except pregnant and lactating women. Recent studies have linked the high consumption of animal origin proteins to certain non-communicable diseases such as cardiovascular diseases, overweight, diabetes, cancers and bone diseases, and premature death (Arnett et al., 2019; de Oliveira Mota et al., 2019; Ohanenye et al., 2020) was reported that the substitution of some red meat with plant-based diets in United States adults lowered the risk of type 2 diabetes by 16–35%, suggesting that inclusion of plant-based foods in the diet has some advantages. The benefit of substituting meats with plant-based proteins includes providing the body with fibres, proteins, phytosterols, and beneficial lipids (Ohanenye et al., 2020).

During seed germination, a series of physiological and biochemical processes occur: (1) the imbibed seeds germinate, the radicle and hypocotyl elongate and the cotyledon expands; (2) the content of anti-nutritional factors decreases; (3) macromolecules (such as polysaccharides and fats) are transformed into small molecules (such as oligosaccharides and free amino acids), which increases their digestibility, and (4) the content of bioactive phytochemicals and the antioxidant capacity increases (Zhang et al., 2020a). Recently, several studies have highlighted the beneficial effects of having LED light during germination on the accumulation of phytonutrients in sprouts (such as phenolic compounds, carotenoids, glucosinolates, photosynthetic pigments and vitamins) and delaying ageing, reducing nutrient loss and extending shelf life (Brazaitytė et al., 2016; Jin et al., 2015; Lee et al., 2017;

Qian et al., 2016). However, the effects of different types of LED lamps on the nutritional composition of cowpea sprouts had not been investigated.

In this study, cowpeas were germinated out under different types of light, including red LED, blue LED, white fluorescent lamps (w) and in darkness (D), to investigate the effects of LED light quality on nutritional, TPC, and antioxidant properties.

9.2 Materials and Methods

9.2.1 Plant materials and growth conditions in LED chambers

Cowpeas and growth conditions were set up as described in 3.1.3.

9.2.2 HPLC analysis for phenolic compounds

The HPLC analysis was carried out as described in 3.2.7

9.2.3 Mineral content

Mineral content was analysed as described in 3.2.8

9.2.4 Total phenolic content and antioxidant properties

Antioxidant properties and TPC were analysed as described in 3.2.9

9.2.5 Total starch content and resistant starch content

Total starch, resistant and soluble, were analysed as described in 3.2.10 and 3.2.11

9.2.6 Soluble, Insoluble and total fibre content

Fibre content was determined as described in 3.2.12

9.2.7 Raffinose series oligosaccharides

Raffinose series oligosaccharides were determined as described in 3.2.13

9.2.8 Phytic acid content

Phytic acid content was determined as described in 3.2.14

9.2.9 Trypsin inhibitory activity

Trypsin inhibitory activity was determined as described in 3.2.15

9.3 Statistical Analysis

Statistical analysis was carried out as described in 3.3

9.4 Results and Discussion

9.4.1 Effect of LED light types on TPC and antioxidant properties of cowpea sprouts

According to the results of this study different types of light had different effects on the total phenolic content (Figure 9.2a). The TPC content of cowpea sprouts grown under white, blue, red and dark was 4.6, 3.9, 4.4 and 4.0 times that of the non-germinated cowpeas, respectively. The highest TPC was found in white lamp light-grown sprouts which was significantly different from those germinated under other LED light conditions. Consequently, from the aspect of the total phenolic, the white light was determined to be the optimal chamber conditions to produce cowpea sprouts with high phenolic content in this study. Conversely, several studies reported that blue and red LED light can increase the phenolic compounds content in pea, buckwheat, cabbage, and broccoli sprouts (van Hung et al., 2020).

Figure 9-1 Appearances of cowpea sprouts after 6 days of germination period under different LED chambers (a) dark (b) blue (c) red (d) white

Light is one of the most important environmental factors for plants, as it provides the source of energy for photosynthesis, and the signal for a multitude of physiological responses. The photosynthetic process is affected by the light quality (wavelength), light quantity (intensity), photoperiod (duration) and direction (Zhang et al., 2020a). LEDs have been reported as a viable alternative to conventional artificial sources due to their benefits such as flexible light quality, longer lifespan, more safety, lower radiant heat emission than other lamps and the fact that they convert more electricity into light compared to conventional artificial light sources (Balasundram et al., 2006; Rashidi et al., 2021). Phenolic compounds are products of secondary metabolism in all higher plants, they are mainly synthesised by the shikimic acid, pentose phosphate and phenylpropanoid pathways (Balasundram et al., 2006). The polyphenols have been documented to provide several health benefits, including antioxidant, anti-carcinogenesis, anti-viral, antimicrobial, anti-inflammatory, anti-mutagenic, and anti-diabetic potential (Liu et al., 2016; Singh et al., 2017b).

The antioxidant activity of the cowpea sprouts was determined using the DPPH free-radical activity, ABTS cation radical scavenging activity, ferric reducing antioxidant power (FRAP) methods. Figure 9.1 shows the effect of different light types on the antioxidant activities of the cowpea sprouts. The DPPH free radical activity of cowpea sprouts under blue LED light, red LED light, white lamplight, and dark, was 1.2, 1.4, 1.5, and 1.2 times, respectively, compared to the raw cowpea sample (Figure 9.1b). The FRAP of the cowpea sprouts exposed to the blue LED light, red LED light, the white lamplight, and dark was 1.3, 1.6, 1.7, and 1.3 times, respectively, compared to the raw cowpea sample. The highest FRAP was found in the white lamp grown sprouts (Figure 9.1 c). The ABTS of cowpea sprouts under the blue LED light, red LED light, the white lamplight, and dark was 3.7, 4.0, 4.0, and 3.8 times, respectively, compared to the control (Figure 9.1 d). Overall, the highest DPPH, FRAP and

ABTS activities were found in white lamp light-grown sprouts. There were no differences observed between the DPPH, FRAP and ABTS activities of the white lamp grown sprouts and that of the sprouts grown under red LED and the white lamp light conditions. Therefore, the maximum antioxidant activity was determined to be in the red LED grown and the white lamp light conditions sprouts.

The higher contents of TPC and antioxidant activities in the cowpea sprouts compared with raw cowpea seeds could be due to the release of the bound phenolic compounds from the cell walls during germination due to enzyme hydrolysation of the binding of polyphenols with other organic substances such as carbohydrate or protein (van Hung et al., 2020). As an effect of the light, the TPC increases in germinated sprouts by improving photosynthesis through efficient absorbance of light by chlorophylls (by primarily photosynthetic pigments), as well as the malonyl-CoA pathway, which is associated with the synthesis of phenolic compounds (Qian et al., 2016). Red and blue LED lights are more efficiently absorbed by chlorophylls than white LED lamps, during the photosynthetic processes and these two types of light spectra have been most studied in plant photobiology (Zhang et al., 2020a). The red light was reported to promote photosynthesis and vegetative growth by increasing the content of chlorophyll, promoting the formation of photosynthetic apparatus (Zhang et al., 2020a).

However, this study results showed that white light was effiecient than the other LED tytes in terms of producing phenolic compounds and antioxidant properties. This could be due the fluctuation of the intensity of the LED lights during the gerimination period.

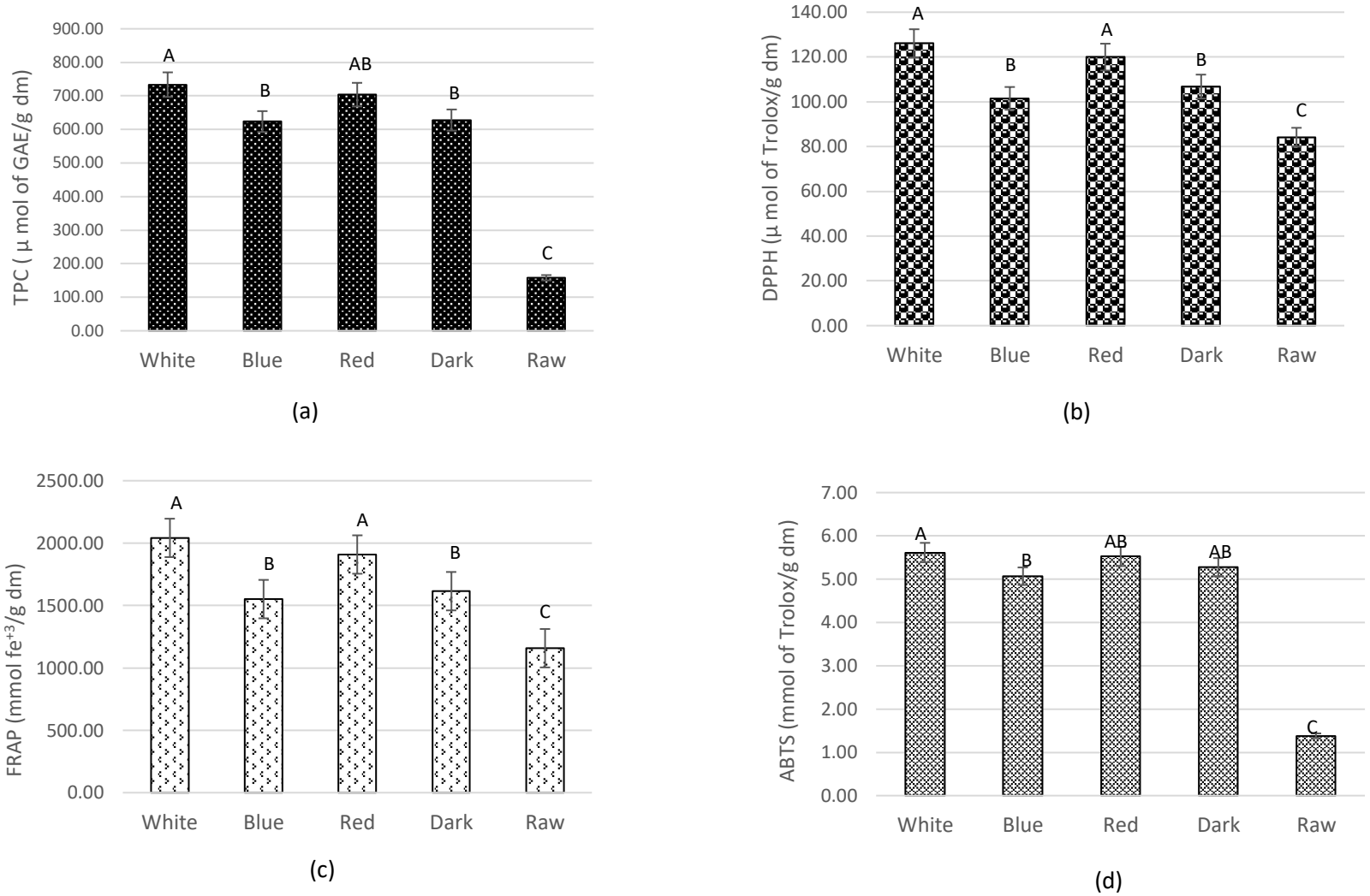


Figure 9-2The effect of light conditions on the total phenolic content (TPC) **(A)** and antioxidant capacity; DPPH assay**(B)**, FRAP assay **(C)** and ABTS assay **(1D)** of raw and germinated cowpea sprouts. Value = means (n=3). Error bars indicate standard deviation. Different capital letters indicate the samples that are significantly different from each other (*P<0.05*)

Table 9-1 Influence of different coloured light on the phenolic compounds of cowpea sprouts determined by HPLC method

Phenolic acids	Raw Seeds (ppm)	LED light types			
		White (ppm)	Blue (ppm)	Red (ppm)	Dark (ppm)
Protocatechuic acid	0.30 ± 0.0	ND	ND	ND	ND
Hydroxybenzoic acid	0.84 ± 0.0^{e}	2.27 ± 0.0^{a}	1.87 ± 0.01^{c}	1.91 ± 0.01^{b}	1.63 ± 0.0^{d}
Procyanidin B2	0.47 ± 0.0	ND	ND	ND	ND
Catechin	0.45 ± 0.0	ND	ND	ND	ND
Cyanidin-3-glucoside	0.10 ± 0.01	ND	ND	ND	ND
Peonidin-3-glucoside	0.15 ± 0.0	ND	ND	ND	ND
Malvidin-3-O-glucoside	0.83 ± 0.0	ND	ND	ND	ND
p-Coumaric acid	0.35 ± 0.0^{c}	0.38 ± 0.0^{b}	0.27 ± 0.0^{d}	0.41 ± 0.0^{a}	0.22 ± 0.0^{e}
Ferulic acid	0.73 ± 0.0	ND	ND	ND	ND
Rutin	ND	2.61 ± 0.01^{a}	1.79 ± 0.06^{d}	1.97 ± 0.02^{c}	2.48 ± 0.01^{b}
Epicatechin gallate	ND	0.44 ± 0.0^{c}	0.63 ± 0.0^{b}	0.74 ± 0.03^{a}	0.47 ± 0.04^{c}
Kaempferol	ND	0.38 ± 0.05^{ab}	0.24 ± 0.01^{bc}	0.39 ± 0.05^{a}	0.23 ± 0.01^{c}
Total	4.22 ± 0.0^{e}	6.08 ± 0.1^{a}	4.80 ± 0.1 ^{d}	5.41 ± 0.0^{b}	5.04 ± 0.0^{c}

ND, not detected; different letters in the same raw respectively indicate the significant differences at *p< 0.05* level; Values shown are mean ± standard deviation (n=3).

Table 9.1 shows the phenolic profile of the raw and geminated cowpea under the different light conditions. The results of this study indicate that the germination significantly increased the total phenolics compared to the raw counterpart. Protocatechuic acid, procyanidin B2, catechin, cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-O-glucoside, and ferulic acid were only detected in raw cowpea samples. According to the study, the accumulation of phenolic acids is affected by cultivation under different growth conditions of light types. The white and red light exposure significantly increased the total phenolics of cowpea sprouts rutin, epicatechin gallate and kaempferol acids were only detected in cowpea

sprouts compared to the dark. Hydroxybenzoric acid and p-Coumaric acid were found in both raw and cowpea sprouts. It was determined that the cowpeas germinated under white light had the highest contents of hydroxybenzoric acid and rutin. According to the results of this study, cowpea germinated under white and red-light types had significantly higher amounts of phenolic acids compared to germination under dark and blue light. This could be due to the mobilization of stored phenolics by the activation of enzymes such as polyphenol oxidase during the sprouting process(Viktorjia & Akvile, 2015). In contrast to these findings Nam et al., (2018) reported that TPC in buckwheat sprouts decreased under red LED light and significantly increased under blue LED light, as compared with white light. Numerous studies have highlighted that light exposure increased the TPC of soybean, Chinese kale and pea sprouts varieties compared to the dark-grown sprouts (Qian et al., 2016; Zhang et al., 2020b). However, Vale et al. (2014) has reported that the total phenolic content of red cabbage increased when grown in the growth chamber at a controlled light cycle (16 h/8 h, light/dark).

The mineral contents in the cowpea raw sample and sprouts under different LED light treatments are shown in Table 9.2. The germination process caused significant changes in total minerals, where the increase in total mineral content after germination was observed in all treatments compared to the raw cowpea sample. A significant increase in B, K, Mn, Mo, Na, P, S, and Zn levels was observed after sprouting compared to the raw sample. The Cr and Cu contents were not significantly changed between raw and sprouts. Phytic acid is an anti-nutritional factor that reduces the bioavailability of minerals by forming insoluble complexes. The mineral content increased due to phytase, a phytate-specific phosphatase enzyme, which is activated during the germination process and hydrolyses the phytic acid, which releases the minerals from the seeds (Liu et al., 2016). The B, K, Na, P, S and Zn

contents increased significantly under red LED light compared to the sprouts under dark conditions. The highest and lowest Ca contents were observed under red LED light (704 mg kg^{-1} dm) and dark (641 mg kg^{-1} dm), respectively. No significant difference was detected in the Cr and Cu contents among treatments. The Fe content ranged from the lowest of 67 mg kg^{-1} dm under blue LED light to the highest of 72 mg kg^{-1} dm under red LED light. The mg content was significantly lower in blue LED sprouts than red LED sprouts. This finding agrees with the literature data concerning Brassicaceae sprouts (Vastakaite & Virsile, 2015).

Studying the impact of germination on the nutritional quality of legumes may provide useful information for optimisation of the use of this legume in food products. The effect of sprouting on nutritionally focused components is shown in Table 9.3. The dietary fibre fractions are important due to their relevant physiological properties such as prevention of obesity, cardiovascular disease, type 2 diabetes, and large intestine cancer. The content of protein in cowpea spouts found in the range of 24-26% is greater than the 21% protein in the whole cowpea seeds. These values are similar to those found in the literature (Oyeyinka et al., 2021; Teka et al., 2020). Blue and red LED grown sprouts had significantly higher amounts of protein compared to dark grown sprouts. Martín-Cabrejas et al. (2008) has reported similar results with cowpea sprouts grown under light and dark conditions which was attributed to loss in dry weight, particularly carbohydrates through respiration during germination.

As seen in the table 9-3, soluble starch, resistant starch and total starch content of cowpea seeds were 45 %, 9 % and 54 % in dry matter basis, respectively. These results were in agreement with those found in the literature for whole cowpea seeds (Jayathilake et al., 2018; Ratnaningsih et al., 2020). Germination modified the content of starch in the cowpea sprouts. The contents of soluble starch, resistant starch and total starch decreased after

germination, between 49 - 50%, 53 - 61% and 50 - 52%, respectively in germinated seeds under light conditions compared to the raw cowpea seeds. Amylase activity during germination is responsible for the reduction in starch content in germinated cowpea sprouts. The starch fractions of cowpea sprouts germinated under the light conditions were not significantly different from those germinated under dark conditions.

Several studies have shown that the germination process can have a significant impact on the dietary fibre fractions and this impact is dependent on germination conditions (Dueñas et al., 2016; Vastakaite & Virsile, 2015). Germination produced a notable increase in the level of SDF and TDF in all cowpea sprouts compared to the raw cowpea seeds. Sprouts germinated in darkness had the highest amount of TDF followed by sprouts germinated under white, red and blue LED light. The IDF fraction was the main fraction of TDF since it represented 51-54% in cowpea sprouts. The dark germinated sprouts had a significantly higher amount of SDF compared to the white, red and blue LED germinated sprouts. The increase in SDF in cowpea sprouts is mainly due to the rise of cellulosic glucose during seed germination. The dietary fibre fractions in this study were not affected by the type of LED light. The reduction in dietary fibre fractions in sprouts under light exposure compared to sprouts germinated in the dark is in concordance with the previous findings by other authors (Martín-Cabrejas et al., 2008; Vale et al., 2015).

Table 9-2 Mineral content of cowpea raw seeds and sprouts germinated under different coloured light

Mineral content (mg/kg of dry mass)	Raw Seeds	LED light type			
		White	Blue	Red	Dark
B	15.17 ± 0.1^{c}	18.23 ± 0.2^{a}	17.28 ± 0.3^{b}	18.66 ± 0.1^{a}	16.87 ± 0.4^{b}
Ca	848.09 ± 27.3^{a}	645.88 ± 16.6^{b}	701.84 ± 106.7^{ab}	704.29 ± 3.0^{ab}	641.75 ± 51.7^{b}
Cr	0.33 ± 0.1^{a}	0.21 ± 0.0^{a}	0.34 ± 0.2^{a}	0.21 ± 0.0^{a}	0.37 ± 0.3^{a}
Cu	10.59 ± 0.6^{a}	12.55 ± 0.2^{a}	20.92 ± 14.8^{a}	13.08 ± 0.3^{a}	12.24 ± 0.2^{a}
Fe	63.24 ± 0.9^{c}	70.19 ± 2.6^{ab}	67.08 ± 1.0^{bc}	72.15 ±1.0^{a}	70.06 ± 2.8^{ab}
K	9985.25 ± 170.4^{e}	12232.86 ± 81.7^{b}	11842.16 ± 176.3^{c}	12833.42 ± 64.2^{a}	11507.75 ± 77.7^{d}
Mg	1947.39 ± 47.4^{b}	2018.09 ± 25.7^{ab}	1951.38 ± 21.5^{b}	2061.13 ± 27.8^{a}	2073.92 ± 45.9^{a}
Mn	17.03 ± 0.3^{a}	15.38 ± 0.2^{b}	14.24 ± 0.1^{c}	14.88 ± 0.3^{bc}	15.3 ± 0.3^{b}
Mo	1.44 ± 0.1^{c}	3.69 ± 0.1^{b}	3.57 ± 0.2^{b}	3.45 ± 0.00^{b}	4.16 ± 0.2^{a}
Na	26.47 ± 0.9^{d}	968.36 ± 8.0^{b}	839.15 ± 8.0^{c}	1154.84 ± 30.8^{a}	825.53 ± 20.0^{c}
P	3852.71 ± 34.3^{d}	5478.43 ± 14.2^{b}	5188.34 ± 49.2^{c}	5691.59 ± 131.6^{a}	5449.13 ± 81.4^{b}
S	2006.45 ± 16.9^{d}	2680.26 ± 18.2^{b}	2508.82 ± 31.0^{c}	2804.82 ± 42.3^{a}	2500.54 ± 35.6^{c}
Zn	40.34 ± 0.2^{c}	52.43 ± 0.8^{ab}	53.22 ± 3.6^{ab}	57.00 ± 0.3^{a}	51.81 ± 1.8^{b}
Total	18814.51 ± 170.1^{d}	24196.56 ± 150.0^{b}	23208.36 ± 185.6^{c}	25429.53 ± 23.89^{a}	23169.44 ± 307.9^{c}

The data are the mean ± SD. The different letters indicate statistically (*P< 0.05*) differences for the treatment in raw.

Raffinose, stachyose and verbascose are raffinose series oligosaccharides (RSOs) found in cowpea. These oligosaccharides are not digested by humans because of the lack of the hydrolytic enzyme, α -1, 6-galactosidase. Microflora in the lower intestinal tract metabolise these oligosaccharides and produce large amounts of gases, which causes flatus production. However, these RSOs are prebiotic, that is, they facilitate the growth of intestinal bifidobacteria (Sreerama et al., 2012b). The RSOs content of cowpea sprouts is significantly lower than the RSOs content of in non-germinated cowpea seeds. The reduction of 79-83% of RSOs during germination is a result of increased activity of the enzyme α-galactosidase which hydrolyses the α -1-6-glycosidic linkages thereby causing an increase in the total soluble sugar content (Martín-Cabrejas et al., 2008). No significant difference was observed between the RSOs content of dark and LED light germinated cowpeas.

The results in Table 9-3 show that, germination caused a significant reduction in TIU and phytic acid; and the percentages of reduction were in the range of 45-51% and 56-64%, respectively. The phytic acid and TIU of cowpea sprouts germinated under light conditions were not significantly different from those germinated under dark conditions. Phytic acid is an anti-nutritional factor that reduces the bioavailability of the minerals. During the germination process, phytate, an endogenous enzyme activity increased; hydrolyse phytate to liberate inorganic phosphates for plant growth. The reduction of TIU is also due to the activation of endogenous proteases. These results agreed with those of Kalpanadevi & Mohan. (2013) who reported the germination of cowpea seeds reduced the antinutrients and improved the *in vitro* protein digestibility.

Table 9-3 The effect of different coloured light on the nutritional components of cowpea sprouts

Treatment	Protein (%)	SDF (% DM)	IDF (% DM)	TDF (% DM)	RSO (millimoles/ 100 grams)	TIU /mg	Phytic Acid (mg/g)	Soluble starch (% w/w DM basis)	Resistant Starch (% w/w DM basis)	Total starch (% w/w DM basis)
Raw seeds	21.04 ± 0.1^{c}	3.15 ± 0.9^{c}	14.57 ± 1.0^{b}	17.72 ± 1.9^{c}	7.92 ± 1.7^{a}	32.51 ± 0.45^{a}	6.73 ± 0.3^{a}	45.31 ± 1.0^{a}	9.19 ± 0.2^{a}	54.50 ± 1.0^{a}
White	26.43 ± 0.7^{a}	17.98 ± 2.1^{ab}	20.04 ± 0.2^{a}	38.02 ± 1.9^{ab}	1.33 ± 0.1^{b}	17.70 ± 1.6^{b}	2.94 ± 0.0^{b}	22.42 ± 0.7^{b}	3.52 ± 0.4^{b}	25.93 ± 0.6^{b}
Blue	24.53 ± 0.1^{b}	14.96 ± 0.3^{b}	18.87 ± 1.7^{ab}	33.83 ± 1.4^{b}	1.30 ± 0.3^{b}	15.92 ± 1.6^{b}	2.36 ± 0.1^{b}	22.99 ± 2.8^{b}	4.25 ± 0.5^{b}	27.24 ± 3.3^{b}
Red	24.91 ± 0.3^{b}	16.12 ± 0.1^{ab}	18.77 ± 0.5^{ab}	34.88 ± 0.6^{b}	1.38 ± 0.1^{b}	16.88 ± 1.3^{b}	2.87 ± 0.0^{b}	22.25 ± 1.6^{b}	3.85 ± 0.4^{b}	26.10 ± 1.4^{b}
Dark	26.17 ± 0.2^{a}	19.77 ± 0.0^{a}	21.86 ± 1.5^{a}	41.64 ± 1.5^{a}	1.64 ± 0.1^{b}	15.86 ± 2.7^{b}	2.51 ± 0.0^{b}	22.76 ± 1.6^{b}	3.91 ±0.7^{b}	26.67 ± 2.2^{b}

Values are mean ± standard deviation (n=3). In each column, the different letters mean statistically (*P< 0.05*) differences in between the treatments.

9.5 Conclusion

During germination, the protein and TDF concentrations of the cowpea sprouts significantly increased with blue and red LED lights compared to the dark conditions. However, the phytic acid, TIA, RSO and starch fractions of cowpea sprouts germinated under the light conditions were not significantly different from those germinated under light conditions. The total phenolic (TPC) and antioxidant properties of the cowpea sprouts germinated under the white and red LED light were significantly higher when compared to the blue and dark environment. The red LED grown cowpea sprouts had the highest content of mineral followed by white, blue and dark conditions, respectively. Therefore, utilising red LED light and the white fluorescent light to germinate cowpea seeds prior to processing source is good practice for the improvement of nutritional, total phenolics and antioxidant properties of cowpea sprouts. Further research is needed to understand the flour functionality properties such as nitrogen solubility, water and oil absorption capacity, forming and emulsifying properties.

Chapter 10

General Discussion

10.1 Aims and Hypothesis

Ready-to-eat extruded snacks with high protein and fibre contents were developed from a composite flour comprising of rice flour, cowpea flour and WPC. To determine the effects of extrusion on nutritional, physicochemical, and textural properties of novel extrudates, a series of parameters were analysed, including physicochemical, textural, nutrient content, *in vitro* starch and protein digestion and antioxidant proprieties and bioactive compounds. To evaluate the influence of extrusion raw blends and extrudates were compared. Additionally, the nutritional properties of germinated cowpea seeds were evaluated under different lighting conditions during the germination process.

Responses to objectives of the research proposal:

- Raw blends composed of different combinations of cowpea, WPC and rice flour were analysed for nutritional and pasting properties.
- Ready-to-eat snacks were developed through the extrusion process and those were analysed for nutritional, physicochemical, and functional properties of extrudates.
- Raw blends and Ready-to-eat snacks were analysed for bioactive components, glycaemic response, and antioxidant properties of extrudates.
- Consumer acceptance of developed extruded snacks was analysed through sensory evaluation.

- Phenolic compounds, flatulence factors, trypsin inhibitory and antioxidant activities were evaluated in cowpea sprouts with various types of LEDs (light emitting diodes), including red (R), blue (B), and white fluorescent lamps (w) and darkness (D).

Answers to the hypothesis of the research proposal:

- Whey-cowpea fortified rice-based extruded snacks were did contain more protein, fibre, and bioactive compounds than rice-based extruded snacks (control).
- The physical, structural, and sensory properties of extrudates were negatively affected by the incorporation of cowpea flour and whey protein concentrate compared to control snacks.
- The levels of nutritional and anti-nutritional factors in cowpea sprouts were manipulated when using various types of LEDs.

10.2 Summary

The dietary preferences of consumers are shifting towards ready-to-eat foods replacing the traditional home-cooked meals due to urbanisation and modified current lifestyles (Sarkar et al., 2018). Most of the available ready-to-eat snacks are made of refined cereal flours, which are relatively high in sugar and salt, and are regarded as energy-dense but nutritionally poor foods (Brennan et al., 2013b). Rising health concerns, along with changing lifestyles and diets, have spurred demand for various snacking options such as high protein and fibre, allergen-free and vegan products. This research focused on improving the protein, fibre, bioactive compounds, and antioxidant properties of rice-based extruded snacks by combining white rice (*O. sativa L.*), cowpea (*V. unguiculata L. Walp.),* WPC in different combinations. The results in this thesis provide novel insights into a new combination of food sources to develop nutritious ready-to-eat snacks with health benefits.

Analysis of the cowpea and WPC fortified raw blends was necessary to determine the effect of the extrusion processing on the components of the experimental formulations (Chapter 4). Fortifying rice flour with cowpea and WPC significantly decreased the peak, breakdown, setback, and final viscosities and significantly increased the pasting temperature compared to the control. This could be due to the formation of inter-protein disulphide bonds due to heating, and it contributes to bonding the surface and exogenous proteins into a matrix surrounding the starch granules against water diffusion (Ding et al., 2021). Starch-protein interactions restrict the starch swelling and reduce the viscosity (Duta & Culetu, 2015), and high amounts of fibre act as a filler, reducing the starch content and thereby reducing its viscosity (Korus et al., 2017). Higher amounts of cowpea flour and WPC resulted in a reduced amount of reducing sugars being released during *in vitro* starch digestion. The proteins in the cowpea flour and the WPC may encapsulate the starch reducing enzyme hydrolysis, while the dietary fibre in the cowpea flour may also reduce the starch digestibility due to its high-water absorbing capacity (Foschia et al., 2015). An increase in the ratio of cowpea flour and WPC increased the protein and fibre contents and decreased the starch content of raw blends. The increased protein content had an impact on protein digestibility.

Nutritional, physicochemical, and textural properties of extrudates were evaluated and compared with the control (Chapter 5). The protein and fibre content in the extrudates significantly increase ($p<0.05$) with cowpea (10-30%) and WPC (5-20%) incorporation compared to the control. The extrudates with higher levels of cowpea and WPC showed a significant increase in bulk density and hardness. According to previous research, dietary fibres directly affect the expansion ratio by binding water more strongly than the starch granules and thus reducing the moisture loss at the die (Lu et al., 2018; Bisharat et al., 2013). In addition, fibre rich raw materials reduce the porosity in extrudates (Lu et al., 2020). Additionally, the increased protein content will affect expansion as the molecular structure

and conformation of protein influence the distribution of water in the matrix, thus also affecting stretching properties during extrusion cooking (Moraru & Kokini, 2003). A slight decrease of 12% was observed in the expansion of 15% cowpea and 5% WPC fortified extrudates compared to the control. The number of peaks during compression increased with incorporations of cowpea and WPC. All cowpea and WPC containing snacks were darker than the control. Browning is the development of a brown colour due to the non-enzymatic reactions through Maillard reactions or caramelisation. Research by Wani & Kumar (2016a) also showed the same trend, that high protein blends of pea flour had an increased brown colour. Significant correlations were found between the protein, fibre, colour values and textural properties. The essential and non-essential amino acid profiles increased in the extrudates, proportionally to the cowpea and WPC fortification.

Chapter 6 reports the effects of extrusion on bioactive compounds (indigestible fibre, α-galactosides, resistant starch, trypsin inhibitory activity and phytic acid), protein digestibility, amino acids, total polyphenols and antioxidant activities of gluten-free rice-based formulations with cowpea and WPC. Raw mixtures of the formulations were compared with the extruded snacks. The addition of 10-30% (w/w) cowpea significantly increased the RSO content (1.0-2.3 mmol/100g) in the raw formulations ($p<0.05$). After extrusion, the RSO content significantly ($p<0.05$) increased two to three folds in all the formulations compared to their raw counterparts. This increase could be associated with improved extraction of RSOs from the extrudate matrix due to the disruption of the cell wall during the extrusion cooking or could be due to the extrusion process itself, altering the structure of other carbohydrates (Arribas et al., 2017). As the cowpea flour proportion increased in the formulations, the trypsin inhibitory units (TIU) content increased significantly ($p<0.05$) to reach 23.83 TIU/mg (at 30% cowpea) in the raw formulations. In general, there was a

significant reduction ($p<0.05$) in TIUs from raw flours to extruded formulations (ranging from 57% to 81% of reduction). Extrusion cooking reduces the trypsin inhibitors, which confirms the thermo-liable nature of protease inhibitors. The phytic acid content (Table 2) ranged from 22.21 to 365.05 mg/100 g and 11.34 to 33.23 mg/100g in raw and extruded formulations, respectively. Regarding the extrusion effect, phytic acid content decreased significantly ($p<0.05$) after processing in cowpea fortified samples. This reduction had a range between 84% and 90 % from the initial concentration of phytic acid in raw formulations. Extrusion increased protein digestibility ($p<0.05$) and amino acid composition in snacks. This could be explained by heat and shear forces used in the extrusion process that can thermally unfold the proteins' structure and increase the surface area for (1) enzymatic reactions, (2) inactivation of the anti-nutritional factors such as phytates, and trypsin inhibitors, and (3) increases in the protein digestibility (Arribas et al., 2017). Extruded and raw samples enriched with cowpea and WPC had an increase in TPC and antioxidant activity. Extrusion significantly reduced the TPC, and antioxidant properties of extruded snacks compared to their raw counterparts. Reduction of TPC and the antioxidant properties could be due to the decomposition or alteration in the molecular structure of phenolic compounds during the extrusion process at temperatures above 80 °C (Zadernowski et al., 1999). The reduced reactivity and a decrease in phenolic compound extractability due to a x degree of polymerisation might be the reasons for the reduction of TPC in extrudates in this study.

Predicted glycaemic response, total phenolic compounds, and antioxidant properties of ready-to-eat extruded snacks formulated by combining rice flour, cowpea flour and WPC before and after *in vitro* digestion process illustrated in Chapter 7. The total reduction of released reducing sugars during the *in vitro* digestion was in the range of 14-41% at the inclusion levels of 10-30% cowpea flour CP and 5-20% of WPC, respectively. This could be due to the higher amount of protein with an increased proportion of cowpea and WPC

possibly creating a stronger protein network, hence reducing the starch availability to enzyme attack; dietary fibre fractions from legume also contributed to this reduction through slow and/or uneven hydration of polysaccharide matrix, which delays/hinders encapsulation of the protein-starch matrix until the later stage of digestion (Foschia et al., 2015). Occurrence/formation of resistant starch in a starchy material with the presence of moisture can act as a plasticiser which facilitates the retrogradation could also contribute to lowering the glycaemic properties (Beigh et al., 2020). After the *in vitro* digestion process, the antioxidant activity of the cowpea and WPC fortified snacks was more than 2-fold higher compared to their extruded counterparts. This could be due to the enzymatic hydrolysis of the starch and protein which breaks down the bonds between phenolic compounds and sugars, cell wall polysaccharides, or amines (Han et al., 2019). Phenolic compounds may undergo degradation and oxidation upon thermal processing and may bind to other components such as proteins that could reduce their extractability. Conversely, changes to cell wall components at high temperature processing or hydrolysis of insoluble-bound phenolic compounds could occur leading to increased extractability (Hachibamba et al., 2013; Wang et al., 2020b).

Chapter 8 reports the results of the sensory evaluation of ready-to-eat snacks. The cowpea and WPC fortified samples had higher scores for overall liking than the control sample made with 100% rice flour. The 15-25% cowpea and 5-15% WPC fortified samples had the highest just-about-right (JAR) scores for penalty analysis for colour and texture attributes. According to Cochran's Q test, panellists perceived and identified texture attributes; soft and crunchy terms were significant for extrudates. Overall, the analysis of all sensory attributes demonstrated that the fortification with 15% cowpea and 5% WPC had higher acceptance by consumers involved in this study. Generally, increases in legume percentage in a cereal-based product affects the sensory quality in terms of overall acceptability including texture,

colour, and flavour; legume flour is linked to “beany” perceptions (Monnet et al., 2019). The principal component analysis revealed the positive associations between the groups, crispiness, and L* value, and hardness b* and a* values, respectively. Fortification of rice-based snacks with cowpea and WPC is a valuable alternative with high protein and a sustainable source of fibre in gluten-free extrudates for consumers.

Intake of dietary phytochemicals rich food through diet or as an ingredient in food processing was proven to reduce the incidence of non-communicable chronic diseases and certain infectious diseases. Cowpea is an important source of bioactive compounds and has shown remarkable and diverse health-promoting properties. Recently, several studies have highlighted the beneficial effects of having LED light during germination on the accumulation of phytonutrients in sprouts (such as phenolics, carotenoids, glucosinolates, photosynthetic pigment and vitamins) and delaying ageing, reducing nutrient loss and extending shelf life (Brazaitytė et al., 2016; Jin et al., 2015; Lee et al., 2017; Qian et al., 2016). Chapter 9 reports the effects of different LEDs on cowpea seed germination. The protein and TDF concentrations of the cowpea sprouts significantly increased with blue and red LED lights compared to the dark conditions. The phytic acid, trypsin inhibitory activity, raffinose series oligosaccharides and starch fractions of cowpea sprouts germinated under the light conditions were not significantly different from those germinated under light conditions. The observation of reduction of the above factors could be due to the activation of endogenous enzymes during the germination process (Kalpanadevi & Mohan, 2013). The total phenolic and antioxidant properties of the cowpea sprouts germinated under the white and red LED light were significantly higher when compared to the blue and dark environment. As an effect of the light, the TPC increases in germinated sprouts by improving the malonyl-CoA pathway, which is associated with the synthesis of phenolic compounds (Qian et al., 2016). Red light has been reported to promote photosynthesis and vegetative growth by increasing

the content of chlorophyll, promoting the formation of photosynthetic apparatus (X. Zhang et al., 2020a). The red LED grown cowpea sprouts had the highest content of mineral followed by white, blue and dark conditions, respectively.

10.3 Conclusion

This study confirmed that the cowpea and WPC fortification of rice flour obtained a clear benefit in terms of enhancing the nutritional properties of rice-based extruded snacks. The cowpea and WPC enriched products had a significant reduction in glucose release during the *in vitro* digestion and increase in the protein digestibility, bioactive compounds and antioxidant capacities compared to the control, which illustrates that there is a great potential of using cowpea and WPC in manipulating the postprandial glucose response of extruded products. *In vivo* analysis is necessary to confirm these functional benefits.

10.4 Recommendation of Future Work

Future work is required to investigate the effect of different extrusion conditions on these nutritional, functional and sensory qualities and find out the optimum operating conditions for extrusion. The germination of cowpea seeds under the different light sources has nutritional benefits over dark conditions. Future research is required to adequately explain the phenolic compounds biosynthesis mechanism and mineral bioavailability in cowpea sprouts that are cultivated using light lamps. It is needed to understand the cowpea flour functionality properties such as nitrogen solubility, water and oil absorption capacity, forming and emulsifying properties.

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Appendix B

Chemicals, Enzymes and Buffers Used for Analysis

B.1 Chemicals, Buffers and enzyme required for starch digestibility analysis:

a) 3,5-dinitrosalicylic (DNS) mixture (0.45 M)

DNS (MW =228.12g/mol) (10 g) was dissolved in 400 mL of 2 M NaOH at room temperature with vigorous stirring. Sodium potassium tartrate tetrahydrate (MW = 282.22 g/mol) (300 g) was dissolved in 500 mL of RO water, then these two solutions were mixed then the volume made 1 L using RO water, and the absorbance was read with help of a spectrophotometer (VWR, V-1200) at 530 nm.

b) Sodium maleate buffer (0.1 M, pH 6)

Maleic acid (11.6g) was dissolved in 800 mL water then the pH was adjusted to pH 6 using 4 M NaOH. Calcium chloride (0.23g) was added to the solution followed by 0.2 g of sodium azide. The volume of the solution was adjusted to 1 L with RO water in a volumetric flask.

c) Sodium acetate buffer (0.1 M, pH 5.2)

Sodium acetate trihydrate (13.6 g) was added to 800 mL water, the pH was adjusted to pH 5.2 using 0.1 M acetic acid, then 4 mL of 1 M $CaCl_2$ was added and made to volume up to 1 L with RO water.

d) Sodium hydroxide solution (4 M)

Sodium hydroxide pellets (32 g) was dissolved in 150 mL RO water and transferred to a 200 mL volumetric flask and made to volume up to 200 mL with RO water.

e) Acetic Acid Solution (0.1 M)

Acetic acid (57.5 mL) was dissolved in 500 mL of RO water and made to volume up to 1000 mL with RO water.

f) Calcium Chloride Solution (1 M)

Calcium chloride (22.0 g) was dissolved in 150 mL of RO water and made to volume up to 200 mL with RO water.

g) Hydrochloric Acid solution (1 M)

Concentrated hydrochloric acid (16.5 mL) was mixed with 100 mL RO water in a 200 mL volumetric flask and made to volume up to 200 mL with RO water.

h) Hydrochloric Acid solution (0.05 M)

Hydrochloric acid (1 mL) was mixed in a 20 mL volumetric flask and made to volume up to 20 mL with RO water.

i) Sodium Bicarbonate solution (1 M)

Sodium hydrogen carbonate (42 g) was dissolved in 400 mL RO water and transferred to a 500 mL volumetric flask and made to volume up to 500 mL with RO water.

j) Glucose Standard Solution (5mg/mL):

D-glucose (1g) was dissolved in 150 mL RO water, transferred to a 200 mL volumetric flask and made volume up to 200 mL with RO water. Stored as 5 mL aliquots in the freezer at -20°C.

k) Glucose standard solution (10 mg/mL):

D-glucose (2 g) was dissolved in 150 mL RO water and transferred to a 200 mL volumetric flask and made volume up to 200 mL with RO water. Mixed well. Stored as 5 mL aliquots in the freezer at -20 °C.

l) Ethanol:

Ethanol (99.5 %) was purchased from Sigma Aldrich, St Louis USA.

m) Pepsin, Amylogluocosidase, Pancreatin and Invertase:

Invertase (300U/mg in 50 % glycerol, stored at -20 °C), amyloglucosidase (3260 u/mg, Megazyme Inc. Wicklow, Ireland, Pepsin (Acros Organics, New Jersey, USA CAS:901-75-6), and pancreatin (EC: 232-468-9, CAS: 8049-47-6, activity: 42362 FIP-U/g, Applichem GmbH, Darmstadt, Germany) were used as enzymes.

B.2 Chemicals and enzymes required for the protein digestibility analysis

a) 0.1 M HCl solution

Concentrated (35 %) hydrochloric acid (8.18 mL) MW (HCl) was mixed in 1000 mL RO water.

b) 0.1 M NaOH solution

Sodium hydroxide (4.0 g) was dissolved in 1000 mL RO water.

c) Trypsin, chymotrypsin and protease enzyme:

Pepsin (1031 U/mg), pancreatin (350 U/mg), Trypsin (2000 U/mg), chymotrypsin (40 U/mg) and protease (5 U/mg) from porcine gastric mucosa, were purchased from Sigma Aldrich, St Louis USA.

B.3 Chemicals and buffers required for total phenolic content (TPC) and antioxidant analysis

n) 0.2 N Folin-Ciocalteu reagent

Folin-Ciocalteu reagent (2 N) was purchased from Sigma Aldrich, St Louis USA. Folin-Ciocalteu reagent (2 N) (20 mL) was placed in a 100 mL volumetric flask and made to volume with RO water.

o) 7.5 % sodium carbonate (Na_2CO_3)

Sodium carbonate (7.5 g) was dissolved in 100 mL RO water.

p) Gallic acid solution (5 mg/mL)

Gallic acid (3,4,5-trihydroxybenzoic acid) (0.5 g) was dissolved in 100 mL volumetric flask with RO water and it was used as a stock solution to prepare the standard series.

q) Methanol (70%)

700 mL of analytical grade methanol was diluted up to 1L with RO water.

r) 200 µM of Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) stock solution

1 mL of 2 mM Trolox (0.025g of Trolox was dissolved in 50 mL of 70% methanol) was diluted to 10 mL with 70% methanol.

s) 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl)

DPPH (3.94 mg) was dissolved in 100 mL of methanol.

t) 7 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) stock solution

ABTS (0.0384 g) was dissolved in 10 mL of RO water.

u) 100 mM potassium persulfate($K_2S_2O_8$) stock solution

$K_2S_2O_8$ (0.27 g) was dissolved in 10 mL of RO water.

v) Phosphate buffered saline solution (PBS)

One tablet of PBS was dissolved in 200 mL of RO water.

w) Acetate buffer (300 mM, pH=3.6)

Glacial acetic acid (16 mL) was added to sodium acetate trihydrate (3.1 g); then the solution was made up to 1 litre using distilled water. The pH of the solution was checked using a pH meter.

x) Hydrochloric acid (40 mM)

HCL (MW=36.46 g/mol, Density=1.2 g/mL, Concentration= 37% w/w) (0.205 mL) was dissolved in 100 mL of RO water.

y) 10 mM TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) solution

TPTZ (0.031 g) was added to 10 mL of 40 mM HCl and dissolved at 50 °C.

z) 20 mM Ferric chloride solution

Ferric chloride (0.054 g) was dissolved in 10 mL of distilled water.

aa) 1 mM Ferrous Sulphate ($Fe_2SO_4.7H_2O$) stock solution

$FeSO_4 \cdot 7H_2O$ (0.139 g) was dissolved in 500 mL of RO water.

B.4 Buffer used for raffinose series oligosaccharides analysis

bb) Sodium acetate buffer (50mM, pH 4.5)

Glacial acetic acid (2.9 mL) was added to 900 mL of distilled water. The pH was adjusted to 4.5 using 1 M sodium hydroxide and the final volume made up to 1L. The buffer solution was stored at 4 °C.

B.5 Enzymes, buffers and chemicals used for fibre analysis

a) Enzymes

α- Amylase, heat-stable (E-BLAAM, Megazyme); 3,000 Ceralpha Units/mL

Protease (E-BSPRT, Megazyme); 50 mg/mL; 350 Tyrosine Units/mL

Amyloglucosidase (E-AMGDF); 200 pNP β-maltoside Units/mL (or 3,300 Units/mL on soluble starch)

b) 0.05 MES/TRIS buffer (pH 8.2 at 24 °C)

2(N-morpholino)ethanesulfonic acid (MES) (19.52 g) and tris (hydroxymethyl) aminomethane (TRIS)(12.2g) were dissolved in 1.7 L of deionised water. 6.0 N NaOH was used to adjust the pH to 8.2 and volume made up to 2L.

c) Hydrochloric acid (0.561 N) solution

6 N HCl (93.5 mL) was added to approx. 700 mL of water in 1 L volumetric flask and volume was made up to 1 L.

B.6 Buffers used for total starch analysis

a) Sodium acetate buffer (100 mM, pH 5.0) plus 5 mM calcium chloride

Glacial acetic acid (1.05 g/mL) (5.8 mL) was added to 900 mL of distilled water and pH was adjusted to 5.0 using 1 M NaOH. Calcium chloride dihydrate (0.74 g) was added and mixed well. Final volume was adjusted to 1L.

b) Sodium acetate buffer (200 mM, pH 4.5) plus 5 mM calcium chloride

Glacial acetic acid (1.05 g/mL) (11.6 mL) was added to 900 mL of distilled water and calcium chloride dihydrate (0.74 g) was added and mixed well. pH was adjusted to 4.5 using 1 M NaOH. The final volume was adjusted to 1L.

c) Sodium acetate buffer (600 mM, pH 3.8) plus 5 mM calcium chloride

Glacial acetic acid (1.05 g/mL) (69.6 mL) was added to 1600 mL of distilled water and pH was adjusted to 3.8 using 4 M NaOH. Calcium chloride dihydrate (1.48 g) was added and mixed well. The final volume was adjusted to 2L.

d) Sodium hydroxide (1.7 M)

Sodium hydroxide pellets (68 g) were added to 900 mL of RO water and dissolved. The final volume was made up to 1 L.

B.7 Buffers used for resistant starch analysis

a) Sodium maleate buffer (100 mM, pH 6.0) plus 5 mM calcium chloride dehydrate

Maleic acid (23.2g) was dissolved in 1600 mL of distilled water and the pH was adjusted to 6.0 with 4M sodium hydroxide. Calcium chloride dihydrate ($CaCl_2.2H_2O$) (1.47g) and sodium azide (0.4g) was added, dissolved, and adjusted the volume up to 2 L.

b) Sodium acetate buffer (1.2 M, pH 3.8)

Glacial acetic acid (1.05 g/mL) (69.6 mL) was added to 800 mL of distilled water and pH was adjusted to 3.8 using 4 M NaOH. The final volume was adjusted to 1L.

c) Sodium acetate buffer (100 mM, pH 4.5)

Glacial acetic acid (1.05 g/mL) (5.8 mL) was added to 900 mL of distilled water and pH was adjusted to 4.5 using 4 M NaOH. The final volume was adjusted to 1L.

d) Potassium hydroxide solution (2 M)

KOH (112.2g) was added to 900 mL of deionised water, dissolved and made up volume to 1L.

B.8 Buffers and solutions used for trypsin inhibitor activity

a) Tris buffer (50 mM, pH 8.2 containing 10 mM $CaCl_2$)

Tris*(hydroxymethyl)aminomethane* (6.06 g) was dissolved with 1.11 g of $CaCl_2$ in 800 mL of RO water. Concentrated HCl was used to adjust the pH to 8.2 and the final volume made up to 1L.

b) HCl solution (1 mM, pH above 2.5 containing 2.5 mM $CaCl_2$)

12 M HCl (83.3 µL) and $CaCl_2$ (0.28 g) were mixed in RO water and the final volume was adjusted to 1L.

c) Stock trypsin solution (4 mg/mL porcine trypsin in HCl solution- above 4,000-8000 BASE units/mL)

Crystalline porcine trypsin (200 mg) was dissolved in 50 mL of the 1 mM HCl solution.

- Working trypsin solution (320 µg/mL, 320-680) – Stock trypsin solution (2 mL) was diluted to a total volume of 25 mL in a volumetric flask using the HCl solution

d) Stock BAPA solution (40 mg benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA))

BAPA (400 mg) was dissolved in 10 mL of dimethyl sulfoxide.

- Working BAPA solution (0.4 mg/mL or 0.92 mM) – Stock BAPA solution(0.25mL) was diluted with pre-warmed to 37 °C assay buffer to a total volume of 25 mL

e) Acetic acid solution (30% acetic acid quenching solution)

30% acetic acid solution was prepared by combining 150 mL of glacial acetic acid with 350 mL of RO water.

B.9 Chemicals used for phytic acid content determination

a) Colour reagent

- Solution A- Ascorbic acid (10% w/v)/ Sulphuric acid (1 M)

Ascorbic acid (10g) was added to 90 mL of RO water and 5.35 mL of concentrated sulphuric acid dissolved in it. The final volume was made up to 100 mL with distilled water.

- Solution B- Ammonium molybdate (5% w/v)

Ammonium molybdate (1.25g) was added to 20 mL of RO water and mixed well. The final volume was made up to 25 mL.

The colour reagent was prepared by adding 1 part of solution B to 5 parts of solution A on the day of use.

b) Trichloroacetic acid (50% w/v)

Trichloroacetic acid (50g) was added to 60 mL of RO water and dissolved. The final volume was made up to 100 mL.

c) Hydrochloric acid (0.66 M)

Hydrochloric acid (54.5 mL) was added to 945.5 mL of RO water.

d) Sodium hydroxide (0.75 M)

Sodium hydroxide pellets (6g) were added to 180 mL of RO water and dissolved. The final volume was made up to 200 mL.

Appendix C Sensory Evaluation

C.1 Lincoln University Human Ethics Committee (HEC) approval letter

Research Management Office

T 64 3 423 0817
PO Box 85084, Lincoln University
Lincoln 7647, Christchurch
New Zealand
www.lincoln.ac.nz

17 July 2020

Application No: 2020-30

Title: Sensory evaluation of gluten-free extruded snack

Applicant: H Dilrukshi

The Lincoln University Human Ethics Committee has reviewed the above noted application. Thank you for your response to the questions which were forwarded to you on the Committee's behalf.

I am satisfied on the Committee's behalf that the issues of concern have been satisfactorily addressed. I am pleased to give final approval to your project.

Please note that this approval is valid for three years from today's date at which time you will need to reapply for renewal.

Once your field work has finished can you please advise the Human Ethics Secretary, Alison Hind, and confirm that you have complied with the terms of the ethical approval.

May I, on behalf of the Committee, wish you success in your research.

Yours sincerely

Grant Tavinor
Chair, Human Ethics Committee

PLEASE NOTE: The Human Ethics Committee has an audit process in place for applications. Please see 7.3 of the Human Ethics Committee Operating Procedures (ACHE) in the Lincoln University Policies and Procedures Manual for more information.

C.2 Research information sheet for sensory analysis

Agriculture and Life Science, Lincoln University

RESEARCH INFORMATION SHEET

You are invited to participate as a subject in a project entitled

Sensory evaluation of gluten- free puffed snack

The aim of this project is:

To evaluate the effects of formulating gluten-free puffed snacks using rice flour, cowpea flour, and whey protein concentrate on sensory quality.

The following people will be excluded from this study:

1. Any person who does not consume puffed snack products.
2. Any person who has allergic or intolerance to rice, cowpea, and whey protein concentrates.

Your participation in this project will involve using your senses to evaluate the food products. You will look at, smell, and taste the food. You will be asked to record your perception on a worksheet after you finish evaluating the sample. At times, you may be asked to give a score of the sample in terms of appearance, texture, smell, and overall acceptance, to say how well you like or dislike a particular attribute of the food, to choose which sample you prefer most. Please find full instruction on a worksheet, and you may ask the researcher if you do not understand. You may also be asked your gender, age, and dietary preference, but it will be kept confidential and anonymous.

Participation should take in 15 to 20 minutes for evaluation. Your participation is voluntary, and you may withdraw from the project without any penalty whatsoever. You may ask the researchers not to include your data until 31.12.2020 through an e-mail.

The results of the project may be published, but you may be assured of your anonymity in this investigation: the identity of any participant will not be made public, or made known to any person other than the researcher, her supervisors and the Human Ethics Committee. To ensure anonymity, your name will not appear on any data sheets or files. The information collected, and data files stored as soft copy on a computer will only be assessed by the researchers on the project. Any result will be disseminated on public (e.g. journal, conference paper) as average data to keep individual data anonymity.

The project is being carried out by H.N.Nadeesha Dilrukshi
Contact details: Nadeesha.Hewanadungodage@lincolnuni.ac.nz
She will be pleased to discuss any concern you have about the participating the project

Main supervisor: Prof. Charles Brennan.
Contact details: Charles.Brennan@lincoln.ac.nz

Co-supervisors: Dr. Margaret Brennan
Margaret.Brennan@lincoln.ac.nz Dr. Damir Torrico
damir.torrico@lincoln.ac.nz

Head of Department: Assoc Prof. Rainer Hoffman
Contact details: 325811 ext. 30604, rainer.hoffman@lincoln.ac.nz

The project has been reviewed and approved by Lincoln University Human Ethics Committee.

Puffed Snack Sensory Tasting Test

I am Nadeesha Dilrukshi, currently a Ph.D. student in the Department of Wine, Food and Molecular Biosciences. I would like to invite you to the sensory tasting test of puffed snacks.

I would highly appreciate if you would like to share about 15 to 20 minutes of your time to taste and rate puffed snacks. If you are interested, please reply directly to this e-mail (nadeesha.hewanadungodage@lincolnuni.ac.nz) to select the time slot that best suits you. The analysis will be conducted on 4th and 5th August in RFH-086 (Sensory evaluation facility).

Time slots available (please choose one):

09.30 - 10.00 am	10.00 - 10.30 am
10.30 - 11.00 am	11.00 - 11.30 am
11.30 - 12.00 pm	12.00 - 12.30 pm
12.30 - 1.00 pm	1.00 - 1.30 pm
1.30 - 2.00 pm	2.00 - 2.30 pm
2.30 - 3.00 pm	3.00 - 3.30 pm
3.30 - 4.00 pm	4.00 - 4.30 pm

There will be six samples of puffed snacks. Ingredients used in all the samples are rice flour, cowpea flour, and whey protein concentrate in different propotions. Participation is on a voluntary basis.

Please do not participate in this test if you cannot eat puffed snacks or any of the above ingredients for any reason. Research Information Sheet and Consent Form are attached to this e-mail for your information. Feel free to contact me directly if further information is needed.

C.3 Sensory evaluation questionnaire

Sensory Evaluation of Gluten-Free puffed Snack

You are going to taste six samples. Please taste the samples from **LEFT** to **RIGHT** and record the number on each page. Rinse your mouth with water before tasting each sample.

This experiment is aiming to evaluate the sensory properties of gluten-free puffed snacks made from white rice flour, cowpea flour and whey protein concentrate. These samples contain above ingredients in different combination and proportion.

A. Participant Profile:

1. Gender:

    a. Male
    b. Female
    c. Prefer not to say
    d. Other

2. Age:

    a. ≥20 years
    b. 21-30 years
    c. 31-40 years
    d. 41-50 years
    e. 51-60 years
    f. ≤61 years

3. Ready-To-Eat Snack consumption frequency:

    a. Every day
    b. Two or three times a week
    c. Sometimes in a week
    d. Two or three times a month
    e. Sometimes in a month
    f. Occasionally

Enter your **FIRST sample number** here _____

1. Appearance

- Size and shape

Please tick **ONE** box that best describes your liking for size and shape of the sample.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

- **Colour**

Please tick ONE box that best describes your liking for size and shape of the sample.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

How would you rate the colour?

Too little	Just -about -right	Too dark

2. Aroma

Please tick **ONE** box that best describes your liking for aroma of the sample.

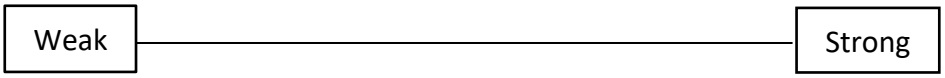
Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

3. Overall Flavour

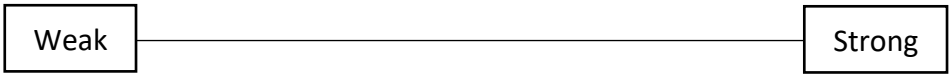
Please tick **ONE** box that best describes your liking for flavour of the sample.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

- **Raw flour flavour**



- **Burnt and bitter**

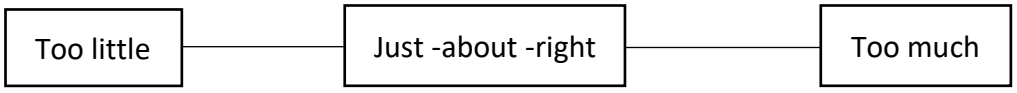


4. Texture

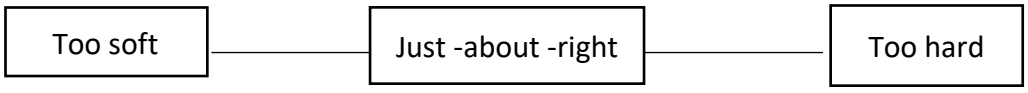
Please tick **ONE** box that best describes your liking for texture of the sample.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

- **Crispness**



- **Hardness**



5. Aftertaste

Please tick **ONE** box that best describes your liking for overall flavour of the sample.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely

6. If this product was available in the market, would you like to purchase?

Yes	No

If there any comment that would you like to share to make further improvement?

_____

_____

_____

_____

_____

Check-all-that-apply (CATA) questions: Please check all emotions elicited by this sample

Satisfied	Relaxed	Sharing	Joyful	Exciting	Balanced	Energetic	Bored	Guilty
Happy	Sad	Calm	Annoyed	Angry	Surprised	Peaceful	Contempt	Disgusted

CATA questions: Please check all emotions elicited by this sample for sensory attributes

Salty	Bitter	Sweet	Sour	umami	Burnt
crunchy	crispy	soft	chewy	mealy	Dairy

Thank you for your participation.