Film functionality of prolamins extracted from barley and sorghum brewers' spent grain

by

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DECLARATION

I, **Anesu Avril Magwere** declare that the dissertation, which I hereby submit for the degree MSc Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ...…………………………...

DATE: ………………………………….

DEDICATION

This dissertation is dedicated to my father, Chamunorwa Magwere. You have always believed in me, never once have you given up. Thank you for being an educator and a pillar in my life, I am forever grateful.

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No man is an island; therefore, I would like to express my sincere gratitude to the following for without their assistance, I would not have made it this far:

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ABSTRACT

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Commercial production of bioplastics is limited due to their high cost and inferior properties compared to synthetic plastics. Brewer's spent grain (BSG) is a low-cost brewery by-product, rich in prolamin protein and fibre with potential as a raw material for protein-based bioplastics. However, the functional properties of BSG prolamins are poorly understood.

This study characterised prolamins extracted from commercial BSG from barley malt (hordein), and from laboratory prepared BSG from waxy (high amylopectin) and high protein digestibility sorghum grain (kafirin). Cast films made from these prolamins were investigated as a model bioplastic and compared with films made from commercial zein and standard kafirin.

Barley BSG gave a lower prolamin extraction yield (9%) than sorghum BSG (37-43%). This was presumably caused by greater disulphide-bonded polymerisation, as shown by SDS-PAGE, of the hordein. This was due to industrial drying of the BSG, and because aqueous ethanol is not the optimum solvent for hordein extraction. BSG kafirin protein purity (81-88%) was higher than BSG hordein (71%) because sorghum BSG was washed to remove residual starch, thus semi-purifying it. SDS-PAGE indicated that kafirin disulphide-bonded polymerisation occurred during the sorghum brewing and BSG drying but to a lesser extent than barley.

Stereomicroscopy and scanning electron microscopy revealed that all films remained intact after soaking but developed pores. Hordein films had the lowest buffer uptake (28%), followed by the kafirins extracted from different sorghum lines (39-96%). Commercial zein films absorbed the most buffer (105%). These findings reflected the degree of polymerisation of the different prolamins, hordein being the most polymerised and commercial zein the least. Tensile properties of plasticised films revealed that hordein films were similar in strength to commercial zein films but the least extensible (126% strain). Kafirin films were the strongest with intermediate strain values.

Composite kafirin-zein films were made with the aim to produce bioplastics of intermediate functionality between the individual prolamins. Buffer uptake for kafirin-zein films increased from 50% with a high kafirin proportion to 85% with a high zein proportion because of zein's lower hydrophobicity. All composite films were less strong than kafirin films and less extensible than zein. As the proportion of kafirin increased, film tensile strength increased, and extensibility reduced due to kafirin's greater disulphide-cross-linking.

This study shows that BSG prolamins can produce bioplastics with higher resistance to moisture uptake and greater tensile strength than commercial zein bioplastics. BSG hordein bioplastics are better moisture barriers than BSG kafirin bioplastics but are less strong and less extensible. Furthermore, BSG kafirin and kafirin-commercial zein composites can make bioplastics with better moisture resistance and extensibility than zein and kafirin, respectively.

TABLE OF CONTENTS

DECLARATION i
DEDICATION ii
ACKNOWLEDGEMENTS iii
ABSTRACT iv
TABLE OF CONTENTS vi
LIST OF TABLES viii
LIST OF FIGURES ix
1. INTRODUCTION 1
2. LITERATURE REVIEW 3
2.1 The process of brewing 3
2.1.1 Lager beer 3
2.1.2 Traditional sorghum beer 5
2.2 Generation of brewer's spent grain 7
2.3 Brewer's spent grain composition 7
2.4 Current uses of brewer's spent grain 8
2.5 Structure and composition of sorghum and barley grains with respect to brewing 8
2.5.1 Sorghum 8
2.5.2 Barley 10
2.6 Characterisation of sorghum, maize and barley prolamin proteins: kafirin, zein and hordein 11
2.6.1 Kafirin and zein subclasses and structure 11
2.6.2 Functional properties of kafirin and zein 13
2.6.3 Hordein subclasses and structure 14
2.6.4 Functional properties of hordein 15
2.7 Effect of brewing on prolamins: heat induced disulphide bonding of prolamin proteins 15
2.8 Prolamin extraction methods 16
2.9 Functionality of prolamins: film formation 18
2.10 Factors influencing prolamin film properties 19
2.11 Conclusions 21
3. HYPOTHESES AND OBJECTIVES 22
3.1 HYPOTHESES 22
3.2 OBJECTIVES 24
4. EXPERIMENTAL DESIGN 25

5. MATERIALS AND METHODS 27
5.1 Materials 27
5.2 Methods 27
5.2.1 Laboratory safety measures 27
5.2.2 Laboratory-scale sorghum lager-type brewing to produce spent grain 28
5.2.3 Extraction of kafirin and hordein 29
5.2.4 Prolamin characterisation 29
5.2.5 Film formation 31
5.2.6 Analyses 34
6. RESULTS AND DISCUSSION 36
6.1 Effects of brewer's spent grain source on protein preparation yield and purity 36
6.2 SDS-PAGE of prolamins under reducing and non-reducing conditions 39
6.3 Nutritional composition of waxy normal protein digestibility (WND) sorghum flour and waxy normal protein digestibility sorghum brewer's spent grain flour 42
6.4 Amino acid composition of different prolamin preparations 44
6.5 Prolamin films 47
6.5.1 Effects of BSG hordein, WHD, WND and NWD BSG kafirin on surface morphology and film buffer uptake 47
6.5.2 Tensile properties of BSG hordein, sorghum BSG kafirins extracted from WHD, WND and NWD lines films 53
6.5.3 Effects of compositing commercial zein and standard kafirin on film surface morphology and buffer uptake 57
6.5.4 Effect of compositing commercial zein and kafirin on their film tensile properties 63
7. GENERAL DISCUSSION 66
7.1 Critical review of methodologies applied in the research 66
7.2 Major research findings 73
7.2.1 Effect of the brewing process on the functionality of BSG hordein and BSG kafirin films 73
7.2.2 Waxy (high amylopectin) and high protein digestibility sorghum lines 78
7.2.3 Effects of compositing zein and kafirin on the buffer uptake, tensile strength and extensibility of their films 79
8. CONCLUSIONS AND RECOMMENDATIONS 84
9. REFERENCES 85
10. APPENDIX 97

LIST OF TABLES

Table 1: Chemical composition of barley and sorghum brewer's grain 8
Table 2: Amounts of prolamin powder added per respective composite film mixture 32
Table 3: Amounts of prolamin powder added per respective composite film mixture 33
Table 4: Effects of spent grain source on purity and yield of protein extracted from sorghum and commercial barley spent grain 38
Table 5: Nutrient composition of waxy normal protein digestibility sorghum and waxy normal protein digestibility sorghum brewer's spent grain 43
Table 6: Amino acid composition of commercial zein, standard kafirin, commercial barley brewer's spent grain hordein and sorghum brewer's spent grain kafirin protein preparations 45
Table 7: Effect of soaking in 0.2M phosphate buffer (pH 6.8) at 39°C for 12 hours on surface area and weight on films from commercial zein, standard kafirin from sorghum flour, hordein from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines 49
Table 8: Effects of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on tensile properties of their films..55
Table 9: Effects of soaking on commercial zein and standard kafirin films and their composites in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39°C on film aqueous buffer uptake........59
Table 10: Effect of compositing commercial zein and kafirin on their film tensile properties 65
Table 11: Summary of the effects of the types of prolamin proteins on the functional properties of their films...70

LIST OF FIGURES

Figure 1: Generalised process for brewing lager beer 4

Figure 2: Generalised process for brewing commercial traditional sorghum beer 6

Figure 3: Longitudinal section of a sorghum kernel (Taylor and Belton, 2002) 9

Figure 4: Cross-section of a barley kernel 10

Figure 5: Proposed α-zein structural models 13

Figure 6: Schematic representation of the wet (casting) and dry (thermoplastic) protein bioplastic film making processes 18

Figure 7: Experimental design to characterise and to determine the functional properties of films made from commercial barley brewer's spent grain and laboratory prepared sorghum brewer's spent grain prolamins 25

Figure 8: Experimental design to determine the effects of standard kafirin and commercial zein composite films on water uptake, tensile properties and film surface appearance 26

Figure 9: SDS-PAGE of commercial zein, kafirin and hordein preparations........ 41

Figure 10**:** Appearance of prolamin films to scale, before and after immersion in phosphate buffer (pH 6.8) for 12 hours at 39°C........ 50

Figure 11: Stereomicroscopy showing the effects of soaking in 0.2M phosphate buffer (pH 6.8) for12 hours at 39°C on prolamin film surface morphology 51

Figure 12: SEM showing the effects of soaking in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39°C on prolamin film surface morphology 52

Figure 13: Effect of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on the maximum strain of prolamin films........ 56

Figure 14: Effect of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on maximum stress of prolamin films........ 56

Figure 15: Visual appearance of commercial zein and standard kafirin composite films to scale, before and after immersion in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39°C 60

Figure 16: Stereomicroscopy showing the effects of soaking on commercial zein and standard kafirin films and their composites in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39°C on film surface morphology 61

Figure 17: SEM showing the effects of soaking on commercial zein and standard kafirin films and their composites in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39^{o}C on film surface morphology 62
Figure 18: Schematic model to show the effect of disulphide bonding and the presence of impurities on the formation of a prolamin film matrix 77
Figure 19: Schematic model to explain the formation of commercial zein and kafirin composite films 81

1. INTRODUCTION

Beer is the fifth widely consumed beverage throughout the world (Aliyu and Bala, 2011). In 2014 alone, the world beer production totalled 180 million tonnes (FAOSTAT, 2014). Its production generates various wastes and by-products, and these include spent grain, spent hops and spent yeast (Dos Santos Mathias, de Melo and Sèrvulo, 2014). Brewer's spent grain can be defined as the solid residue that remains after the extraction of wort (fermentable sugars) during the brewing process (Dos Santos Mathias et al., 2014). On average, brewer's spent grain can represent up to 85% of the total by-products generated by the brewing industry (Aliyu and Bala, 2011). The average annual global production of brewer's spent grain is some 39 million tonnes (wet basis) with its main applications being animal feed (Lynch, Steffen and Arendt, 2016).

Today, the brewing industries of both developed and less developed countries are increasingly recycling waste and by-products from the brewing process to reduce pollution (Nigam, 2017). The major application for brewer's spent grain is cattle feed (Mussatto, 2014). However, the volumes of brewer's spent grain generated in breweries is vast compared to the small market for it as animal feedstock (Kerby and Vriesekoop., 2017). Consequently, brewer's spent grain has been investigated in paper manufacture, energy production and as a component that is added in brick making, among other uses (Mussatto, 2014). These applications have been studied in an effort to reduce environmental degradation caused by industrial waste pollution (Nigam, 2017).

Since brewer's spent grain is high in fibre (30-50%) and proteins (19-30%), particularly prolamin-type proteins (Taylor and Taylor, 2018) and is produced all year round in large quantities, it should not be regarded as having little economic value (Lynch et al., 2016). Thus, there is a need to develop value-added products from brewer's spent grain in order to maximise its value (Robertson, I'Anson, Treimo, Faulds, Brocklehurst, Eijsink. and Waldron, 2010). One of the potential applications for brewer's spent grain is to utilise its prolamin proteins to make biodegradable bioplastics. Bioplastics are plastics made from natural polymers and biodegradable plastics are plastics in which their degradation results from the action of naturally occurring microorganisms such as bacteria, fungi, and algae (ASTM International, 2003). Bioplastics can made from prolamins and have potential function in both food and non-food applications (Krochta, 2002). To date, there are few prolamin bioplastics available due to their high cost and inferior water barrier properties, lower tensile strength and elongation as compared to synthetic plastics (Muhiwa, Taylor and Taylor., 2017). This is because prolamins are far less uniform in structure than synthetic plastics (Taylor, Anyango and Taylor, 2013a). Prolamins extracted from brewer's spent grain have the potential to improve the economic viability and hence increase the production of commercial protein-based bioplastic products (Muhiwa et al., 2017).

The aims of this study were twofold; firstly, to characterise the prolamin proteins extracted from brewer's spent grain from commercial barley-based lager beer brewing and from laboratory-scale traditional sorghum beer brewing with different types of sorghum (waxy-high amylopectin and high protein digestibility) and secondly, to determine the functional properties of cast films prepared from these prolamin proteins with the cast films being used as a model bioplastic material.

2. LITERATURE REVIEW

This literature review focuses on lager and traditional sorghum beer, how the beer is produced, brewer's spent grain generation and composition, prolamin protein extraction methods and the chemistry and structure of prolamins. It reviews the possible modification of prolamin functionality by the brewing process and the formation of commercial zein: kafirin composites with varying mass ratio contributions. Additionally, it discusses how these modifications may affect the functional properties of the bioplastics made from these prolamins.

2.1 The process of brewing

2.1.1 Lager beer

Beer is a diverse beverage that offers consumers a variety of tastes depending on the ingredients and production processes applied (Dos Santos Mathias et al., 2014). Beer can be defined as a non-distilled, yeast fermented alcoholic product of brewer's wort made from malted cereal (barley and sorghum), which may be supplemented with starchy adjuncts such as maize or rice. Beers include lagers, ales and stouts.

Lager beer is a clear beer with an ethanol content ranging between 3 and 8% (v/v) (Waites, Morgan, Rockey and Higton, 2001). Traditionally, lager beer was made using barley malt, but more recently, non-barley larger beers have been developed using commercial enzymes and other cereals such as rice and sorghum (Taylor, Dlamini and Kruger, 2013b). In spite of these changes, the following main stages remain the same in lager beer production. These are milling, adjunct cooking, mashing, wort (fermentable sugars) preparation, yeast fermentation and maturation (Figure 2.1) (Pires and Brányik, 2015). Barley malt is milled, added to cooked starchy adjuncts, usually unmalted cereal such as barley, maize or sorghum (Pires and Brányik, 2015). This is followed by mashing. The mashing stage primarily enzymatically hydrolyses the cereal starch to produce a fermentable sugars, non-fermentable sugars and partially degrades proteins to polypeptides and amino acids (Mussatto, Dragone and Roberto, 2006). This liquid is referred to as wort. During mashing, the temperature of the mixture (cooked starchy adjuncts and malt) is slowly increased from 37°C to 78°C to promote the enzymatic hydrolyses of the cereal and malt (Gupta, Abu-Ghannam and Gallaghar, 2010). This process of mashing takes 1-2 h (Pires and Brányik, 2015). The malt is a source of carbohydrate and protein enzymes such as amylases and proteases (Sammartino, 2015). Other starch degrading enzymes such as α-glucosidase and limit dextrinase are also present in the malt.

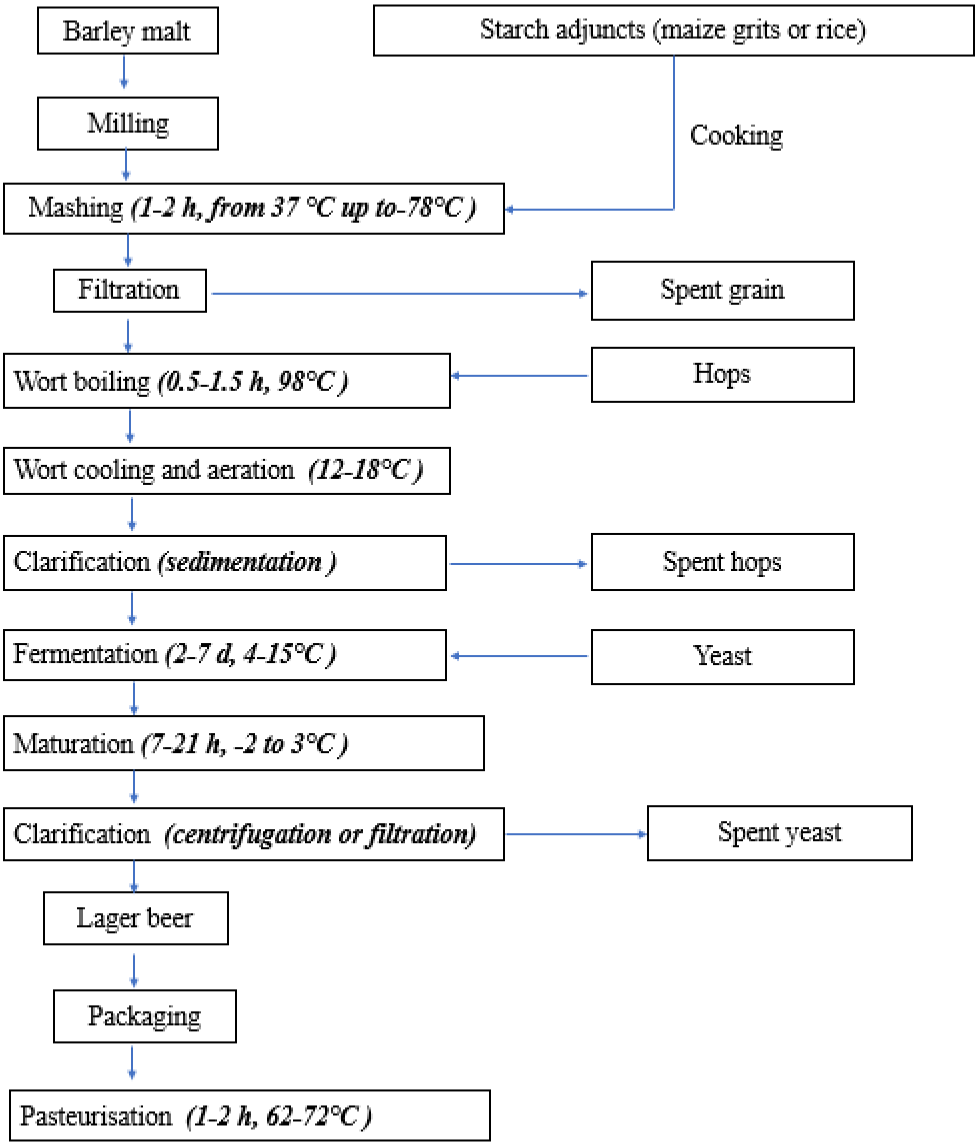

Figure 1: Generalised process for brewing lager beer (adapted from Waites et al., 2001; Pires and Brányik, 2015)

After the mashing process has been completed, wort is filtered from the spent grains. At this stage, hops are added to the wort and boiled for 30 min to 1.5 h at 98°C. Boiling the wort causes the inactivation of the enzymes from the malt and the formation of aroma and colour compounds and the isomerisation of the α-acids of hops. The wort then undergoes clarification by sedimentation or centrifugation to remove the spent hops (Waites et al., 2001). The wort is aerated, cooled to 12-18°C and yeast (*Saccharomyces cerevisiae*) is added (Pires and Brányik, 2015). When yeast is added to the wort, ethanol and carbon dioxide are produced through the fermentation, which takes between 2 and 7 days at 4-15°C (Bokulich and Bamforth, 2013). The lager beer is then matured for several days or months at 8°C or lower (Waites et al., 2001). After maturation, the lager beer undergoes further clarification at -2-3°C for 1-2 h, is packaged and then normally pasteurised at 62-72°C for 1-2 h (Waites et al., 2001).

2.1.2 Traditional sorghum beer

Traditional sorghum beer, also referred to as opaque beer, is pinkish-brown in colour, cloudy in appearance with a sour-fruity taste and is consumed whilst still microbiologically active (Mawonike, Chigunyeni and Chipumuro, 2017). Its alcohol content is low(4.5% (v/v)) and contains 5-7% suspended solids and its pH is pH 3.3-4 (Lyumugabe, Gros, Nzungize, Bajyana and Thonart, 2012). Commonly, pigmented red tannin type sorghum is used to brew the beer. These pigments which are anthocyanins, are primarily responsible for the beer colour (Taylor, 2003a).

Brewing variations exist because of differences in regional beer tastes and availability of brewery equipment. However, the brewing process of traditional sorghum beer includes milling, souring, cooking, mashing and alcoholic fermentation (Lyumugabe et al., 2012). Taylor (2003b) describes commercial traditional sorghum beer production (Figure 2.2). The initial stage is the cooking of cereal adjunct (sorghum or maize) mixed with soured sorghum malt for 1-2 h. The soured sorghum malt is produced by incubating for two days at 48-50°C a 10% sorghum malt slurry inoculated with a lactic acid bacteria culture, *Lactobacillus delbrueckii (leichmannii)* from a previously successful sour. These lactic acid bacteria carry out a lactic acid fermentation to produce lactic acid (Taylor, 2003a). The lactic acid lowers the mash pH to approximately pH 4. The low pH provides an optimum environment for proteases action (Bokulich and Bamforth, 2013). Furthermore, the low pH inhibits the growth of pathogens and slows down the rate of microbial spoilage (Taylor, 2003b).

After cooking, the mash is cooled to approximately 60°C. Sorghum malt which is a source of α- and β amylases, is then added to the cooked cereal grits and the mixture is incubated at 50-65°C for 2 h. Often, additional sorghum malt is added to the mash after the mash has been recooked at 80-100°C for 20 min. The high temperature pasteurises the mash and completely gelatinise sorghum malt starch. The mash is then strained to separate the wort from the residual solids (spent grain). The wort is cooled to 28°C and active dry yeast (*Saccharomyces* *cerevisiae*) is added to begin alcoholic fermentation. The fermentation produces ethanol and carbon dioxide. The fermentation process is 8-24 h long.

Traditional sorghum beer differs significantly from lager beer, mainly due the properties of the sorghum tropical cereal grain used (Taylor, 2003b). The β-amylase activity of sorghum malt is generally lower than that of barley malt and as a result, lager beer contains higher alcohol levels ranging between 3-8% (v/v) than the 2-4.5% (v/v) range of traditional sorghum beer (Waites et al., 2001; Taylor, 2003a). The other difference lies in the *Saccharomyces* strains used. Traditional sorghum beer is made using a top-fermenting strain, *Saccharomyces cerevisiae* whilst lager beer production makes use of a bottom-fermenting strain, *Saccharomyces cerevisiae* (Bokulich and Bamforth, 2013).

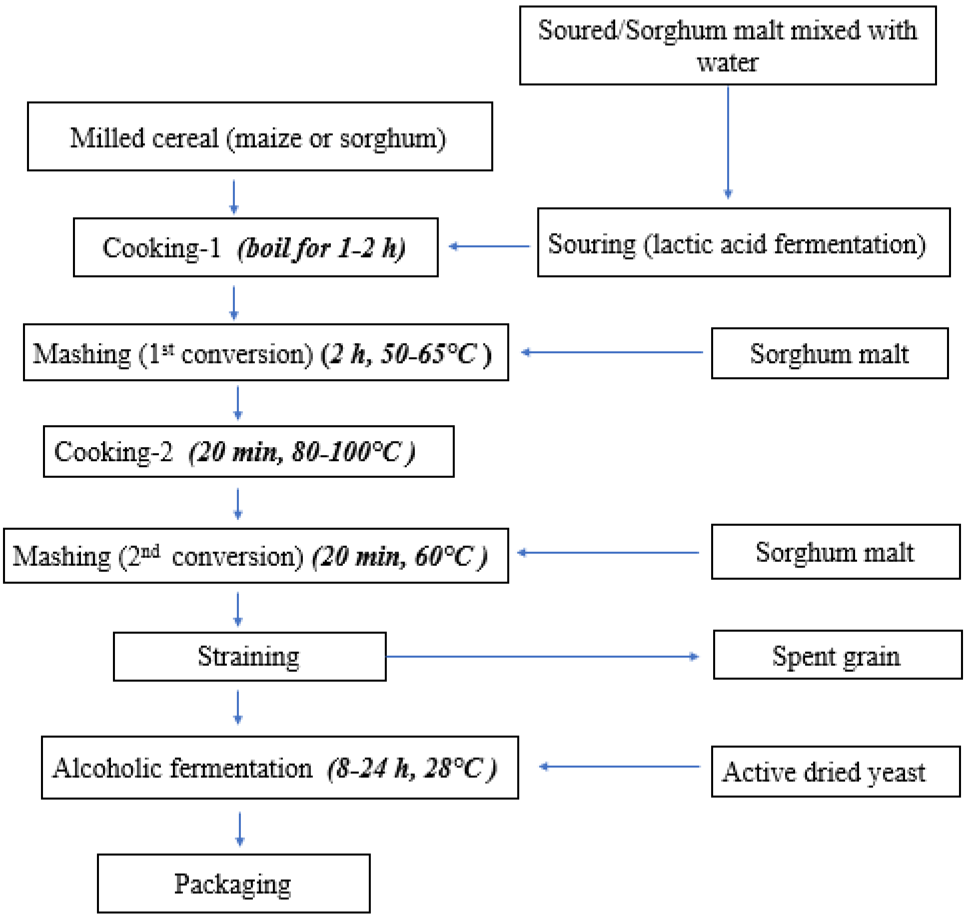

Figure 2: Generalised process for brewing commercial traditional sorghum beer (adapted from Taylor, 2003b)

2.2 Generation of brewer's spent grain

During the brewing process, the starchy endosperm of the cereal undergoes enzymatic degradation (Lynch et al., 2016). This results in the liberation of fermentable sugars (maltotriose and maltose), non-fermentable dextrins, polypeptides, proteins and amino acids. The fermentable sugars are respired anaerobically by yeast fermentation into ethanol and carbon dioxide. The solid residue that remains after the wort is extracted is referred to as brewer's spent grain (Dos Santos Mathias et al., 2014). As stated, brewer's spent grain is a brewing industry co-product that is rich in prolamin-type protein and fibre (Taylor and Taylor, 2018).

2.3 Brewer's spent grain composition

The chemical composition of brewer's spent grain varies because it is dependent on the type and quality of adjuncts and malt used in the brewing processes, species and variety of grains, harvest time and the brewing process (Buffington, 2014). Brewer's spent grain mainly consists of grain husks, pericarp (bran) and other residual components of the original grain not hydrolysed into soluble compounds (Xiros and Christakopoulos, 2009). Barley and sorghum brewer's spent grain have different chemical compositions (Table 2.1). Sorghum spent grain has significantly higher protein (38-41%, dry basis) than barley spent grain (19-30%, dry basis) (Holmes, Cahill, Smart and Cook, 2013; Lynch et al., 2016). Although both spent grains are rich in fibre, barley spent grain contains a higher percentage of cellulose (12-25%), hemicellulose (20-25%) and lignin (12-28%) (dry basis) than sorghum spent grain, which contains 10-12% cellulose, 16-19% hemicellulose and 9-10% lignin (dry basis).

Table 1: Chemical composition of barley and sorghum brewer's grain

Components	Barley brewer's spent grain (% of dry material)	Sorghum brewer's spent grain (% of dry material)
Cellulose	12-25	10-12
Hemicellulose	20-25	16-19
Lignin	12-28	9-10
Protein	19-30	38-42
Ash	2-5	4-5
Lipid	10	9-10

(Adapted from Lynch et al., 2016; Holmes et al., 2013)

2.4 Current uses of brewer's spent grain

Primarily, brewer's spent grain is used as animal feed (Mussatto, 2014). Its other actual and investigated uses are a fertiliser, for energy and bioethanol production, in paper manufacturing and as a brick component (Cooray, Lee and Glatz, 2017; Mussatto, 2014). However, as stated, these applications do not fully utilise the large volumes of brewer's spent grain generated in breweries. Thus, there is need to develop other applications.

2.5 Structure and composition of sorghum and barley grains with respect to brewing

2.5.1 Sorghum

Sorghum is a cereal crop indigenous to the savannah regions of Africa (Holmes et al., 2013). The sorghum kernel (Figure 2.3) is a caryopsis (Serna-Saldivar and Espinosa-Ramíres, 2019). There is a wide genetic variability in sorghum and the five sorghum races have different kernel shapes and sizes, as well as weight ranging from 3 to 80 mg. The sorghum kernel comprises three anatomical parts: the endosperm (84.2%), the germ (9.4%) and the pericarp (6.5%).

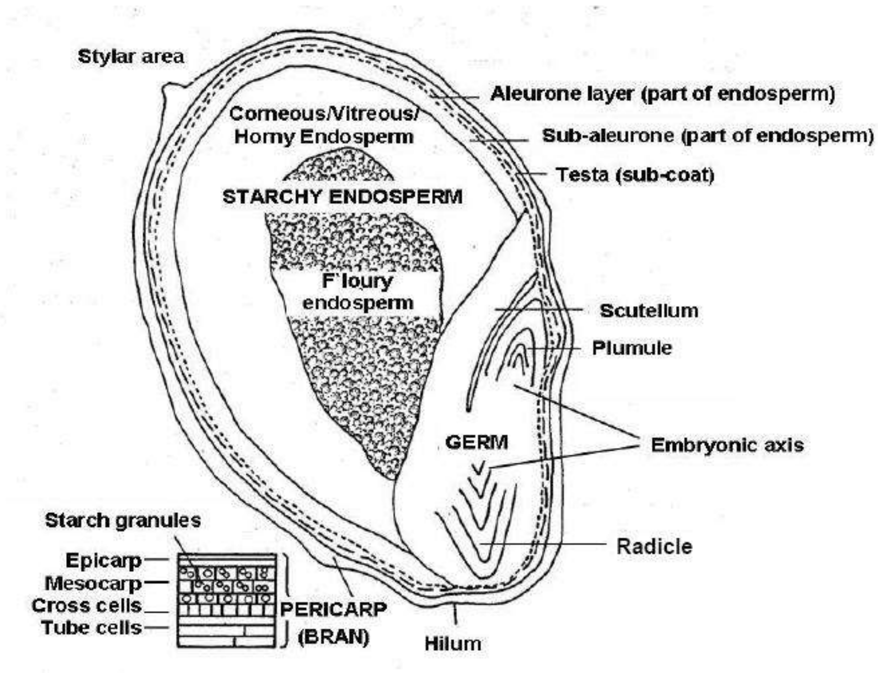

Figure 3: Longitudinal section of a sorghum kernel (Taylor and Belton, 2002)

Starch is a major component in the sorghum grain (60-75%), comprising 67-76% amylopectin and 24-33% amylose (Ratnavathi, 2018). Sorghum grains may exist as waxy, non-waxy and heterowaxy endosperm types. The waxy genotype has a waxy appearance and it contains nearly 100% amylopectin (Serna-Saldivar and Espinosa-Ramíres, 2019). Both the waxy and heterowaxy cultivars have starch with reduced proportions of amylose compared to non-waxy cultivars. During the brewing process, sorghum starch is hydrolysed by both the α- and β-amylase contained in the sorghum malt to produce fermentable sugars (Sammartino, 2015).

Sorghum grain contains 7-15% protein and the proteins are located in the endosperm (80.9%), pericarp (4%) and germ (14.9%) (Serna-Saldivar and Espinosa-Ramíres, 2019). Variations in protein content are due to differences in environmental conditions, genetic factors and cultivation practices (Bean, Ioerger, Smith and Blackwell, 2011). Kafirins, which are prolamins are the largest protein fraction comprising 70-80% of the sorghum protein (Ratnavathi, 2018). In brewing, proteins provide amino acids for yeast nutrition and contribute to mouthfeel, colour, foam and flavour of the beer (Sammartino, 2015). Proteins are hydrolysed by proteases and peptidases which are from the sorghum malt.

2.5.2 Barley

The barley kernel (Figure 2.4), like the sorghum grain, is also classified as a caryopsis (Gubatz and Weschke, 2014). The kernel also comprises three main parts: the embryo (germ), endosperm (aleurone and starchy endosperm), grain coverings (seed coat and pericarp layers plus a husk, which normally remains attached to the pericarp) (Lynch et al., 2016).

The starch is located in the starchy endosperm and accounts for 50-70% of the grain weight. (Gubatz and Shewry, 2011). Barley starch comprises amylose (20-30%) and amylopectin (65-77%). Barley proteins constitute 8-15% of the kernel weight, with the hordeins, which are prolamins, being the most abundant proteins (40-50%) of the total proteins (Gupta et al., 2010). The functionality of barley starch, protein and hydrolytic enzymes during brewing is essentially the same as that of sorghum (Section 2.5.1).

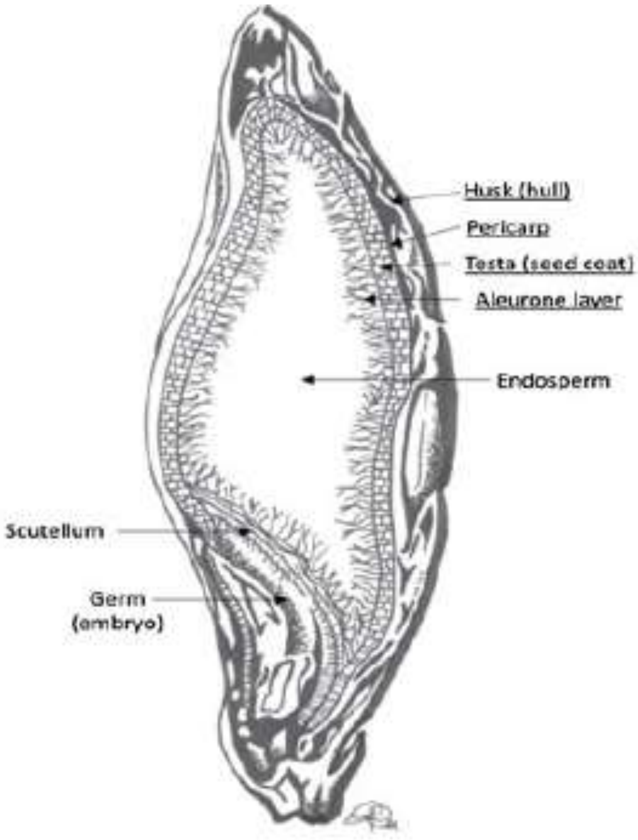

Figure 4: Longitudinal section of a barley kernel (Lynch et al., 2016)

2.6 Characterisation of sorghum, maize and barley prolamin proteins: kafirin, zein and hordein

In cereal grains, the majority of the proteins that store nitrogen for the development of the seedlings are prolamins (Momany, Sessa, Lawton, Selling, Hamaker, and Willett, 2006). The prolamins are located in the cells of the starchy endosperm (Guerrieri and Cavaletto, 2018). Prolamins contain high amounts of glutamine and proline; thus, the name prolamin, whilst they contain low levels of lysine and tryptophan (Belton, Delgadillo, Halford and Shewry, 2006). Prolamins are soluble in alcohol-water mixtures but are insoluble in water. Zein, hordein and kafirin, are the prolamins of maize, barley and sorghum, respectively.

2.6.1 Kafirin and zein subclasses and structure

Subclasses

Zein and kafirin show extensive homology (DeRose, Ma, Kwon, Hasnain, Klassy and Hall, 1989), and as a result will be considered together. Both zein and kafirin comprise α-, β-, γ- and δ-sub-classes (Belton et al., 2006). These classifications are based on their structure, DNA sequencing, amino acid composition and sequences, molecular masses and immunochemical cross-reactions (El Nour, Peruffo and Curioni, 1998 Belton et al., 2006).

The α-kafirin subclass accounts for 80-84% of the kafirin in the corneous endosperm and 66-71% in the floury endosperm (Bean, Zhu, Smith, Wilson, Ioerger and Tilley, 2019). Alpha-zein comprises 70% of the total zein in the maize grain (Lawton, 2002). The α-zein and α-kafirin contain low levels of cysteine, whereas the β- and γ-kafirin and the β- and γ-zein subclasses which contain considerably higher levels of cysteine (El Nour et al., 1998). The α-zeins are generally found as disulphide-linked dimers and can be divided by SDS-PAGE into two major subclasses; 19 K and 22 K zeins (Momany et al., 2006; DeRose et al., 1989). Beta-zein (10-15% of total zein fraction) contains seven cysteine residues in its amino acid sequence, whilst β-kafirin (7-13% of total kafirin) contains 10 cysteine residues (Shewry and Tatham, 1990; Belton et al., 2006).

The γ-prolamins of both kafirin and zein are readily soluble in water when they are in a monomeric form (Belton et al., 2006). They are insoluble in the native state because of the presence of inter-chain disulphide bonds. The γ-zein and γ-kafirin have similar hexapeptide repeats of amino acid sequences Proline-Proline-Proline-Valine-Histidine-Leucine close to the N-terminus (Tatham, Field, Morris, I'Anson, Cardle, Dufton and Shewry, 1993).

Structure of α-zein and α-kafirin

Currently, both the α-zein and α-kafirin secondary structures are not yet clear but some models have been proposed (Figure 2.5). Argos, Pedersen, Marks and Larkins (1982) proposed a model based on circular dichroism analysis for the structure of α-zein. The model predicts a high proportion of tightly coiled α-helix because of nine repetitive sequences, which form a cylindrical shape (Figure 2.5A). The α-helices are antiparallel and are separated by α-turn region. Glutamine residues in the turn regions, both at the top and bottom of the cylinder, interact via hydrogen bonds causing the rod-shaped zein molecules to tightly pack within the protein bodies, the organelles of zein and kafirin storage in the starchy endosperm.

This Argos et al. (1982) model was modified by Garratt, Oliva, Caracelli, Leite and Arruda (1993) so that it could accommodate all α-prolamins, including α-kafirin considering its extensive homology with α-zein (Figure 2.5B). This 3-dimensional model was based on pairs of amino acid repeat units forming anti-parallel α-helices. However, unlike the Argos et al. (1982) model, these anti-parallel α-helices are arranged in a hexagonal net with alternating groups of hydrophobic and polar amino acids. Matsushima, Danno, Takezawa, and Izumi (1997), proposed another model where α-zein is made up of α-helices that are stacked up in a linear manner in the direction of the long axis forming a compact rectangular prism. Hydrophilic glutamine-rich loops are at the top and bottom of the prism, whilst the faces of the prism (containing the α-helices) show hydrophobic properties (Figure 2.5C). Bugs, Forato, Bortoleto-Bugs, Fischer, Mascarenhas, Ward and Colnago (2004) proposed structural models of α-zein based on an extended hairpin structure. This model consists of two anti-parallel α-helices formed from coiled coils to form a super helical conformation with polar charged and hydrophobic amino acids distributed along the helical surfaces (Figure 2.5D).

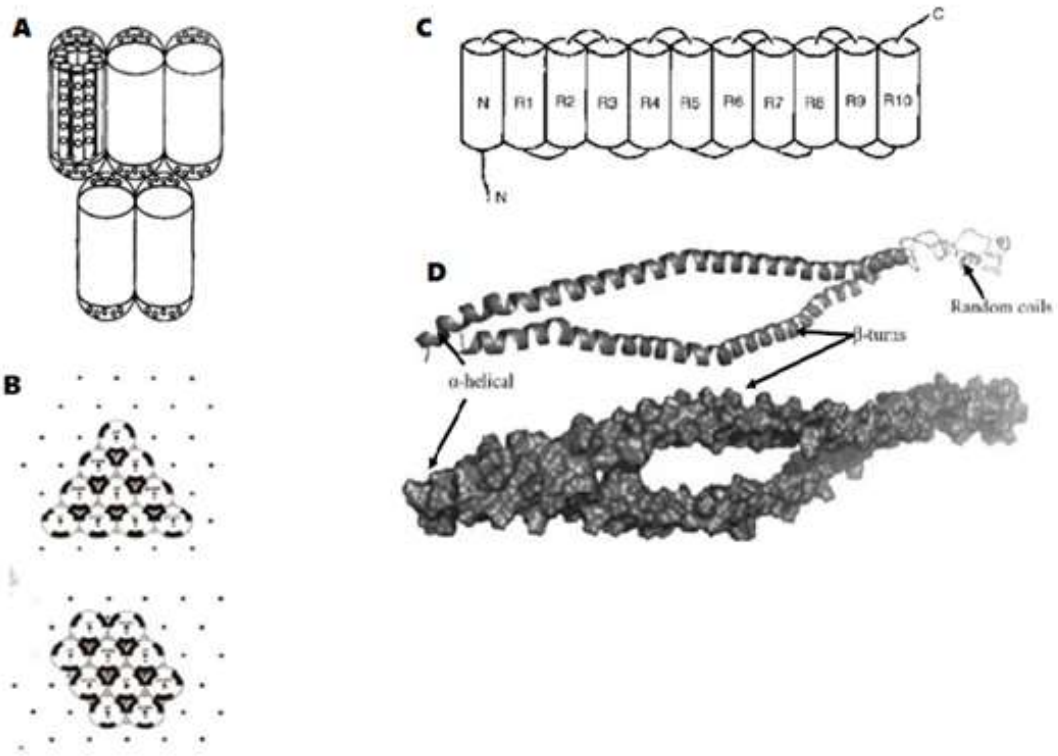

Figure 5: Proposed α-zein structural models. A- Argos et al. (1982); B-Garratt et al. (1993); C-Matsushima et al. (1997); D-Bugs et al. (2004) (from Belton et al., 2006)

2.6.2 Functional properties of kafirin and zein

Despite the fact that they are very hydrophobic proteins, both kafirin and zein possess some hydrophilic character with kafirin being relatively more hydrophobic than zein (Belton et al., 2006). Free energy of hydration values calculated from their amino acid composition show that α-zein (-139.78 kcal/mol) and α-kafirin (-140.36 kcal/mol) have similar degrees of total hydrophobicity. Differences are present for the γ-prolamins (Duodu, Taylor, Belton and Hamaker, 2003). The γ-kafirin (-113.63 kcal/mol) is more hydrophobic than γ-zein (-124.52 kcal/mol). Thus, over all kafirin is considered to be more hydrophobic than zein.

Kafirin contains a higher proportion of cysteine rich β- and γ-kafirin subclasses than zein (Belton et al., 2006). This allows kafirin to form more disulphide bonds than zein (Duodu, Nunes, Delgadillo, Parker, Mills, Belton and Taylor, 2002). Crossing waxy and high protein digestibility-high lysine (HDHL) lines produces sorghum lines with both high protein digestibility and waxy traits (Elhassan, Oyuntoyimbo, Taylor and Taylor, 2018). As previously mentioned, the waxy sorghum lines are composed of nearly 100% amylopectin (Rooney, 1996). The HDHL line contains low levels of the β- and γ-kafirin subclasses (Benmoussa, Chandrashekar, Ejeta and Hamaker, 2015) as compared to normal sorghum lines. Consequently, its kafirin has less disulphide cross-linking than normal sorghum kafirin.

2.6.3 Hordein subclasses and structure

Subclasses

Hordeins can be classified based on their molecular mass and amino acid components into three broad groups: the sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) (Finnie and Svensson, 2014). They are further classified into A, B, γ, C and D hordeins (Balakireva and Zamyatnin, 2016). The A hordeins have a low molecular weight (15 kDa) and are not considered to be true storage hordeins but probably breakdown products of larger hordeins, alcohol-soluble albumins or globulins (Baxter, 1981). The B and γ- hordeins are sulphur-rich, the C-hordeins are sulphur-poor and the D hordeins belong to the HMW group (Finnie and Svensson, 2014).

The HMW (D hordeins) comprise 6-10% of total hordein fraction and are made up of interchain disulphide bonded polypeptide subunits (Shewry and Tatham, 1990); thus, existing only in a polymeric form (Finnie and Svensson, 2014). HMW hordeins have a molecular weight less than 100 kDa (Balakireva and Zamyatnin, 2016). The S-rich B hordeins comprise 80% of the total hordein fraction whereas the γ-hordeins comprise only 5% (Gupta et al., 2010). The B and γ-hordeins usually occur in both monomeric and polymeric forms (Finnie and Svensson, 2014). The molecular weights of these sulphur-rich hordeins range from 32-45 kDa (Balakireva and Zamyatnin, 2016). The B-hordeins can be characterised by SDS-PAGE into three bands (B1, B2 and B3 hordeins). The B2 and the B3 hordeins are the major fractions whilst the B1 hordeins are the minor fraction. There are three types of the γ-hordeins; γ1-, γ2- and γ3-hordeins. The majority of the γ-hordeins exist as monomers with intrachain disulphide bonds (Shewry and Tatham, 1990). However, the γ1- and γ2-hordeins can form intermolecular disulphide bridges. The C hordeins comprise 10-20% of total hordein fraction (Balakireva and Zamyatnin, 2016). Two C-hordeins types, C1 and C2 have been identified by SDS-PAGE analysis. These hordeins have molecular weights that range from of 55 kDa to 70 kDa (Shewry and Tatham, 1990; Balakireva and Zamyatnin, 2016).

Structure of hordein

The amino acid composition of hordeins shows that they contain large regions of repeating short-sequence motifs in their primary structure (Finnie and Svensson, 2014). These repeating short-sequence motifs enable hordeins to form super secondary structures that are based on β-reverse turns and poly-L-proline structures. Different investigations have been carried out to determine the secondary structure of C hordeins. Methods that were used included, circular dichroism spectroscopy, computer models and small angle X-ray scattering (SAXS) (Tatham and Shewry, 2012). The primary structure of C hordein results in repetitive β-turns that form a loose spiral, worm-like secondary structure. Structural predictions indicate that HMW D-hordeins have an extended rod-like structure (Balakireva and Zamyatnin, 2016). This structure is made up of α-helices at the N- and C-terminal domains and central repetitive domains form regularly repeated β-turns (Shewry and Tatham, 1990).

2.6.4 Functional properties of hordein

One third of the total amino acids that make up hordein are hydrophobic resulting in its overall hydrophobic nature (Xia, Wang and Chen, 2011). Therefore, hordein bioplastic films have better barrier properties than films made from starch, which is hydrophilic (Xia et al., 2011). All the hordein sub-classes, except for the S-poor prolamins, contain cysteine residues, which gives them an ability to form disulphide bonds (Balakireva and Zamyatnin, 2016).

2.7 Effect of brewing on prolamins: heat induced disulphide bonding of prolamin proteins

Regardless of the type of brewing process used, the properties of hordein and kafirin undergo changes as a result of the wet-cooking that occurs (Holmes et al., 2013). Barley spent grain hordein become more cross-linked than barley grain hordein as a result of heat induced disulphide cross-linking (Baxter, 1981, Celus, Brijs and Delcour, 2006). Similar to barley, wet-cooking sorghum, as occurs during the brewing process, increases the proportion of disulphide cross-linked kafirins (Duodu et al., 2002). This also results in a conformational change from the original α-helical structure to a more β-sheet structure.

These disulphide cross-linkages result in the formation of high molecular weight polymers that are insoluble in aqueous alcohols (Hamaker, Mohamed, Habben, Huang and Larkins, 1995;

Cookman and Glatz, 2009). This poor solubility caused by prolamin cross-linking results in lower extraction yields (Muhiwa et al., 2017). Furthermore, additional prolamin cross-linking affects the physical properties of films made from these prolamins, for example increasing tensile strength, reducing water permeability, flexibility and extensibility (Byaruhanga, Erasmus and Taylor, 2005; Xia et al., 2011; Wittaya, 2012).

2.8 Prolamin extraction methods

Extraction conditions for prolamins are chosen based on the desired end use of the protein (Bean et al., 2019). Small-scale laboratory extraction is used for research into prolamin characterisation and functional properties. Large-scale extraction, currently for zein only, is for commercial purposes. A number of laboratory extraction procedures have been developed utilising 70% ethanol plus sodium metabisulphite as a reducing agent and possibly NaOH (Bean et al., 2019). The reducing agent is added to break the disulphide bonds within the cross-linked proteins to increase protein solubility, resulting in a higher protein yield (Wall and Paulis, 1978). During kafirin extraction, NaOH can be used for pH adjustment, which results in kafirin precipitation (Taylor, Taylor, Dutton and de Kock, 2005a).

Hamaker et al. (1995) extracted zein and kafirin using 1% sodium dodecyl sulphate, the reducing agent 2-mercaptoethanol (2%), an alkaline buffer (pH 10) and 70% ethanol or 60% tert-butanol. This method caused a precipitation of the non-prolamin proteins, whilst the prolamins remained in solution. This method proved to be highly repeatable and the zein and kafirin fractions had a preparation purity of 50-56% and 68-73% respectively.

The use of tert-butanol plus the reducing agent dithiothreitol to extract prolamins for food coatings was found to be a highly efficient method, however, high cost and toxicity rendered it unsuitable for food use (Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton, 2005). These authors developed an industrially applicable kafirin extraction method using food compatible chemicals. Extraction of kafirin from dry-milled sorghum using 70% (w/w) aqueous ethanol, 0.5% (w/w) sodium metabisulphite with 0.35% (w/w) NaOH at 70°C yielded the most favourable results. This method increased kafirin yield and resulted in bioplastic films with good sensory and high tensile properties. Addition of NaOH to aqueous ethanol improved kafirin solubility and thus increased kafirin yield.

Taylor et al. (2005a) developed an extraction method that replaced aqueous ethanol with glacial acetic acid. Kafirin extractions were performed using aqueous alcohol extractants (70% (w/w) ethanol at 70°C and 55% (w/w) isopropanol at 40°C), each containing sodium metabisulphite and sodium hydroxide. These were compared against glacial acetic acid with or without sodium metabisulphite at 25°C. The researchers found that 70% (w/w) ethanol at 70°C and 55% (w/w) isopropanol at 40°C containing sodium metabisulphite were the most effective extractants for kafirin with a recovery of 74.4-81.3%. Kafirin extracted using aqueous ethanol had the highest purity (74.6%), followed by aqueous isopropanol (73.1) and lastly, glacial acetic acid with metabisulphite pre-soak (42.8%). The purity of kafirin extracted using glacial acetic acid with metabisulphite was increased to 68.0% by washing out the salt. It was hypothesised that the efficiency of glacial acetic acid was because of its low dielectric constant (6.1) that allows it to dissolve hydrophobic proteins such as kafirin.

Wang, Tilley, Bean, Sun and Wang (2009) investigated extraction of kafirin from sorghum distillers dried grains with solubles (DDGS) using the following solvents: acetic acid, hydrochloric acid and ethanol, sodium hydroxide and ethanol, all under reducing conditions. Sodium metabisulphite was used as the reducing agent. The extraction yields obtained were 44.2%, 24.2% and 56.8% for acetic acid, HCl-ethanol and NaOH-ethanol, respectively. The protein purities were 98.9%, 42.3% and 94.9% for acetic acid, HCl-ethanol and NaOH-ethanol under reducing conditions, respectively. The secondary structures of the kafirin extracted by all three methods indicated a predominance of α-helices and a smaller proportion of β-sheet in their secondary structure.

Lau, Johnson, Stanley, Mikkelsen, Fang, Halley and Steadman (2015) extracted kafirin from distillers dried grains using of 70% (w/w) aqueous ethanol, 0.5% (w/w) sodium metabisulphite with 0.35% (w/w) sodium hydroxide at 70°C. The distilled dried grains were washed three times using 5-6 volumes of water at 50°C to remove most of the water-soluble substances. The kafirin preparation extracted had an 82.3% protein content.

It can be concluded that the effectiveness of prolamin extraction methods is dependent on multiple factors that include the exact nature of the solvent, solvent temperature and the hydration-state of the sample, as was proposed by Katoch (2011). Thus, extraction methods affect the yield, purity and property of the prolamins as was found by Wang et al. (2009). Extracting prolamins from brewery co-products instead of whole grain flour is potentially more effective as starch would have been removed during conversion thus the prolamins are semi-purified (Lau et al., 2015).

2.9 Functionality of prolamins: film formation

As stated, prolamins can be used to make biodegradable bioplastics, in particular films which can potentially serve as environmentally friendly options to synthetic polymer products (Belton et al., 2006; Anyango, Taylor and Taylor, 2011).

Two processes can be used to make protein films (Figure 2.6). For the wet process (casting method), the protein is dissolved in a suitable solvent, and poured onto a flat surface and dried to evaporate the solvent (Cuq, Gontard and Guilbert, 1998; Taylor et al., 2013a). Cast prolamin films can serve as model bioplastics to determine the functionality of the extracted proteins in terms of, for example, water uptake, water stability, breakdown under stimulated digestion, tensile and sensory properties (Taylor, Taylor, Dutton, and de Kock, S 2005b; Muhiwa et al., 2017). For the dry (thermoplastic) method, the protein is heated at high temperature until it melts, and shear force is applied under low moisture conditions (Cuq et al., 1998). Once melted, the protein is shaped into a film by extrusion or thermo-moulding).

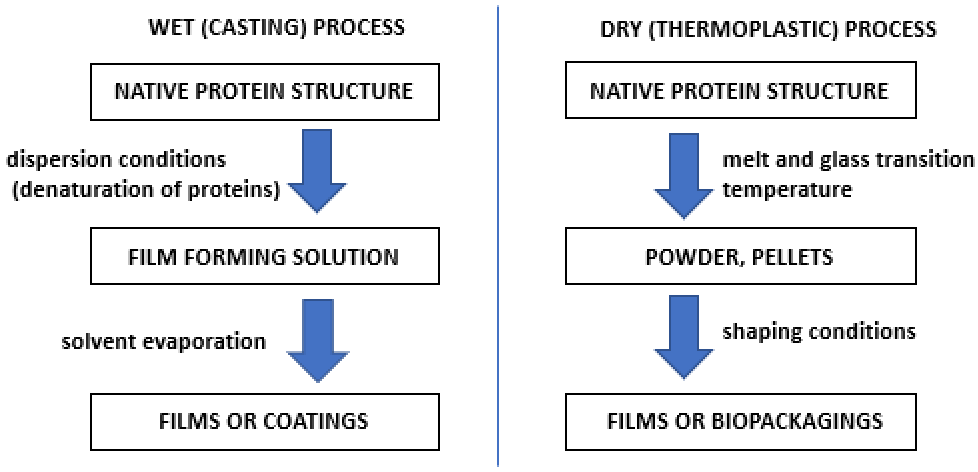

Figure 6: Schematic representation of the wet (casting) and dry (thermoplastic) protein bioplastic film making processes (adapted from Cuq et al., 1998)

This research employedthe wet (casting) process. Cast protein-based films are commonly made from solutions or dispersions of the protein as the solvent (e.g. ethanol, water, acetic acid or ethanol-water mixtures) evaporates (Wittaya, 2012). In some instances, pH and/ or heat may be necessary to accomplish this (Wittaya, 2012; Taylor et al., 2013a).

The formation of protein films occurs in three stages: a) rupture of low-energy intermolecular bonds that stabilise polymers in the protein extract state, b) arrangement and orientation of the polymers to form a shape and c) removal of the solvent resulting in the formation of a three-dimensional network stabilised by new interactions and bonds (Cuq et al., 1998). In their native state, proteins are bound by a combination of ionic, hydrogen, disulphide and hydrophobic bonds (Wittaya, 2012). When they are denatured either by heat, pH changes or solvents they form extended protein structures necessary for film formation (Wittaya, 2012). The precise nature of the interactions between the proteins that account for the film formation is unknown (Gao et al., 2005). However, upon extension of structures, the proteins undergo chain to chain interactions by hydrogen, ionic, hydrophobic and covalent bonding in the film matrix (Wittaya, 2012). This increase in disulphide cross-linking results in a tight and compact network and structure of proteins (Guo Lu, Cui, Jia, Bai and Ma, 2012). An increased polymer chain to chain interaction results in stronger but less flexible and less permeable films (Wittaya, 2012).

2.10 Factors influencing prolamin film properties

The various prolamins have differing functionality because of differences in the number and proportion of subclasses and the hydrophobic amino acids present (Taylor et al., 2013a). Compared to zein films, kafirin films have been shown to have higher tensile strength and better water barrier properties (Da Silva and Taylor, 2005). This is because kafirin contains more cysteine residues than zein (Belton et al., 2006). Therefore, kafirin can form more disulphide bonds than zein and this results in kafirin films with greater tensile strength than zein films (Xiao, Chen and Huang, 2017).

Prolamin film functionality can be improved by the modification of the polymer network through cross-linking of polymer chains (Wittaya, 2012). Such modification can be done using physical, chemical or enzymatic methods (Taylor et al., 2013a). Byaruhanga et al. (2005) used microwave heating to physically modify kafirin films. The heating induced intermolecular cross-linking that increased tensile strength, reduced water vapour permeability and strain of the kafirin films. As discussed in Section 2.7, wet-cooking also induces cross-linking which may increase tensile strength of prolamin films. Anyango et al. (2011) used glutaraldehyde as a means of cross-linking kafirin films. The carbonyl groups of glutaraldehyde and free amino acid groups of the kafirin polypeptide formed covalent cross-linking. The cross-linking resulted in kafirin films with increased tensile strength. Larré, Denery-Papini, Popineau, Deshayes, Desserme and Lefebvre (2000) enzymatically modified gluten films using transglutaminase. Transglutaminase catalysed inter- and intra- cross-linking. The resultant films had improved tensile strength and elongation at break.

Additionally, modification of protein films can be done by forming composites to enhance their barrier and mechanical properties (Krochta, 2002). Protein-based film composites can be made by combining the protein polymer with compatible polysaccharides, lipids or other proteins (Emmambux, 2004). This is done with the aim of taking advantages of the properties of each compound to improve the overall film (Falguera, Quintero, Jimenez, Munoz and Ibarz, 2011). Cho, Park and Rhee (2002) laminated whey and casein biopolymers, which are hydrophilic, with zein, which is more hydrophobic. This resulted in a decrease in water vapour permeability because of the increase in hydrophobicity due to zein.

Prolamin films are usually brittle and stiff because of the extensive protein to protein chain interactions caused by disulphide cross-linking, hydrophobic, hydrogen and electrostatic forces (Krochta, 2002). These properties can also be modified by the addition of plasticisers. Film polypeptide interactions can be reduced by the addition of plasticisers (Xia et al., 2011). Plasticisers can be defined as non-volatile, small-molecular-weight compounds that, when added to bioplastic materials, improve the flexibility and mechanical properties (Banker, 1966). It is hypothesised that the plasticiser is trapped within the protein matrix and this reduces the protein to protein chain interaction (intermolecular forces) and increases free volume (Wittaya, 2012). The polymer and the plasticiser form a complex that is held together by intermolecular secondary valence forces (Banker, 1966). This reduction in protein to protein chain cohesion results in improved flexibility and elongation, but it also reduces film strength and barrier properties (Krochta, 2002).

There are two types of plasticisation, external and internal plasticisation (Banker, 1966). External plasticisation is achieved by adding a substance which results in structural and energy modifications within the polymer itself thus, reducing cohesion of the protein chains (Wittaya, 2012). Internal plasticisation is caused by modification of the internal chemical structure of the polymer (Banker, 1966). An advantage of external plasticisers over internal plasticisers is that there is a wide variety to select from, which gives a chance to select and regulate the amount of the plasticiser added for the desired product (Sommer, 1985). Examples include; glycerol, glycol and polyethylene. However, since external plasticisers are not chemically attached to the polymers by primary bonds like the internal plasticisers, they can be lost by evaporation, migration or extraction (Vieira, Da Silva, Dos Santos and Beppu, 2011). Internal plasticisers normally have bulk structures that provide polymers with more space between each other, allowing them more flexibility than the small sized external plasticisers.

2.11 Conclusions

Brewer's spent grain, a coproduct generated from the brewing process, is rich in prolamin-type protein and can be potentially used as a prolamin source for making bioplastics. These bioplastics could partially replace synthetic plastic, which in turn would reduce the level of plastic pollution. However, during the brewing process, prolamins undergo wet-cooking, which results in the formation of disulphide bonds leading to the formation of high molecular weight proteins. This changes the prolamin's extractability, purity and functionality. Little work has been done using brewer's spent grain as a prolamin source for bioplastics. There is need for research on the comparative functionality of BSG hordein versus BSG kafirin and zein bioplastics. Additionally, compositing protein-based films has shown potential to change the functionality of the resultant film. Understanding the bioplastic functionality of brewer's spent grain prolamins and composites with zein in a film model will help identify their most suitable applications.

3. HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

Hypothesis 1

Bioplastics can be made from prolamins extracted from brewer's spent grain. Brewer's spent grain is similar in composition to distillers dried grains and solubles (DDGS) in that both are ethanol production co-products and are rich in protein due to the conversion of their carbohydrates into fermentable sugars (Wang et al., 2009; Lynch et al., 2016). Kafirin extracted from sorghum DDGS has been used to make bioplastics (Muhiwa et al., 2017). Therefore, BSG can be used to make bioplastic films.

Hypothesis 2

Bioplastic films made from brewer's spent grain hordein, and kafirins from sorghums with waxy and high protein digestibility traits will have higher tensile strength and lower water uptake than standard kafirin and commercial zein bioplastic films. During the brewing and drying processes, brewer's spent grain undergoes wet heat-treatment potentially resulting in cross-linking of the prolamin proteins in the spent grain (Celus et al., 2006; Duodu et al., 2003). A comparison of prolamins extracted from sorghum brewer's spent grain compared with prolamins extracted from sorghum grain showed that more disulphide cross-linked dimers were present in the brewer's spent grain prolamin than those extracted from sorghum grain (Muhiwa et al., 2017). Similarly, barley hordeins extracted from brewer's spent grain have more disulphide cross-links than hordeins extracted from barley malt and barley grain (Celus et al., 2006; Baxter, 1981). When made into films the presence of a higher degree of disulphide cross-linking enables the formation of a protein film network which has increased tensile strength and reduced permeability of water (Byaruhanga et al., 2005). It is assumed that the increased disulphide cross-linking in barley brewer's spent grain hordein may also result in hordein films with increased tensile strength and reduced water permeability. Hence, the brewer's spent grain prolamin films have more resistance to water uptake and increased tensile strength in comparison to standard kafirin and commercial zein prolamin films.

The high protein digestibility sorghum lines contain lower levels of cysteine-rich β- and γ-kafirin compared to the normal protein digestibility lines (Elhassan et al., 2018) and so would be expected to form less disulphide cross-linking than normal protein digestibility sorghum lines when wet-cooked. As a consequence, it would be expected that films made from kafirin extracted from high protein digestibility sorghum lines would have lower tensile strength and have higher water uptake than films made from kafirin extracted from sorghum lines with normal protein digestibility.

Hypothesis 3

Mixing kafirin and commercial zein prolamins will form composite bioplastics with intermediate properties of kafirin and commercial zein. Kafirin and commercial zein have different tensile and water uptake properties. Kafirin films have higher tensile strength, are less extensible and have lower water uptake than commercial zein films (Da Silva and Taylor, 2005). These differences are due to the presence of more cysteine rich β- and γ-subclasses in kafirin than in commercial zein, resulting in higher levels of disulphide cross-linking in kafirin than commercial zein. A mixture of these two prolamins will form a composite bioplastic material with more desirable functional properties than the individual prolamins, that is higher tensile strength than commercial zein bioplastic and increased extensibility than that of kafirin bioplastic.

3.2 OBJECTIVES

Objectives

1. To extract and characterise kafirin and hordein from brewer's spent grain with the aim of determining their composition compared to that of standard kafirin and commercial zein, which have been used for making protein bioplastics.
2. To compare the functional properties of hordein extracted from commercial barley brewer's spent grain and kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility sorghum lines brewer's spent grain with commercial zein and standard kafirin, using cast films as a model bioplastic.
3. To determine the functional properties of composite kafirin-commercial zein bioplastics with the aim of forming composite films with ratio combinations that result in intermediate properties of films from the individual prolamins.

4. EXPERIMENTAL DESIGN

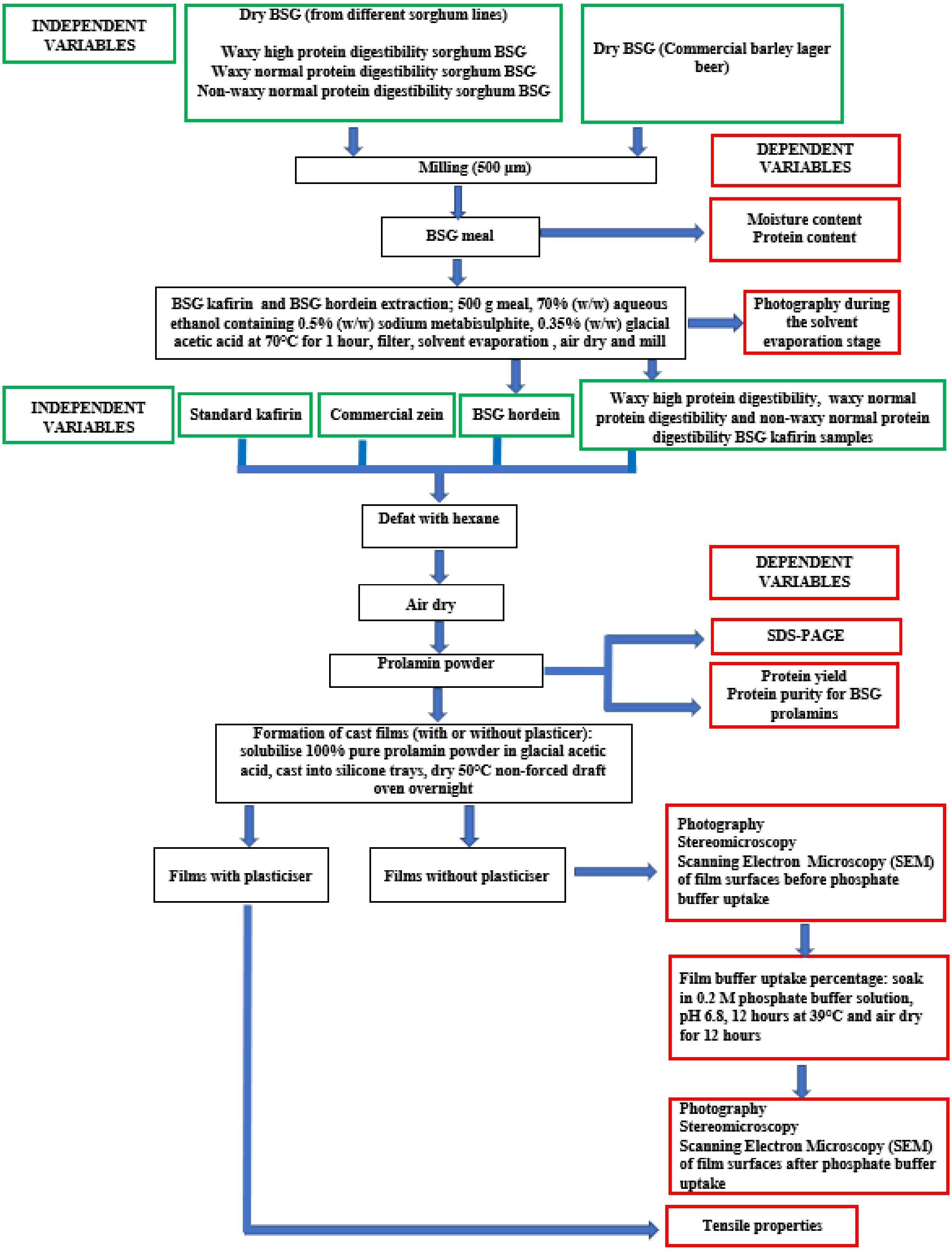

Figure 7: Experimental design to characterise and to determine the functional properties of films made from commercial barley brewer's spent grain and laboratory prepared sorghum brewer's spent grain prolamins

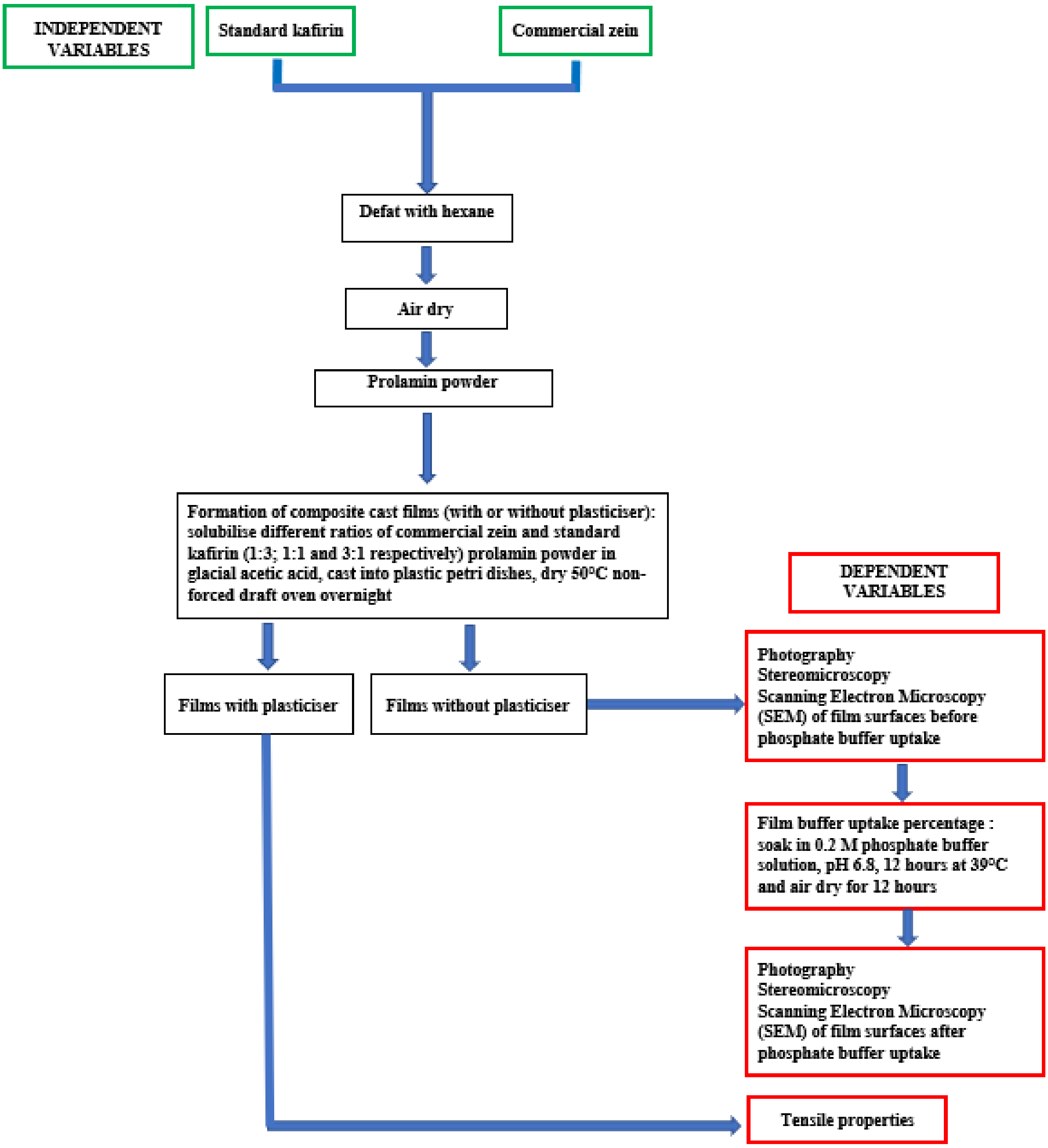

Figure 8: Experimental design to determine the effects of standard kafirin and commercial zein composite films on water uptake, tensile properties and film surface appearance

5. MATERIALS AND METHODS

5.1 Materials

The following prolamin proteins were used in this study:

Commercial zein (mainly α-zein) obtained from Sigma-Aldrich, Johannesburg, South Africa (product code Z3625).

Standard kafirin (containing all kafirin subclasses as present in the grain) extracted in the laboratory from white tan-plant sorghum grain cultivar PANNAR PEX 602/606.

Hordein extracted in the laboratory from air-dried barley malt plus liquid adjunct lager beer spent grain that was kindly provided by ABInBev, Sandton, South Africa.

Three different kafirins extracted from sorghum brewer's spent grain obtained by laboratory-scale sorghum lager beer-type brewing performed using three sorghum lines with different quality traits: waxy-high protein digestibility (WHD), waxy-normal protein digestibility (WND) and non-waxy normal protein digestibility (NWD). The lines were developed from crosses between parental lines RT x 2907 (waxy) and P850029 (high protein digestibility) by Texas A&M University and grown at the Ukulima Research Farm in Limpopo Province, South Africa in a controlled field trial.

5.2 Methods

5.2.1 Laboratory safety measures

This research was carried out in a manner that ensured a safe working environment. Prior to working in the laboratory, a signed laboratory safety agreement was submitted to the Department of Consumer and Food Sciences to show that the researcher had read and understood the Departmental laboratory safety rules. The rules included the wearing of appropriate protective clothing such as safety eyewear, latex gloves, laboratory coats and protective shoes. Additionally, knowledge of proper chemical care (labelling, storage and disposal) was essential. Special care was taken when glacial acetic acid was used because of its volatility and flammability (Merck Chemical Database, 2018). Exposure to glacial acetic acid may result in severe burns and eye damage. Glacial acetic acid was used under a fume hood. Ethanol, which is also highly flammable, was used in closed glassware without exposing it to open flames (Merck Chemical Database, 2018). Exposure to ethanol may cause serious eye irritation. Hexane liquid and vapour are highly flammable, and inhalation may case irritation to the respiratory tract (Merck Chemical Database, 2018). It was used under a fume hood. After use, it was disposed of according to the Department's waste solvent disposal protocol. Exposure to sodium metabisulphite may cause irritation to the skin and eyes (Merck Chemical Database, 2018), therefore the chemical was always handled with protective clothing. All experiments were carefully designed beforehand, detailed protocols written and authorised by the supervisors. Training was given to the researcher to ensure they had the correct knowledge of the procedures as well as the operation of the equipment.

5.2.2 Laboratory-scale sorghum lager-type brewing to produce spent grain

Tap water (2.5 L) containing 1.0 g calcium chloride (80 ppm) was preheated to 50^{o}C in a stainless-steel pan. Additional tap water (1.5 L) was measured into a stainless-steel bucket. Whole grain sorghum flour (800 g) milled to a maximum 500 µm particle size using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden), was transferred into the bucket and the contents thoroughly mixed to form a slurry. The pH of the mash was adjusted to pH 6.0 with a few drops of orthophosphoric acid. Termamyl SC enzyme (8 mL), which was kindly donated by Novozymes SA, Benmore, South Africa, was added to the slurry and thoroughly mixed. The slurry was then mixed with the 2.5 L water in the stainless-steel pan with stirring. The temperature of the mash was increased to 85^{o}C at approximately 1^{o}C/min with continuous stirring. The mash temperature was then held at 85^{o}C for 5 min. During mashing, a sample was taken at 5-min intervals, and iodine solution was used to check whether the mash still contained starch. If the mash turned purple, it would indicate the presence of residual starch and its absence would be indicated by a lack of colour change of the brown colour of the iodine solution. The mash was carefully filtered through one layer of cotton filter cloth on a 30 cm stainless-steel sieve placed on top of a stainless-steel bucket. After all the liquid (wort) was drained through, the cotton cloth was then made into a "bag" and twisted to squeeze out the additional free liquid in the spent grain. The wort was discarded. The prolamin-rich spent grain was carefully washed with tap water to remove any residual wort. The drained spent grain was spread over a 30 cm sieve to make a thin layer. The sieve containing the spent grain was dried in a forced draft oven at 50^{o}C until completely dry. When dried, the spent grain formed a fine powder and crumbled between the fingers.

5.2.3 Extraction of kafirin and hordein

Kafirin and hordein were extracted from sorghum and barley brewer's spent grain, respectively as described by Elhassan et al. (2018) with modifications. The extractant consisted 0.5% (w/w) sodium metabisulphite (12.5 g), 0.35% (w/w) glacial acetic acid (8.75 g), distilled water (730 g) and 70% (w/w) ethanol (1750 g). The spent grains were milled using a laboratory hammer mill to a maximum 500 µm particle size. The milled spent grain meal (500 g) was weighed into a 5L plastic bucket with a tight-fitting lid and a central hole for the rod of the stirring element. The extractant was added to the brewer's spent grain meal in the bucket, closed with a lid and the extraction was carried out at 70°C, in a water bath with vigorous stirring for 1 h. The supernatant was collected after centrifugation for 5 min at 1000 g at 25°C. The residue of the extracted brewer's spent grain meal was washed with another 500 g extractant. This was followed by immediate centrifugation for 5 min at 1000 g at 25°C and the clear supernatant was collected and combined. The combined supernatants were placed in an open stainless-steel tray in a fume hood. The ethanol in the supernatant was allowed to evaporate overnight. The protein which had precipitated after ethanol evaporation, was transferred into a beaker and washed with a minimal amount of cold distilled water (8˚C) and filtered under vacuum using a Buchner funnel and two layers of Whatman No. 4 filter papers (150 mm diam.). The prolamin preparation was then dried at 25°C in the fume hood. The dried prolamin preparation was weighed and then milled using an IKA A11 Basic mill (air-cooled knife-type mill) (Staufen, Germany). The dried, milled prolamin powder was then defatted using 100% (w/w) hexane. The mass of the hexane used was three times the weight of dry protein preparation in grams. Hexane was added to the dry protein preparation and stirred manually every 5 min for 1 h and then allowed to stand for 15 min at 25°C. Thereafter, the hexane was decanted. This process was repeated three times. On the third time, a vacuum filter was used to filter out the hexane from the protein preparation. The protein preparation was dried in the fume hood overnight. After weighing, it was then milled again using the IKA A11 Basic mill. The defatted prolamin preparations were stored in ziplock-type polyethylene bags at 10°C prior to film formation and analysis.

5.2.4 Prolamin characterisation

5.2.4.1 Moisture content

The moisture content of the protein preparations was determined by Approved Method 44-15A, Air oven method (AACC, 2000).

5.2.4.2 Protein content

The protein content was measured using the Dumas total combustion method, AACC Approved method 46-30 (AACC, 2000), using (N × 6.25) conversion factor. The percentage yield of the protein preparation was calculated as the mass of total protein recovered divided by the total amount of protein present in the brewer's spent grain (dry basis) multiplied by 100.

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

Hordein and the kafirin preparations extracted from brewer's spent grain, standard kafirin and commercial zein preparations were characterised by SDS-PAGE under both reducing and non-reducing conditions as described by Anyango et al. (2011). An XCell SureLock Mini-Cell electrophoresis unit (Invitrogen Life Technologies, Carlsbad, California) was used with Invitrogen NuPAGE 4-12% Bis-Tris pre-prepared gels, 1 mm thick with 15 wells. The loading was equivalent to 10 µg of 100% protein per sample well. An Invitrogen Mark 12 molecular weight standard was used (5 µg). Electrophoresis was carried at a constant voltage of 200 V, 80 A and 10 Watts per gel for 1 h. After running, the proteins were stained with Coomassie Brilliant Blue R250 overnight and then destained. Gels were stored in 3% acetic acid before scanning on a flatbed scanner.

5.2.4.4 Total amino acid composition of protein preparations

The total amino acid composition for brewer's spent grain kafirin, brewer's spent grain hordein, standard kafirin and commercial zein preparations were analysed by the Southern African Grain Laboratory, Pretoria, South Africa. The methodology used was as follows:

I) SAGL IN-HOUSE METHOD 028: total amino acid analysis

Fifteen amino acids were analysed, namely Aspartic acid, Glutamic acid, Serine, Glycine, Histidine, Arginine, Threonine, Alanine, Proline, Tyrosine, Valine, Isoleucine, Leucine, Phenylalanine, Lysine. The samples were analysed in duplicate by the AccQ-Tag method using a Waters Acquity H-Class UPLC with Empower software (Waters, Millipore Corp., Milford, MA). Samples (100 mg) were hydrolysed with 6 N HCl for 24 hours and then derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbomate (AQC) to produce stable derivatives. These amino acids were then analysed by reverse phase UPLC.

II) SAGL In-house method 015: cystine and methionine

The samples were analysed by the Pico-Tag method using a Waters Breeze HPLC with Empower software (Waters, Millipore Corp., Milford, MA). For cystine and methionine, the sample was first oxidized with performic acid over night at 0ºC. Performic acid is a powerful oxidizing reagent that converts cysteine and cystine quantitatively to cysteic acid and methionine to methionine sulfone. The reaction was stopped with hydrogen bromide and the sample was dried. The sample was then treated as for all other amino acids. Samples (200 mg) were hydrolysed with 6 N HCl for 24 hours and then derivatized with phenylisothiocyanate (PITC) to produce phenyltiocarbamyl (PTC) amino acids. These amino acids were then analysed by reverse phase HPLC.

5.2.4.5 Nutrient composition

The basic nutrient composition (moisture, crude protein, ash, fat, total dietary fibre, total iron and total zinc) of sorghum brewer's spent grain and sorghum flour were determined by the Southern African Grain Laboratory, Pretoria, South Africa using standard methods.

5.2.5 Film formation

5.2.5.1 Prolamin film formation without plasticiser for buffer uptake analyses

Cast prolamin films were prepared as described by Muhiwa et al. (2017) with modifications. Commercial zein, standard kafirin, brewer's spent grain hordein and brewer's spent grain kafirin protein preparations were separately weighed into 100 mL Erlenmeyer flasks. These weights were equivalent to 1.2 g of pure 100% protein dry basis. For the composite films, commercial zein and standard kafirin preparations were separately weighed according to their ratios (1:1, 1:3 and 3:1 respectively) and each ratio combination added to a 100 mL Erlenmeyer flask (Table 2). The total weights for each composite mixture were equivalent to 1.2 g of pure 100% protein dry basis.

Table 2: Amounts of prolamin powder added per respective composite film mixture

Commercial zein		Standard kafirin		Prolamin powder ratio of commercial zein to kafirin
Mass (g)	Percentage contribution to film (%)	Mass (g)	Percentage contribution to film (%)	
0	0	1.26	100	
0.43	25	0.95	75	1:3
0.86	50	0.63	50	1:1
1.28	75	0.32	25	3:1
1.71	100	0	0	

Glacial acetic acid (8.8 g) (w/w) was added to the 100 mL Erlenmeyer flask containing 1.2 g (100% protein equivalent, dry basis) prolamin protein, together with a magnetic stirrer. The combined weight of the flask, glacial acetic acid and stirrer was recorded to two decimal places. The flask was closed with aluminium foil (60 x 60 mm) before heating to 65-70°C (using a stirrer hot plate) for 10 min, with continuous stirring to solubilise the prolamin powder. The solution was cooled for 5 – 10 min at ambient temperature (23°C). The flask and contents were weighed, and glacial acetic acid was added to replace the evaporated solvent.

Aliquots (3 g) (w/w) of protein solution were transferred into rectangular silicone baking trays (28 x 69 mm), followed by gently swirling for 30 secs to evenly distribute the contents at the bottom of the silicone tray. The silicone trays containing protein solution were placed on a level surface (to ensure an even film thickness) in a forced draft oven with fan disconnected for the solution to dry overnight at 50°C. After drying, films were carefully removed from the baking trays, scanned using a flatbed scanner, placed in ziplock-type polyethylene bags and stored at 8°C.

5.2.5.2 Prolamin film formation with plasticiser for tensile properties analyses

Prolamin films used to analyse tensile properties (strain, stress and force) were made as described by (Emmambux, Stading and Taylor, 2004) with modifications. Commercial zein, standard kafirin, brewer's spent grain hordein and brewer's spent grain kafirin protein preparations equivalent to 1.44 g of pure 100% protein (dry basis) were separately weighed into a 100 ml Erlenmeyer flask. For the composite films, commercial zein and standard kafirin preparations were separately weighed according to their ratios (1:1, 1:3 and 3:1 respectively) and each ratio combination added to a 100 mL Erlenmeyer flask (Table 3). The total weights for each composite mixture were equivalent to 1.44 g of pure 100% protein dry basis.

Table 3: Amounts of prolamin powder added per respective composite film mixture

Commercial zein		**Standard kafirin**		**Prolamin powder ratio of commercial zein to kafirin**
Mass (g)	**Percentage contribution to film (%)**	**Mass (g)**	**Percentage contribution to film (%)**	
0	0	1.52	100	
0.52	25	1.14	75	1:3
1.03	50	0.76	50	1:1
1.55	75	0.38	25	3:1
2.06	100	0	0	

Glacial acetic acid (9.0 g) (w/w) then 0.6 g plasticiser (1:1:1 (w/w/w) mixture of polyethylene glycol, glycerol and lactic acid) were added to the 100 mL Erlenmeyer flask containing 1.44 g (100% protein equivalent, dry basis) prolamin protein, together with a magnetic stirrer. The combined weight of the flask, glacial acetic acid, plasticiser and stirrer was recorded to two decimal places. The flask was closed with aluminium foil (60 x 60 mm) before heating to 65-70°C (using a stirrer hot plate) for 10 min, with continuous stirring to solubilise the prolamin powder. The solution was cooled for approximately 10 min at ambient temperature (23°C). The flask and contents were weighed, and glacial acetic acid was added to replace the evaporated solvent. Aliquots (4 g) (w/w) of protein solution were transferred into 9 cm diameter plastic petri dishes, followed by gently swirling for 30 secs to evenly distribute the contents at the bottom of the 9 cm diameter plastic petri dish. Petri dishes were used instead of silicone trays in order to increase the number of strips that could be obtained per film for the analyses of tensile properties. The dishes without lids, containing protein solution were placed in a forced draft oven (with fan disconnected and on a level surface to ensure an even film thickness) to dry overnight at 50°C. After drying, films were carefully removed from the petri dishes, scanned using a flatbed scanner, placed in zip-lock bags and stored at 8°C.

5.2.6 Analyses

5.2.6.1 Film tensile properties

A modified method based on ASTM International, (2002) standard D882 was used. The plasticised films were conditioned to 50% relative humidity as follows; the films in petri dishes were placed in a desiccator resting on a perforated tray. Saturated calcium nitrate solution was placed under the perforated tray. The saturated calcium nitrate solution was made by adding 121.2 g calcium nitrate to 100 mL of water at 20^{o}C. The films were left in the desiccator for 24 h to allow conditioning to 50% relative humidity. After conditioning, the films were cut into strips (60 mm x 5 mm) using a sharp scalpel and a cardboard template. The thickness of the film strips was measured at three random places using a micrometer and recorded. The mean film thickness was calculated. The film strips were individually mounted on the grips (Tensile rig grips code A/TG) so that 40 mm of the film was exposed. The films were subjected to tension with a crosshead speed of 0.4 mm/s. The tensile properties were analysed using a Shimadzu EZ-L texture analyser, (Tokyo, Japan). The maximum force and distance at break were marked and the stress and strain calculated as follows:

Stress (σ) = F (maximum force)/ A (area of film strip) = N/mm^2

Area of film strip = (width x thickness)

% Strain (ε) = increase in length of film strip x 100

The results were expressed as stress at maximum force, maximum stress, maximum strain, force at break, stress at break and strain at break.

5.2.6.2 Film buffer uptake

Films made without plasticisers were used for buffer uptake analysis. The analysis was performed as described by Muhiwa et al. (2017) with modifications. Film weight (mg), dimensions (length by width in mm) were measured using a ruler and recorded prior to soaking. The appearance prior to soaking was analysed using a flatbed scanner, stereomicroscopy and scanning electron microscopy. The films were immersed in 0.2M sodium phosphate buffer (pH 6.8) for 12 h at 39^{o}C with gentle agitation in a water bath set at 30 resolutions per min. The films were removed from the buffer and the surface water was blotted with a paper towel. The blotted films were then immediately weighed, and their dimensions measured using a ruler.

Thereafter, the blotted films were air-dried for 12 h. The appearance of the dried films was analysed using a flatbed scanner, stereomicroscope and a scanning electron microscope. Percentage buffer uptake of the films was calculated as:

$$\%\ \text{buffer uptake} = \frac{\text{mass of films after immersion in buffer} - \text{initial mass of films before immersion in buffer}}{\text{initial mass of films before immersion in buffer}} * 100$$

5.2.6.3 Stereomicroscopy

The prolamin films were placed on a white tile and viewed both before and after buffer uptake experiment using a Discovery V20 Stereomicroscope (Zeiss, Goettingen, Germany) and photographed.

5.2.6.4 Scanning electron microscopy (SEM)

The surface morphology of the prolamin films was analysed both before and after the buffer uptake experiment using a Zeiss Gemini Ultra plus Field Emission SEM (Zeiss, Oberkochen, Germany). Rectangular strips of the film (approximately 10mm x 5mm) were cut using a sharp knife and mounted on aluminium stubs using double-sided carbon tape and coated three times with carbon using Emitech K950X carbon coater (Ashford, UK). The films were viewed and photographed.

5.2.6.5 Statistical analyses

Each experiment was performed in duplicate and repeated at least twice. The data were analysed using IBM SPSS Statistics 20 software (SPSS, Chicago, IL).). One-way analysis of variance (ANOVA) was used to determine significant differences of functional properties amongst the different prolamin types using films as models. One-way ANOVA was also used to determine any significance difference of functional properties of the different composite film ratios. Significance differences were determined by Fisher's Least Significance Difference test (LSD) at a 95% level of confidence.

6. RESULTS AND DISCUSSION

6.1 Effects of brewer's spent grain source on protein preparation yield and purity

The sorghum flours contained different amounts of protein (11.7-13.6%, dry basis) (Table 4) which is in agreement with the range of 7-15% sorghum grain protein content reported by (Serna-Saldivar and Espinosa-Ramíres, 2019). As expected, their respective BSG flours had higher protein contents (40.1-42.2%, dry basis) which is similar to the reported range of 38.0-41.2% (dry basis) by Holmes et al. (2013). Similarly, barley flour protein content of 12.5% as reported by USDA National Nutrient Database (2018) was lower than that of the barley BSG (23.6%, dry basis). During brewing, carbohydrates were converted into fermentable sugars, thus, concentrating the remaining grain components including proteins (Lynch et al., 2016). The barley BSG had lower protein content (23.6%, dry basis) than all sorghum BSG lines (40.1-42.2%, dry basis). Commercial brewing mashing processes are highly efficient in terms of protein solubilisation, as proteins are essential for yeast nutrition, beer flavour, mouthfeel, foam and colour (Sammartio, 2015). Barley BSG was produced by a commercial brewing process which was probably more efficient in terms of protein solubilisation than the laboratory-scale brewing process for sorghum beer. This resulted in barley BSG with a lower protein content than sorghum BSG.

Barley BSG had the lowest moisture content (3.3%) of the BSG samples (Table 4). This was probably because it was dried using an industrial drying process that used a much higher drying temperature than that of the oven-drying method used for sorghum BSG. Many breweries use a two-step process in drying their wet BSG (Aliyu and Bala, 2011). Initially, the BSG is pressed to lower its moisture content to less than 60%. This is followed by heat drying to ensure the moisture content lowers to below 10%. Traditionally, the heat drying is done using direct rotary-drum driers but other methods such as superheated steam using temperatures as high as 180^{o}C, can be used (Mussatto et al., 2006; Tang, Cenkowski and Izydorczyk, 2005). Laboratory-scale produced sorghum BSG was dried using an oven at 50^{o}C. For oven-drying, temperatures beyond 60^{o}C are not used to avoid unpleasant odours (Mussatto et al., 2006).

The yield of BSG hordein was the lowest protein yield (8.8%, protein basis) whilst the yield of the BSG kafirins were higher (37.3-43.8%, protein basis). Hence, the extractions were more effective for kafirin than hordein. The prolamin extractions were carried out using 70% ethanol as the solvent at 70^{o}C. However, Shewry, Field, Kirkman, Faulks and Miflin (1980) reported that hordein was more efficiently extracted and dissolved by 50% propan-1-ol compared to 70% ethanol at either 4, 20 or 60°C. Therefore, the choice of solvent used for hordein extraction could have resulted in a lower yield. Disulphide cross-linking in prolamins can be induced by wet-cooking and heat drying (Duodu et al., 2003; Celus et al., 2006; Muhiwa et al., 2017). Both BSG kafirin and BSG hordein were exposed to wet-cooking during brewing and to heat drying as method of preservation. BSG hordein was probably dried using a much higher temperature than BSG kafirin thereby inducing more disulphide cross-linking in BSG hordein. As a result, BSG hordein would have a higher degree of disulphide cross-linking than BSG kafirin, and consequently making the protein more difficult to extract, resulting in a lower BSG hordein yield.

BSG hordein preparation had lower protein purity (71.4%, dry basis) than the BSG kafirin preparations (80.6-88.8%, dry basis) (Table 4). Sorghum BSG was washed to remove residual starch prior to drying and kafirin extraction. Iodine tests were performed to ensure the complete removal of starch. The removal of residual starch or soluble non-starch polysaccharides would have semi-purified the prolamins (Lau et al., 2015). This is in agreement with the findings of Muhiwa et al. (2017) who obtained improved protein preparation purity when meals were washed prior to extraction. Barley BSG was not washed prior to extraction, therefore, the extracted hordein preparations could have contained residual starch thus, lowering its purity.

Table 4: Effects of spent grain source on purity and yield of protein extracted from sorghum and commercial barley spent grain

BSG source	Moisture content of BSG (g/100 g)	Grain flour protein content (g protein/100 g) (db)	BSG protein content (g protein/100 g) (db)	Yield of defatted protein preparation (g/100 g) (db)	Protein preparation purity (%) (db)
Barley	$3.30^a \pm 0.04$	12.48*	$23.60^a \pm 0.86$	$8.78^a \pm 1.44$	$71.40^a \pm 0.53$
Waxy high protein digestibility sorghum	$5.33^b \pm 0.06$	$11.69^a \pm 0.10$	$40.13^b \pm 0.37$	$37.29^b \pm 0.04$	$80.62^b \pm 0.72$
Waxy normal protein digestibility sorghum	$9.62^c \pm 0.08$	$13.63^c \pm 0.13$	$44.61^d \pm 0.46$	$43.80^b \pm 5.55$	$88.83^d \pm 0.40$
Non-waxy normal protein digestibility sorghum	$10.44^d \pm 0.30$	$12.80^b \pm 0.17$	$42.15^c \pm 0.27$	$41.35^b \pm 2.93$	$82.92^c \pm 0.30$

Values presented as mean values of three repetitions (n= 3) ± standard deviation, db = dry basis, BSG =brewer's spent grain;

Values in the same column with different superscript letters differ significantly ($p < 0.05$). *Value from USDA National Nutrient Database (2014)

6.2 SDS-PAGE of prolamins under reducing and non-reducing conditions

SDS-PAGE under reducing and non-reducing conditions was used to characterise commercial zein, standard kafirin, hordein from BSG and kafirins extracted from BSG from WND, WHD and NWD sorghum lines (Fig. 9). SDS-PAGE under reducing conditions of commercial zein (Fig. 9, track 14) revealed the presence of two distinctive bands at approximately 19 kDa and 25 kDa, which were identified as α_1- and α_2-zein, respectively (Xiao, Yunqi, Gonzalez, Xia and Huang, 2015). This shows that commercial zein was predominantly α-zein due to the absence of bands corresponding to β- (17 kDa) and γ-zein (27 kDa and 18 kDa) (Paraman and Lamsal, 2011). The absence of the cysteine-rich β- and γ-subclasses in commercial zein results in it having low levels of cysteine residues available for disulphide bonding.

Under non-reducing conditions, BSG hordein bands had the lowest intensity of all prolamins, despite the fact that all SDS-PAGE wells were loaded with 10 μg of protein (Fig. 9, track 12). This indicated the presence of protein polymers so large (> 100 kDa) that they could not enter the separating gel, as explained by Emmambux and Taylor (2009). These polymers were probably the D-hordeins (> 100 kDa) and disulphide-bonded sulphur-rich B-hordeins, whose polymerisation was induced by wet-cooking (Finnie and Svensson, 2014; Xia et al., 2011), which occurred during brewing and industrial drying. As expected, reduced BSG hordein had bands with greater intensity than the non-reduced BSG hordein bands (Fig. 9, track 16). This indicated that the disulphide bonds in the polymers had been reduced resulting in monomeric polypeptides that were small enough to enter the separating gel. The reduced BSG hordein bands (Fig. 9, track 16) corresponded to the A- (approximately 15 kDa), B- (approximately 32-45 kDa) and C-hordeins (approximately 40-75 kDa) (Balakireva and Zamyatnin, 2016).

Under non-reducing conditions the electrophoretic band pattern of kafirins extracted from BSG from WND, WHD and NWD sorghum lines under non-reducing conditions were essentially identical (Fig. 9, tracks 2, 3 and 4). The bands appeared at approximately 20-27 kDa, 45-50 kDa, 65 kDa and higher. This showed the presence of both α_1 and α_2-kafirin (24 kDa and 25 kDa, respectively) (Xiao et al., 2015). Bands corresponding to γ-kafirin (27 kDa) subclass (Belton et al., 2006) were also present. Elhassan et al. (2018) reported that under reducing conditions, WHD kafirin did not have a band on 18.5 kDa, identified as β-kafirin, yet this band was present in WND and NWD kafirins. In this study a very low intensity band corresponding to β-kafirin at 16 kDa (Oria, Hamaker, Axtell and Huang, 2000) was present in all three BSG kafirins, including from the WHD line (Fig. 9, tracks 7, 8 and 9). The low intensity of the WND and NWD BSG's β-kafirin bands was possibly due to the formation of reduction-resistant oligomers during the wet-cooking process (Duodu et al., 2002). In the WHD BSG kafirin, the synthesis of β-kafirin had been supressed (Elhassan et al., 2018) and consequently only a faint band was present.

SDS-PAGE under non-reducing conditions, showed that the relative concentration of dimers and oligomers in the sorghum BSG kafirins was higher than in the standard kafirin (Fig. 9, tracks 6, 7, 8 and 9). This indicated a higher level of disulphide-bonded polymerisation in sorghum BSG kafirins. This was probably as a result of heat-induced polymerisation (Zhao, Bean, Ioerger, Wang and Boyle, 2008; Duodu et al., 2003) during the adjunct cooking and mashing steps of the brewing process. This is in agreement with the work of Muhiwa et al. (2017) who found that DDGS kafirin had a relatively greater concentration of dimers than kafirin extracted from sorghum meal. They concluded that this was because DDGS kafirin had been exposed to high temperatures during processing which induced a higher level of disulphide cross-linked polymerisation. Under reducing conditions, the monomers (<28 kDa) had increased band intensities, but the dimers (40-50 kDa) had decreased band intensities, whilst there was a total disappearance of the oligomers (>65 kDa). This was due to the reduction of the polymers into monomeric polypeptides, as described for the BSG hordein.

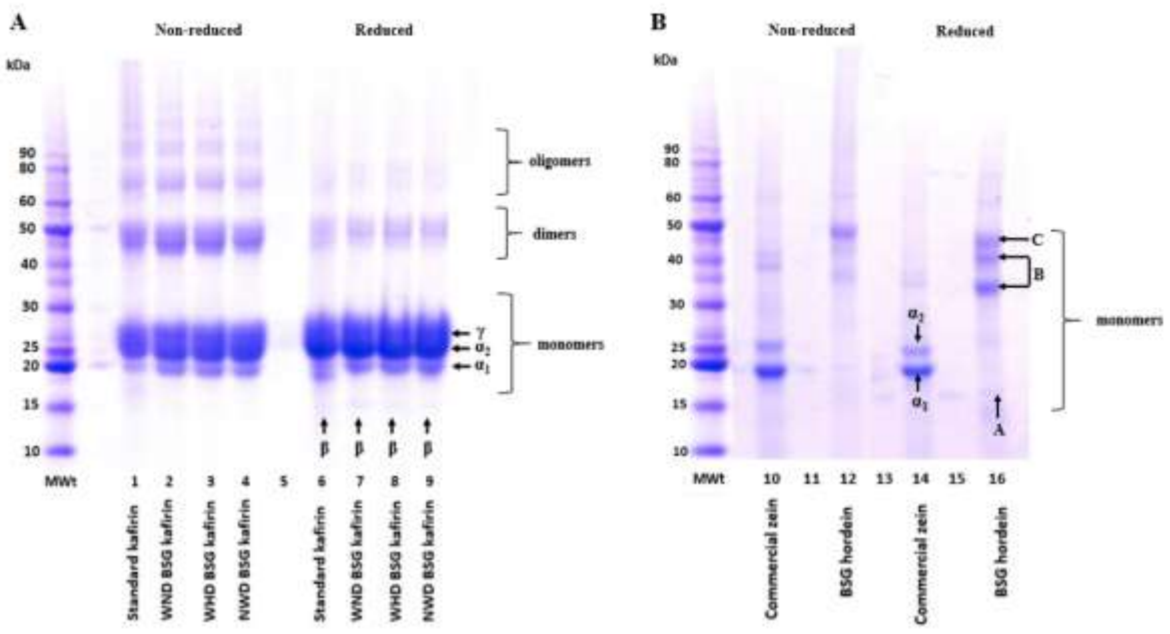

Figure 9: SDS-PAGE of commercial zein, kafirin and hordein preparations. A-SDS-PAGE of standard kafirin, brewer's spent grain (BSG) kafirin preparations under non-reducing and reducing conditions. B- SDS-PAGE of BSG hordein and commercial zein preparations under non-reducing and reducing conditions BSG kafirin was extracted from 3 sorghum lines; waxy normal protein digestibility (WND), waxy high protein digestibility (WHD) and non-waxy normal protein digestibility (NWD). MWt = Molecular weight standards

6.3 Nutritional composition of waxy normal protein digestibility (WND) sorghum flour and waxy normal protein digestibility sorghum brewer's spent grain flour

WND BSG flour was oven-dried at 50°C, resulting in its lower moisture content (8.3%) than the sorghum flour from which it came (11.4%) (Table 5). The nutrient content of WND sorghum BSG flour was higher in all cases (except carbohydrates) than its WND sorghum flour. This is in agreement with Holmes et al. (2013) who analysed the composition and ultrastructure of sorghum BSG. WND BSG flour contained 231% more protein and 279% more total dietary fibre than WND sorghum flour. Similarly, WND BSG flour had higher amounts of ash (minerals) (81%), fat (285%), iron (213%) and zinc (342%) than WND sorghum flour. These increases in nutrient contents were because of the enzymatic degradation of starch into fermentable sugars (wort) during brewing (Lynch et al., 2016). The removal of wort would leave the insoluble material, including insoluble proteins and dietary fibre in higher concentration. Hence, both red sorghum spent grain and WND BSG were rich in dietary fibre and protein (Table 5). However, red sorghum spent grain and WND BSG had different nutritional compositions. Such differences are probably due to the variety of grain, type and quality of malt and processes used during brewing (Buffington, 2014).

Table 5: Nutrient composition of waxy normal protein digestibility sorghum and waxy normal protein digestibility sorghum brewer's spent grain (g/100 g or mg/1000 g; dry basis)

Nutrient	Waxy normal protein digestibility sorghum flour	Waxy normal protein digestibility sorghum brewer's spent grain flour	Red sorghum spent grain (data from Adewusi and Ilori, 1994)
Moisture (g/100 g)	11.4	8.3	-
Protein (N x 6.25) (g/100 g)	12.8	42.5 (231%)	23.4
Total dietary fibre (g/100 g)	8.5	32.2 (279%)	54
Ash (g/100 g)	2.0	3.6 (81%)	0.78
Carbohydrate (g/100 g)	73.4*	9.0*	-
Fat (g/100g)	3.3	12.7 (285%)	-
Total iron (mg/1000 g)	30.7 ± 5.3	96.2 ± 16.7 (213%)	1.2
Total zinc (mg/1000 g)	19.2 ± 3.4	85.1 ± 15.0 (342%)	3.4

Samples were analysed by the Southern African Grain Laboratory

*Values calculated by difference

(-) data unavailable

The percentage increases of nutrient content from waxy normal protein digestibility sorghum flour to waxy normal protein digestibility sorghum brewer's spent grain flour are shown in brackets

6.4 Amino acid composition of different prolamin preparations

All the protein preparations were high in proline and glutamic acid (from glutamine) but low in lysine (Table 6), as reported in the literature for the isolated prolamins (Belton et al., 2006; Finnie and Svensson, 2014). Both BSG hordein (2.4 g/100 g) and BSG kafirin (2.2-2.5 g/100 g) had higher cysteine content than commercial zein (1.3 g/100 g) and standard kafirin (1.1 g/100 g). The variation in cysteine levels between the different isolated prolamins was probably due to species and varietal differences, or differences in environmental conditions and cultivation practices (Bean et al., 2011).

Kafirins extracted from WHD, WND and NWD BSG contained similar amounts of cysteine (2.2-2.5 g/100 g). It was expected that WHD BSG kafirin would have a lower cysteine content than the WND and NWD BSG kafirin types due to the suppression of synthesis of the cysteine-rich β- and γ-kafirin in this line (Elhassan et al., 2018). It is speculated that the suppression of the β- and γ-kafirin expression may have triggered the synthesis of compensatory kafirin subclasses (Da Silva, Taylor and Taylor, 2011), adding to the total cysteine content of WHD BSG kafirin.

WHD, WND and NWD BSG kafirin had the highest amounts of hydrophobic amino acids (isoleucine, leucine, phenylalanine, valine, cysteine, glycine, alanine, proline and tyrosine) amounting to 138, 130 and 122 g/100 g. These were followed by standard kafirin (61.4 g/100 g, as is basis), commercial zein (56.4 g/100 g) and BSG hordein (49.1 g/100 g). The greater hydrophobicity of all the kafirin than commercial zein is in agreement with literature (Duodu et al., 2003). Hordein has also been reported to be less hydrophobic than zein (Wang, Tian, Chen, Temelli, Liu and Wang, 2010).

Table 6: Amino acid composition of commercial zein, standard kafirin, commercial barley brewer's spent grain hordein and sorghum brewer's spent grain kafirin protein preparations (g/100 g protein)

Amino acid	Commercial zein protein preparation	Standard kafirin protein preparation	BSG hordein protein preparation	WHD kafirin BSG protein preparation	WND kafirin BSG protein preparation	NWD kafirin BSG protein preparation
Aspartic acid (including asparagine)	$4.0^{b} \pm 0.0$	$6.2^{c} \pm 0.1$	$2.0^{a} \pm 0.0$	$12.3^{f} \pm 0.1$	$11.1^{e} \pm 0.1$	$10.7^{d} \pm 0.0$
Threonine	$2.6^{c} \pm 0.0$	$2.5^{b} \pm 0.0$	$1.9^{a} \pm 0.0$	$5.8^{f} \pm 0.0$	$5.4^{e} \pm 0.0$	$5.1^{d} \pm 0.0$
Serine	$5.1^{e} \pm 0.2$	$4.4^{b} \pm 0.0$	$3.2^{a} \pm 0.0$	$10.6^{d} \pm 0.0$	$9.7^{c} \pm 0.1$	$9.4^{c} \pm 0.2$
Glutamic acid (including glutamine)	$20.3^{a} \pm 0.5$	$26.6^{b} \pm 0.2$	$39.7^{c} \pm 0.2$	$54.7^{f} \pm 0.2$	$49.7^{e} \pm 0.5$	$48.1^{d} \pm 0.3$
Phenylalanine	$5.9^{a} \pm 0.1$	$6.2^{a} \pm 0.1$	$7.2^{b} \pm 0.1$	$15.3^{e} \pm 0.1$	$14.4^{d} \pm 0.2$	$13.4^{c} \pm 0.0$
Valine	$3.3^{a} \pm 0.0$	$4.6^{b} \pm 0.0$	$3.6^{a} \pm 0.0$	$9.9^{e} \pm 0.0$	$9.2^{d} \pm 0.2$	$8.9^{c} \pm 0.0$
Methionine	$2.2^{b} \pm 0.1$	$1.3^{a} \pm 0.0$	$1.2^{a} \pm 0.0$	$2.3^{b} \pm 0.2$	$2.3^{b} \pm 0.2$	$2.4^{b} \pm 0.0$
Alanine	$7.9^{b} \pm 0.0$	$11.0^{c} \pm 0.0$	$1.7^{a} \pm 0.0$	$23.6^{f} \pm 0.1$	$21.9^{e} \pm 0.2$	$20.8^{d} \pm 0.1$
Proline	$9.4^{a} \pm 0.0$	$9.0^{a} \pm 0.0$	$19.9^{c} \pm 0.1$	$21.5^{d} \pm 0.0$	$20.9^{d} \pm 0.4$	$18.8^{b} \pm 0.1$
Cysteine	$1.3^{a} \pm 0.0$	$1.1^{a} \pm 0.1$	$2.4^{b} \pm 0.1$	$2.5^{b} \pm 0.1$	$2.4^{b} \pm 0.3$	$2.2^{b} \pm 0.3$

Table 6 continued

Histidine	$1.4^{c} \pm 0.0$	$1.0^{a} \pm 0.0$	$1.3^{b} \pm 0.0$	$2.8^{f} \pm 0.0$	$2.7^{e} \pm 0.0$	$2.5^{d} \pm 0.0$
Arginine	$1.7^{b} \pm 0.0$	$1.2^{a} \pm 0.0$	$2.2^{c} \pm 0.0$	$3.1^{e} \pm 0.0$	$3.1^{e} \pm 0.0$	$2.8^{d} \pm 0.0$
Glycine	$1.6^{b} \pm 0.0$	$1.3^{a} \pm 0.0$	$1.3^{a} \pm 0.8$	$3.2^{e} \pm 0.0$	$2.9^{d} \pm 0.0$	$2.7^{c} \pm 0.0$
Isoleucine	$3.3^{a} \pm 0.0$	$4.4^{b} \pm 0.0$	$3.7^{a} \pm 0$	$10.0^{e} \pm 0.0$	$9.5^{d} \pm 0.2$	$8.7^{c} \pm 0.0$
Leucine	$16.7^{b} \pm 0.1$	$17.8^{c} \pm 0.1$	$6.3^{a} \pm 0.0$	$39.0^{f} \pm 0.1$	$36.6^{e} \pm 0.4$	$34.5^{d} \pm 0.2$
Lysine	$0.1^{a} \pm 0.0$	$0.2^{b} \pm 0.0$	$0.7^{c} \pm 0.0$	$0.19^{b} \pm 0.0$	$0.1^{a} \pm 0.0$	$0.2^{b} \pm 0.0$
Tyrosine	$4.8^{b} \pm 0.0$	$4.7^{b} \pm 0.0$	$3.0^{a} \pm 0.0$	$10.6^{e} \pm 0.1$	$10.1^{d} \pm 0.0$	$9.6^{c} \pm 0.0$

The amino acid composition of the prolamins was determined by the Southern African Grain Laboratory; Hordein was extracted from commercial barley brewer's spent grain. BSG= brewer's spent grain; WHD= waxy high protein digestibility sorghum; WND= waxy normal protein digestibility sorghum; NWD= non-waxy normal protein digestibility sorghum.

Values represented as mean values of two repeat analyses (n=2) ± standard deviation. Values in the same row with different superscript letters differ significantly ($p< 0.05$)

6.5 Prolamin films

6.5.1 Effects of BSG hordein, WHD, WND and NWD BSG kafirin on surface morphology and film buffer uptake

Before soaking, commercial zein, standard kafirin, BSG hordein, WHD, WND and NWD BSG kafirin films had predominantly smooth surfaces. This was observed visually, by photography, stereomicroscopy and SEM (Figs. 10, 11 and 12, respectively). After soaking, all the films remained intact, had increased in surface area with some developing pores, cracks and folds (indicated by arrows). As observed by SEM, the surface of the unsoaked BSG hordein films were rougher than any of the other unsoaked films (Fig. 3). This was probably due to the poorer solubility of the BSG hordein. The poorer solubility was likely due to heat-induced polymerisation or the use of aqueous ethanol, which is not an optimum solvent for hordein, as explained in Section 6.1. Heat-induced polymerisation in BSG hordein resulted in high molecular weight polymers, which would be difficult to dissolve. The soaked BSG hordein films did not develop pores or folds but swelled and developed cracks (Figs. 2 and 3). This may have been due to the presence of higher molecular weight polymers, which would have greater resistance to expansion during buffer uptake, hence causing cracks (Njila, 2017). This resistance in expansion could also explain the very low increase in film surface area (Table 7 and Fig. 10).

Stereomicroscopy clearly revealed that WHD BSG kafirin and commercial zein films were the smoothest films (Fig. 11). WHD BSG kafirin completely dissolved in acetic acid, probably because it contained less disulphide cross-linkages due to the suppression of β- and γ-kafirin synthesis (Elhassan et al., 2018) than the BSG hordein, NWD and WND BSG kafirin films. Similarly, commercial zein which is predominately α-zein (Anderson and Lamsal, 2011) probably had less disulphide cross-linking and dissolved completely. Soaking the films probably caused dislodging of the impurities in the film matrices, thus, developing pores as described by Muhiwa et al. (2017).

BSG kafirin films developed numerous pores after soaking (Fig. 12). Muhiwa et al. (2017) explained this could be attributed to the poor solubility of kafirin in the film casting solution owing to heat-induced polymerisation. When examined by SEM (Fig. 12) WHD BSG kafirin films had the smallest pores and had loose flakes. The flaking of WHD BSG kafirin films was possibly due them having a less strong film matrix than the WND and NWD BSG kafirin films due to a lower level of disulphide cross-linking.

BSG hordein films absorbed 28% of the buffer solution whilst the BSG kafirin films absorbed more (50-96%) (Table 7). SDS-PAGE showed that BSG hordein was the most polymerised probably due to its exposure to heat during brewing and drying (Fig. 9). Such extensive disulphide-polymerisation would result in a more resistant structure to both moisture uptake and film expansion. This is in contrast to the findings of Xia et al. (2011) who concluded that heat-treating hordein did not enhance film moisture resistance. This was possibly because Xia et al. (2011) made their films with a plasticiser (glycerol). Glycerol reduces the polymer to polymer interactions in a film matrix and has a high capacity to interact with water which improves water permeation through the film (Wittaya, 2012; Xia et al., 2011). Additionally, glycerol could have leached out as described by Anyango et al. (2011), leaving holes in the films, thus increasing the surface area for greater water uptake.

WHD, WND, NWD BSG kafirin films had higher buffer uptake percentages (49.8-96.2%) than standard kafirin films (39.1%) (Table 7). This was contrary to expectations since BSG kafirin had more heat-induced disulphide bonds as was indicated by SDS-PAGE (Fig. 9). These results are also contrary to those of Muhiwa et al. (2017) who found a reduction in water uptake of kafirin films due to heat-induced disulphide bond formation. A possible explanation for these findings was the presence of microcracks in BSG kafirin films (Fig. 12). The microcracks were a result of the BSG kafirins film's greater thickness (Table 7) and poorer flexibility (due to disulphide-bonded polymerisation) than standard kafirin film (Da Silva and Taylor, 2005). These microcracks increased the film surface area, therefore, allowing more buffer uptake.

NWD BSG kafirin films had a much higher level of buffer uptake than WND and WHD BSG kafirin films (Table 7). This was despite their apparent similar levels of disulphide bond polymerisation, as indicated by SDS-PAGE (Fig. 9). NWD BSG kafirin contained the lowest amount of hydrophobic amino acids as previously discussed in Section 6.4. This could probably explain its higher buffer uptake.

Table 7: Effect of soaking in 0.2M phosphate buffer (pH 6.8) at 39ºC for 12 hours on surface area and weight on films from commercial zein, standard kafirin from sorghum flour, hordein from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines

	Before immersion in buffer			After immersion in buffer			
Film type	**Film weight (mg)**	**Film thickness (mm)**	**Film surface area (cm^2)**	**Film weight (mg)**	**Film surface area (cm^2)**	**Increase in film surface area (%)**	**Film aqueous buffer uptake (%)**
Commercial zein	$354.48^a \pm 4.97$	$0.12^b \pm 0.02$	$18.18^a \pm 0.27$	$725.60^d \pm 10.61$	$32.45^d \pm 0.61$	$78.55^d \pm 5.14$	$104.75^f \pm 5.52$
Standard kafirin	$346.08^a \pm 5.12$	$0.09^a \pm 0.06$	$17.83^a \pm 0.26$	$481.50^a \pm 12.38$	$20.53^a \pm 0.45$	$15.13^a \pm 2.71$	$39.15^b \pm 4.09$
Commercial BSG Hordein	$556.20^d \pm 5.06$	$0.19^c \pm 0.04$	$17.82^a \pm 0.23$	$713.65^c \pm 11.00$	$19.48^a \pm 0.47$	$9.34^a \pm 2.26$	$28.31^a \pm 1.27$
Waxy, high protein digestibility BSG kafirin	$458.38^c \pm 4.51$	$0.20^c \pm 0.02$	$18.07^a \pm 0.09$	$771.20^e \pm 6.01$	$26.88^c \pm 1.06$	$48.81^{bc} \pm 6.47$	$68.25^d \pm 0.87$
Waxy normal protein digestibility BSG kafirin	$424.43^b \pm 10.08$	$0.17^c \pm 0.01$	$18.04^a \pm 0.45$	$635.87^b \pm 13.29$	$24.51^b \pm 0.32$	$35.91^b \pm 4.57$	$49.83^c \pm 1.19$
Non-waxy normal protein digestibility BSG kafirin	$434.33^b \pm 4.64$	$0.18^c \pm 0.05$	$18.42^a \pm 0.29$	$852.23^f \pm 8.53$	$28.54^c \pm 1.80$	$55.11^c \pm 11.61$	$96.23^e \pm 2.38$

Values presented as means of two repeat analyses (n=2) ± standard deviation. BSG=Brewer's spent grain. Values in the same column with different superscript letters differ significantly ($p<0.05$)

Figure 10: Appearance of prolamin films to scale, before and after immersion in phosphate buffer (pH 6.8) for 12 hours at 39°C; Commercial zein films, A before and G after immersion; Standard kafirin films, B before and H after immersion; Barley BSG hordein films, C before and I after immersion; Waxy high protein digestibility (WHD) BSG kafirin films, D before and J after immersion; Waxy normal protein digestibility (WND) BSG kafirin films, E before and K after immersion; Non-waxy normal protein digestibility (NWD) BSG kafirin films, F before and L after immersion . R=folds, C=cracks

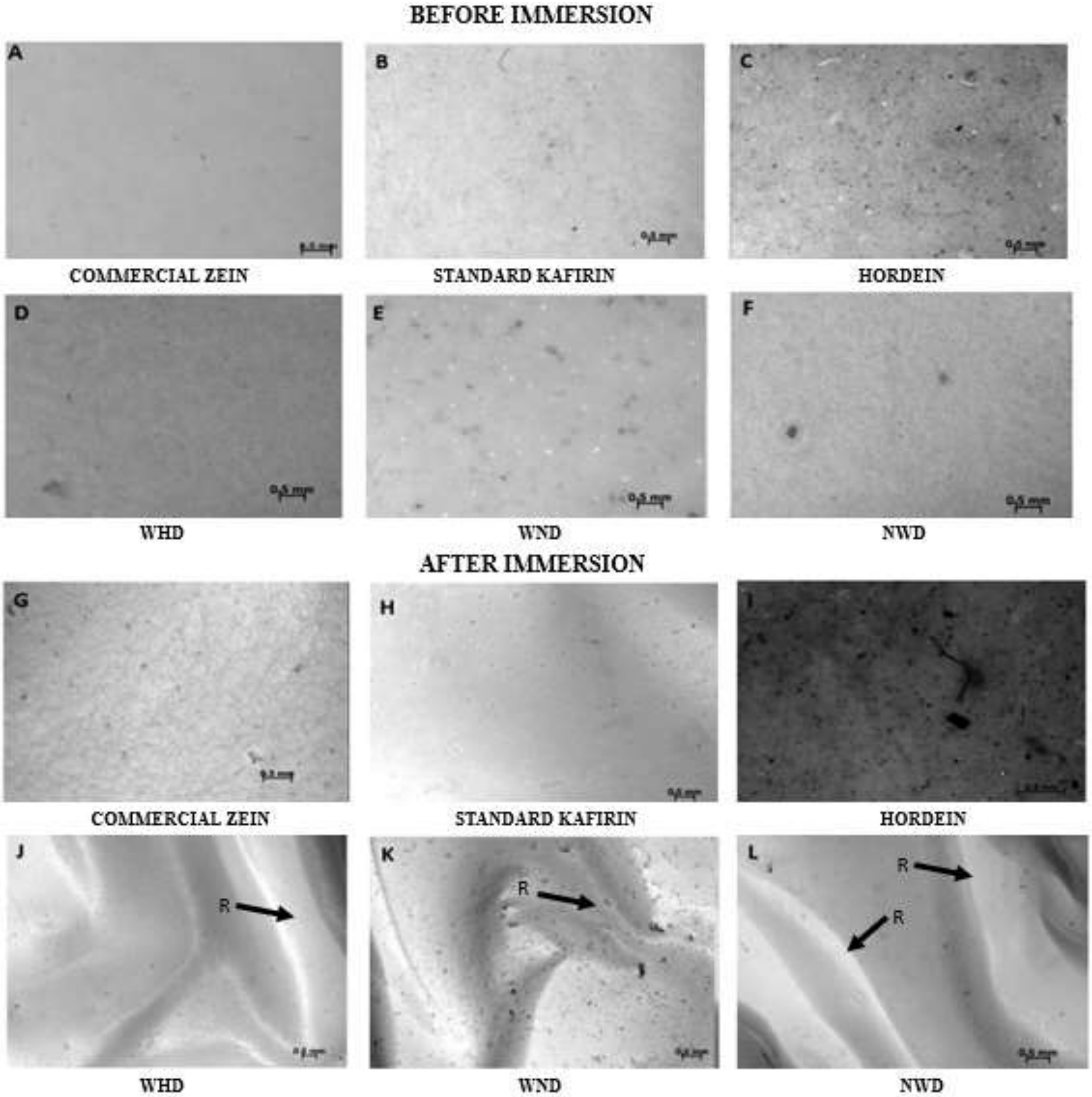

Figure 11: Stereomicroscopy showing the effects of soaking in 0.2M phosphate buffer (pH 6.8) for12 hours at 39°C on prolamin film surface morphology. Commercial zein films, A before and G after immersion; Standard kafirin flour films, B before and H after immersion; Brewer's spent grain (BSG) hordein films, C before and I after immersion; Waxy high protein digestibility (WHD) BSG kafirin films, D before and J after immersion; Waxy normal digestibility (WND) BSG kafirin films, E before and K after immersion; Non-waxy normal protein digestibility (NWD) BSG kafirin films, F before and L after immersion; R=flakes

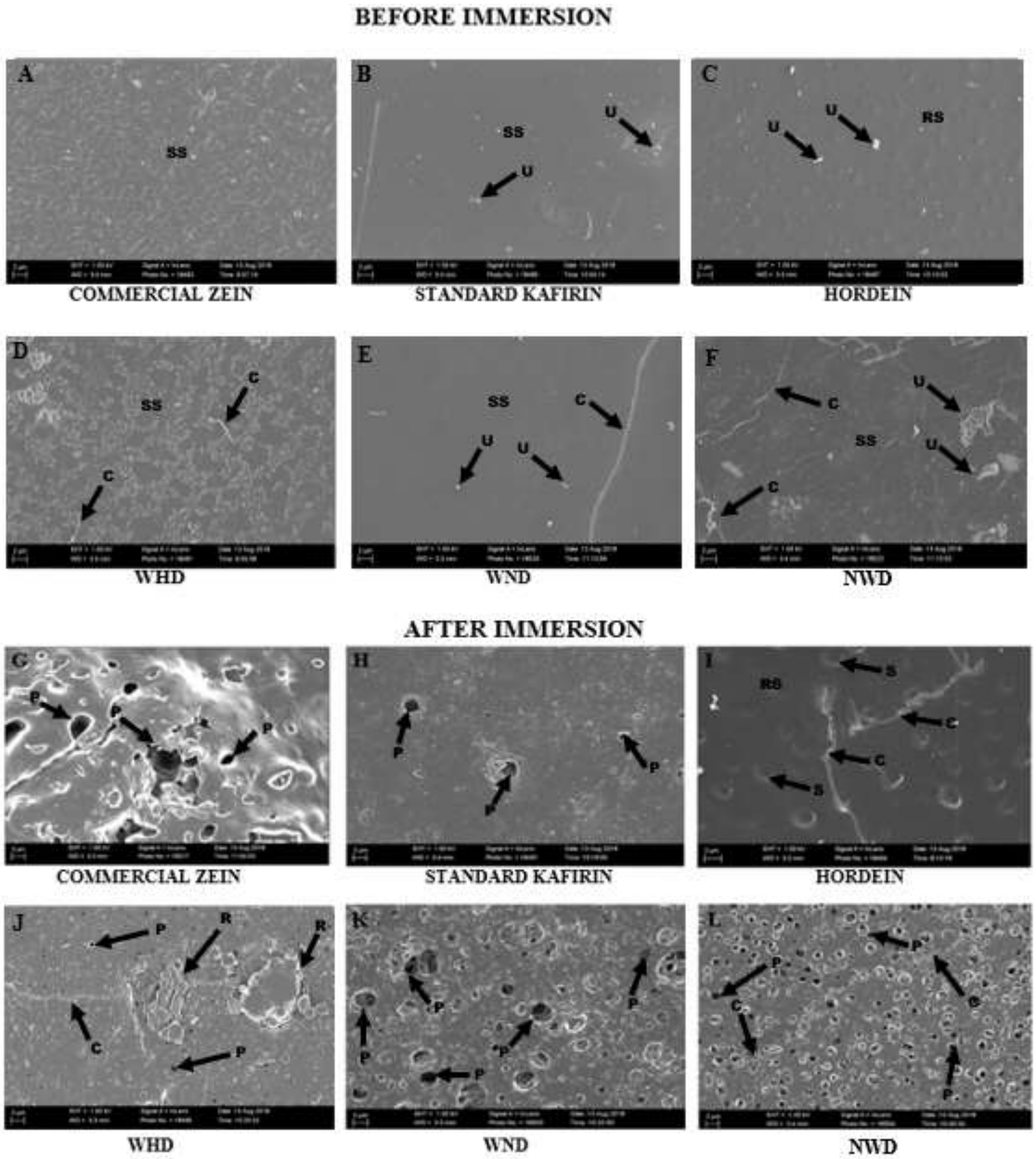

Figure 12: SEM showing the effects of soaking in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39^{o}C on prolamin film surface morphology. Commercial zein films, A before and G after immersion; Standard kafirin flour films, B before and H after immersion; Brewer's spent grain hordein films, C before and I after immersion; Waxy high protein digestibility (WHD) brewer's spent grain (BSG) kafirin films, D before and J after immersion; Waxy normal digestibility (WND) BSG kafirin films, E before and K after immersion; Non-waxy normal protein digestibility (NWD) BSG kafirin films, F before and L after immersion; SS= smooth surface; RS= rough surface; U=undissolved protein; P=pores; C=cracks; R=flakes

6.5.2 Tensile properties of BSG hordein, sorghum BSG kafirins extracted from WHD, WND and NWD lines films

WHD, WND and NWD BSG kafirin films had significantly ($p<0.005$) lower tensile strength (stress at break) and greater strain, 0.73-0.87 N/mm^2 and 167-191%, respectively than standard kafirin films, 1.55 N/mm^2 and 144%, respectively (Table 8, Figs. 13 and 14). BSG kafirins were heat-treated during the brewing process. The heat treatment induced disulphide-polymerisation as evidenced by SDS-PAGE (Section 6.2, Fig 9). Therefore, BSG kafirins were expected to be stronger and less extensible, as found by Byaruhanga et al. (2005). These authors found that films made from microwave heated kafirin had more than double maximum tensile strength and about one third lower strain percentage than films made from non-microwave heated kafirin. Standard kafirin was extracted from a different sorghum variety than the BSG kafirins which were extracted from sorghum lines with genetically identical parents (Elhassan, Emmambux, Hays, Peterson and Taylor, 2015). As shown by SDS-PAGE, the BSG kafirins and standard kafirin had similar β- and γ-kafirin band intensities (Fig. 9). However, SDS-PAGE does not quantify the amount of kafirin sub-classes present. Hence, standard kafirin may have contained higher amounts of β- and γ-kafirin leading to a higher level of disulphide cross-linking in the films. In addition, standard kafirin had a higher protein purity (95%) than the BSG kafirins (80.6-88.8%) (Section 6.1, Table 4). The presence of more impurities in BSG kafirin films would cause a disruption in the homogeneity and continuity within the film protein matrices as described by Buffo, Weller and Gennadios (1997). This would result in a lower tensile strength of the BSG kafirin films than the standard kafirin films.

BSG kafirins had significantly ($p<0.05$) higher tensile strength (stress at break) and lower strain than commercial zein (Table 8, Figs. 13 and 14). This was due to the presence of β- and γ-kafirin, which with heat-induced disulphide polymerisation caused during the brewing process, resulting in higher levels of disulphide cross-linking in the BSG kafirin films, as observed by Byaruhanga et al. (2005) and Da Silva and Taylor (2005). Apart from their exposure to heat, BSG kafirin films were more cross-linked than commercial zein because commercial zein is predominately α-zein (Lawton, 2002) and so largely lacks the cysteine-rich β- and γ-zein subclasses.

As previously discussed in Section 6.2, of all BSG prolamins, BSG hordein had the highest level of disulphide-bonded polymerisation. As a result, BSG hordein films should have had the strongest protein matrix. Based on this, BSG hordein films were expected to be the strongest and least extensible films. On the contrary, BSG hordein films were less strong than the WHD, WND and NWD BSG kafirin films. This can be explained by the lower protein preparation purity of BSG hordein (71.4%) than BSG kafirins (80.6-88.8%) (Table 4) which would result in reduced tensile strength, as is explained above.

There was no statistical difference in tensile strength and extensibility amongst the WHD, WND and NWD BSG kafirin films (Table 8, Figs. 13 and 14). However, it is assumed that WHD BSG kafirin had a lower level of disulphide bonding than the WND and NWD BSG kafirins. This was due to the suppression of the cysteine-rich β- and γ-kafirin synthesis in the WHD BSG kafirin (Da Silva et al., 2011; Elhassan et al., 2018). As a result, WHD BSG kafirin was expected to be the least strong and most extensible BSG kafirin. The difference in the findings could be explained by the SDS-PAGE data in Section 6.2. SDS-PAGE revealed that all BSG kafirins had similar levels of heat-induced disulphide polymerisation. This probably accounted for the similar tensile properties.

As expected, low-density polyethylene (LDPE) had significantly greater tensile (stress at break, 3.53 N/mm^2) and strain (429%) than all prolamin films, which at best were 1.55 N/mm^2 and 290%, respectively (Table 8, Figs. 13 and 14). Prolamin bioplastics possess inferior functional properties compared to synthetic plastics (Krochta, 2002). This is because the prolamin bioplastic structure uniformity is determined to an extent by the degree of repetition of the amino acid sequences (Taylor et al., 2013a). This is far less uniform than the ethene repeat units of LDPE plastic.

Table 8: **Effects of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on tensile properties of their films**

Prolamin type	Thickness (µm)	Maximum force (N)	Maximum stress (N/mm^2)	Maximum strain (%)	Force at break (N)	Stress at break (N/mm^2)	Strain at break (%)
Commercial zein	$110.0^d \pm 7.9$	$0.40^a \pm 0.06$	$0.32^a \pm 0.03$	$297^d \pm 19$	$0.38^a \pm 0.05$	$0.31^a \pm 0.03$	$290^d \pm 17$
Standard kafirin	$73.3^b \pm 3.2$	$1.31^d \pm 0.05$	$1.77^c \pm 0.05$	$151^{ab} \pm 4$	$1.15^c \pm 0.09$	$1.55^c \pm 0.06$	$144^b \pm 4$
Commercial BSG hordein	$114.0^d \pm 5.0$	$0.51^{ab} \pm 0.06$	$0.32^a \pm 0.09$	$126^a \pm 2$	$0.46^{ab} \pm 0.04$	$0.29^a \pm 0.08$	$119^a \pm 4$
Waxy high protein digestibility BSG kafirin	$99.0^c \pm 4.0$	$0.83^c \pm 0.09$	$0.83^b \pm 0.10$	$195^c \pm 6$	$0.73^b \pm 0.04$	$0.73^b \pm 0.06$	$191^c \pm 6$
Waxy normal protein digestibility BSG kafirin	$93.0^c \pm 5.6$	$0.76^b \pm 0.06$	$1.01^b \pm 0.18$	$194^c \pm 3$	$0.64^{ab} \pm 0.20$	$0.87^b \pm 0.34$	$184^c \pm 18$
Non-waxy normal protein digestibility BSG kafirin	$80.0^{bc} \pm 3.6$	$0.76^b \pm 0.11$	$0.76^b \pm 0.11$	$174^b \pm 5$	$0.72^b \pm 0.14$	$0.73^b \pm 0.15$	$167^{bc} \pm 12$
Low-density polyethylene (LDPE)	$35.3^a \pm 0.0$	$1.63^e \pm 0.21$	$4.63^d \pm 0.88$	$444^e \pm 9$	$1.33^c \pm 0.15$	$3.53^d \pm 0.06$	$429^e \pm 8$

Values presented as mean values of at least three independent experiments (n= 3) ± standard deviation. BSG= Brewer's spent grain

Values in the same column with different superscript letters differ significantly ($p< 0.05$)

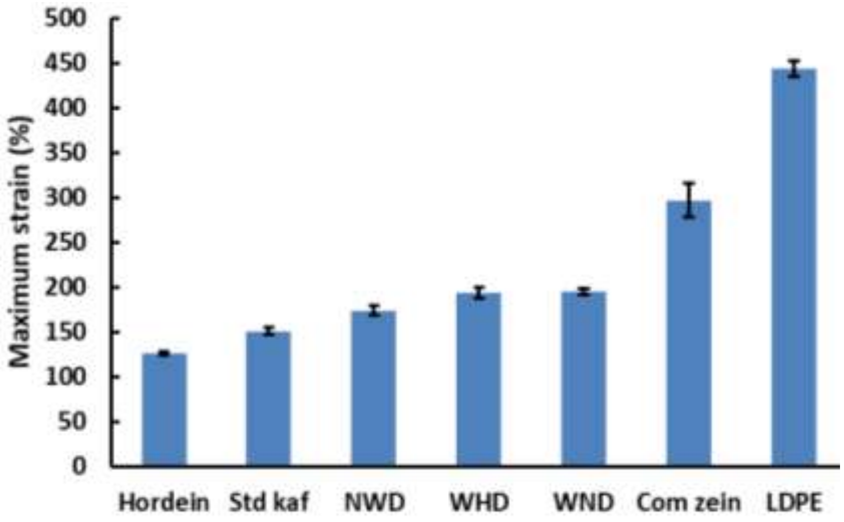

Figure 13: Effect of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on the maximum strain of prolamin films. Values presented as mean values of at least three independent experiments (n= 3) ± standard deviation. Error bars=standard deviation of mean (n=3). BSG=Brewer's spent grain

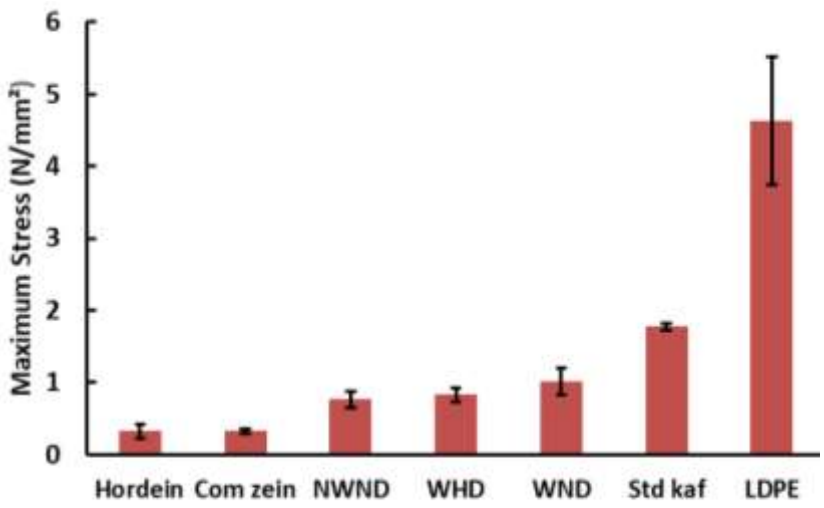

Figure 14: Effect of commercial zein, standard kafirin, hordein extracted from commercial barley BSG and sorghum BSG kafirin extracted from waxy high protein digestibility, waxy normal protein digestibility and non-waxy normal protein digestibility lines on maximum stress of prolamin films. Values presented as mean values of at least three independent experiments (n= 3) ± standard deviation. Error bars=standard deviation of mean (n=3). BSG=Brewer's spent grain

6.5.3 Effects of compositing commercial zein and standard kafirin on film surface morphology and buffer uptake

As previously shown, kafirin and commercial zein films possessed different functional properties. Kafirin films were more resistant to buffer uptake, stronger and less extensible than commercial zein films. Therefore, these two prolamins were combined at different ratios to form composite bioplastic films with the aim of obtaining more desirable functional properties than with the individual prolamins.

Prior to soaking, all films were smooth with no apparent imperfections, as observed visually by photography, stereomicroscopy and SEM (Figs. 15, 16 and 17, respectively). Muhiwa (2016) found that films produced from kafirin and zein preparations with fewer impurities were much smoother than those produced from less pure prolamin preparations. It could be that the films produced in this current study were smooth due to the high protein purity of commercial zein (92%) and standard kafirin (95%). On the contrary, Anyango et al. (2011) observed rough surfaces on kafirin films, which they attributed to poor kafirin solubility due to heat-induced disulphide cross-linking. However, both the standard kafirin and commercial zein used in this current research were not heat-treated. Therefore, they solubilised much easier to produce smooth films.

All the film types remained intact after soaking. However, they all became rough on the surface, developed pores and folds (indicated by arrows) (Figs. 15, 16 and 17). There was a gradual increase in the size and number of pores in the films as the proportion of commercial zein in the zein-kafirin composite films increased (Fig. 17). The 3:1 ratio zein-kafirin films had the greatest number as well as the largest pores (Fig. 17I). In contrast, 1:3 ratio zein-kafirin films had substantially fewer and smaller pores (Fig. 17G). As explained, commercial zein is predominantly α-zein (Anderson and Lamsal, 2011), whilst standard kafirin contains all subclasses including the cysteine-rich β- and γ-kafirins (Duodu et al., 2003). The presence of the β- and γ-kafirin caused the standard kafirin to be more disulphide cross-linked than commercial zein, as shown by SDS-PAGE (Fig. 9). Consequently, zein-kafirin composite films with a higher proportion of standard kafirin probably had a stronger protein network and thus, developed smaller pores. This is in agreement with the findings of Muhiwa (2016) who found that commercial zein films had larger pores than kafirin films. The stronger protein network of the kafirin films might have restricted the expansion of the films after soaking. This is supported by the fact that the surface area of the films decreased from 52.4% to 25.5% with increasing kafirin concentration (Table 9). In fact, there was an increase in buffer uptake of zein-kafirin composite films with an increasing proportion of zein (Fig. 15), from 49.6% at 1:3 zein: kafirin to 85.3% at 3:1 zein: kafirin. This was probably because zein is less hydrophobic than kafirin (Duodu et al., 2003), as explained in Section 2.6.3. Furthermore, commercial zein probably had a lower level of disulphide cross-linking than standard kafirin, as explained above. Therefore, zein-kafirin composite films comprising 75% commercial zein would have a less strong protein network than those comprising 75% standard kafirin. The less strong protein network in the 75% commercial zein films would presumably allow an easier movement of the buffer into the films. In apparent contrast to the findings above, Tsai and Weng (2019) found that increasing zein proportion in zein-whey protein composite films increased their moisture resistance. This was because zein is a hydrophobic protein relative to whey protein (Wittaya, 2012). Hence, the inclusion of zein increased the overall film hydrophobicity.

Table 9: Effects of soaking on commercial zein and standard kafirin films and their composites in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39°C on film aqueous buffer uptake

Film formulation relative percentage and (mass ratio)		Before immersion in buffer			After immersion in buffer			
Commercial zein	Standard kafirin	Film weight (mg)	Film thickness (µm)	Film surface area (cm^2)	Film weight (mg)	Film surface area (cm^2)	Increase in film surface area %	Film aqueous buffer uptake (%)
0	**100**	$329^a \pm 4$	$183.2^b \pm 7.3$	$17.8^a \pm 0.2$	$447^a \pm 18$	$21.1^a \pm 0.1$	$18.0^a \pm 4.9$	$35.8^a \pm 4.3$
25 (1)	**75 (3)**	$336^b \pm 5$	$187.4^{bc} \pm 2.8$	$17.8^a \pm 0.2$	$504^b \pm 6$	$22.3^a \pm 0.3$	$25.5^a \pm 2.8$	$49.6^a \pm 3.3$
50 (1)	**50 (1)**	$346^c \pm 6$	$191.0^c \pm 1.5$	$17.6^a \pm 0.1$	$578^c \pm 8$	$25.5^b \pm 0.9$	$44.9^b \pm 5.5$	$67.0^b \pm 3.6$
75 (3)	**25 (1)**	$359^d \pm 3$	$184.1^b \pm 2.9$	$17.8^a \pm 0.3$	$666^d \pm 10$	$27.2^c \pm 0.4$	$52.4^b \pm 3.4$	$85.3^c \pm 2.4$
100	**0**	$363^e \pm 3$	$170.7^a \pm 1.2$	$17.6^a \pm 0.1$	$770^e \pm 8$	$28.5^c \pm 0.3$	$61.8^c \pm 0.4$	$111.8^d \pm 3.0$

Values presented as mean values of two independent experiments (n= 2) ± one standard deviation, Values in the same column with different superscript letters differ significantly ($p < 0.05$). Film aqueous buffer uptake was calculated as the percentage difference between film weight after immersion and before immersion in buffer

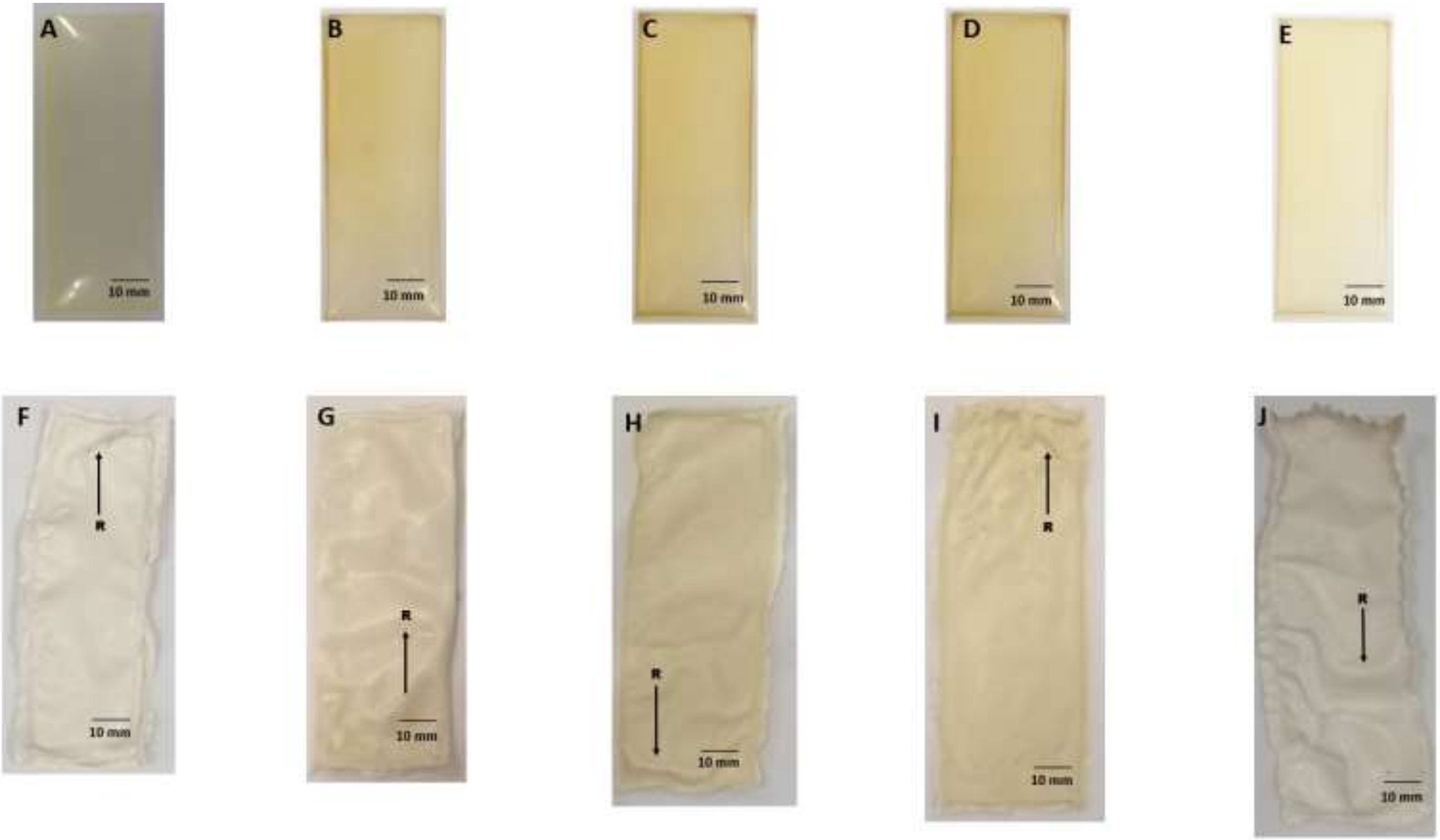

Figure 15: Visual appearance of commercial zein and standard kafirin composite films to scale, before and after immersion in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39°C. 100% standard kafirin films, A before and F after immersion; 25% commercial zein- 75% standard kafirin, B before and G after immersion; 50% commercial zein-50% standard kafirin, C before and H after immersion; 75% commercial zein-25% standard kafirin, D before and I after immersion; 100% commercial zein, E before and J after immersion. R=fold

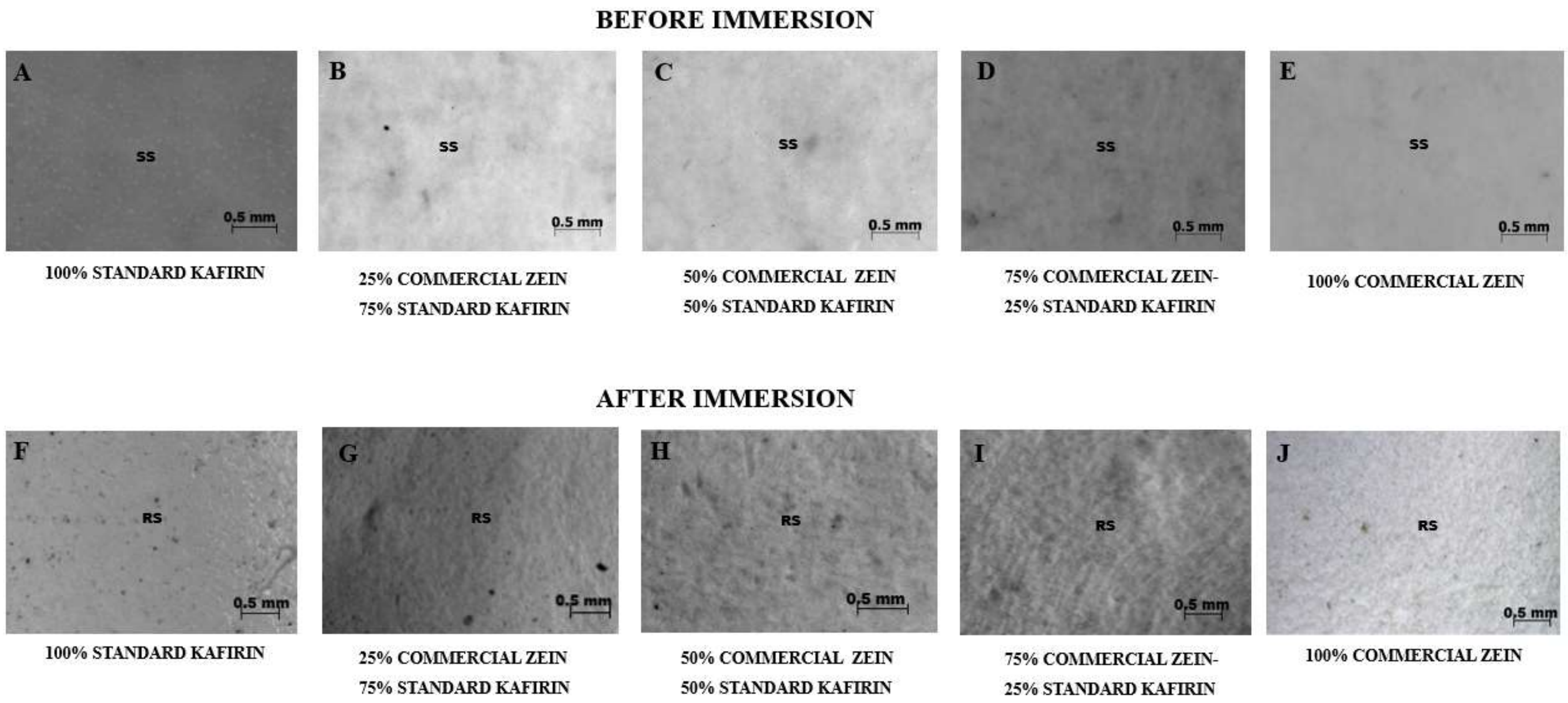

Figure 16: Stereomicroscopy showing the effects of soaking on commercial zein and standard kafirin films and their composites in 0.2M phosphate buffer (pH 6.8) for 12 hours at 39°C on film surface morphology. 100% standard kafirin, A before and F after immersion; 25% commercial zein-75% standard kafirin, B before and G after immersion; 50% commercial zein-50% standard kafirin, C before and H after immersion; 75% commercial zein-25% standard kafirin, D before and I after immersion; 100% commercial zein, E before and J after immersion. SS=smooth surface, RS=rough surface

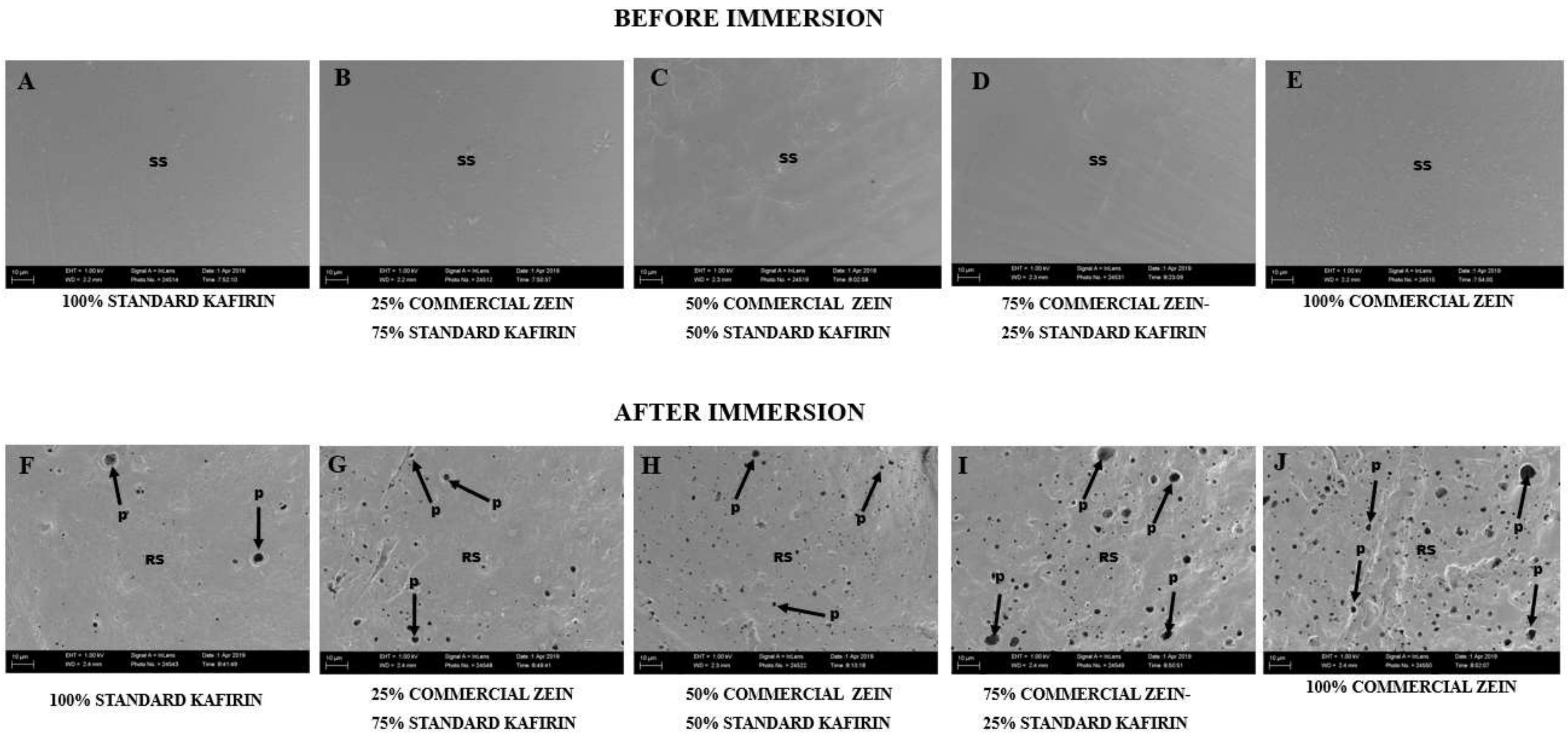

Figure 17: SEM showing the effects of soaking on commercial zein and standard kafirin films and their composites in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39°C on film surface morphology. 100% standard kafirin, A before and F after immersion; 25% commercial zein-75% standard kafirin, B before and G after immersion; 50% commercial zein-50% standard kafirin, C before and H after immersion; 75% commercial zein-25% standard kafirin, D before and I after immersion; 100% commercial zein, E before and J after immersion. SS=smooth surface, RS=rough surface, P=pores

6.5.4 Effect of compositing commercial zein and kafirin on their film tensile properties

The mean tensile stress (1.44 N/mm^2) and strain (138%) (Table 10) values for the films made from the standard kafirin were similar to those of kafirin films found by Byaruhanga et al. (2005). The results were similar probably because the films made in this current study and by Byaruhanga et al. (2005) were made using a similar methodology: drying temperature (50^{o}C), casting solvent (glacial acetic acid) and plasticiser (a 1:1:1 mixture of polyethylene glycerol, glycerol and lactic acid). Film drying temperature, type of casting solvent and plasticiser used are factors that are known to influence the functional properties of films (Lawton, 2004; Taylor et al., 2005b). However, commercial zein films had lower tensile strength (0.24 N/mm^2) and greater strain percentage (287%) than that reported by Buffo et al. (1997). The commercial zein used by Buffo et al. (1997) was obtained from a different source to that used in this current research. Numerous patents have described different methods to extract zein, which differ in pHs, solvents and temperatures used (Lawton, 2002). Such variations in zein extraction methodology may affect its extent of cross-linking, which in turn would affect the tensile properties of the resultant films.

There was an increase in tensile strength of the zein-kafirin composite films as the proportion standard kafirin amount increased (Table 10). The strongest composite films (0.80 N/mm^2) had a 1:3 zein: kafirin ratio. Their tensile strength was on average 29% greater than that of the weakest films of zein: kafirin 3:1 ratio (0.62 N/mm^2). On the contrary, the percentage strain of the composite films increased with an increasing proportion of commercial zein, from 180% at 1:3 zein: kafirin to 278% at 3:1 zein: kafirin. As explained previously, SDS-PAGE (Fig. 9) showed that standard kafirin had a greater level of disulphide cross-linking than commercial zein. Commercial zein was less polymerised probably because it consists mainly of α-zein (Lawton, 2002) and largely lacks the cysteine-rich β- and γ-zein, resulting in far less disulphide bonding. In contrast, standard kafirin contains both β- and γ-kafirin, which are rich in cysteine (Duodu et al., 2003) and can form disulphide bonds. Therefore, the films that contained more standard kafirin than commercial zein probably had a higher level of disulphide bonding. However, the greater extent of disulphide cross-linking in standard kafirin also caused a decrease in film extensibility (percentage strain), probably by decreasing protein chain mobility (Emmambux et al., 2004). These findings are in agreement with those of Da Silva and Taylor (2005). These workers concluded that due to a higher level of disulphide cross-linking, kafirin films were less extensible but much stronger than zein films. Similarly, Anyango et al. (2011) found that glutaraldehyde cross-linking resulted in increased kafirin film tensile strength. Notably, however, LDPE was 48% more extensible than the most extensible prolamin film (commercial zein) and its tensile stress was 177% greater than that of the prolamin strongest film (standard kafirin) (Table 10). As previously mentioned in Section 6.5.2, protein-based bioplastic films are inferior in functionality than LDPE (Krochta, 2002). This is because LDPE is made up of ethylene repeat units, whilst prolamins are made up of 20 different amino acids (Shewry and Halford, 2002).

Table 10: Effect of compositing commercial zein and kafirin on their film tensile properties

Film formulation (relative percentage and mass ratio)		Thickness (µm)	Maximum force (N)	Maximum stress (N/mm^2)	Maximum strain (%)	Force at break (N)	Stress at break (N/mm^2)	Strain at break (%)
Commercial zein	**Standard kafirin**							
0	**100**	85.3^b ± 3.8	1.16^b ± 0.10	1.58^c ± 0.14	138^a ± 13	1.06^b ± 0.17	1.44^c ± 0.13	134^a ± 4
25 (1)	**75 (3)**	105.3^c ± 4.0	0.94^b ± 0.16	0.94^b ± 0.06	180^b ± 7	0.80^b ± 0.05	0.80^b ± 0.04	175^b ± 10
50 (1)	**50 (1)**	91.3^b ± 7.5	1.07^b ± 0.21	0.90^b ± 0.31	220^c ± 4	0.91^b ± 0.16	0.77^b ± 0.29	211^c ± 8
75 (3)	**25 (1)**	131.7^d ± 6.0	0.87^b ± 0.08	0.67^b ± 0.06	278^d ± 7	0.80^b ± 0.03	0.62^b ± 0.09	271^d ± 2.4
100	**0**	115.7^c ± 3.1	0.39^a ± 0.07	0.28^a ± 0.09	292^d ± 10	0.34^a ± 0.08	0.24^a ± 0.08	287^d ± 13
Low-density polyethylene (LDPE)		33.7^a ± 0.0	1.71^c ± 0.16	4.56^d ± 1.02	447^e ± 33	1.49^c ± 0.20	3.99^d ± 1.03	424^e ± 3

Values presented as mean values of three independent experiments (n= 3) ± standard deviation

Values in the same column with different superscript letters differ significantly ($p < 0.05$)

7. GENERAL DISCUSSION

This discussion will firstly critically evaluate the major experimental methodologies applied in this investigation. Secondly, the important findings obtained from the research will be evaluated. Lastly, suggestions for further research work based on these findings will be made.

7.1 Critical review of methodologies applied in the research

Hordein was extracted from commercial BSG from barley malt. Kafirin was extracted from laboratory prepared BSG from waxy (high amylopectin) and high protein digestibility sorghum grain. Therefore, BSG hordein and BSG kafirin were generated using different brewing processes. As a result, the barley BSG and sorghum BSG were subjected to different brewing conditions. Lager beer mashing temperature is from 37°C to 78°C (Gupta et al., 2010) and with a duration of 1-2 h (Pires and Brányik, 2015), whereas the sorghum was heated to 85°C for 5 min. In addition, the drying conditions used for the barley BSG could have been as high as 180°C, if superheated steam was used (Tang et al., 2005). Sorghum BSG was oven-dried at 50°C. Such differences in heat treatment levels (temperature and time) would affect the degree of heat-induced disulphide-bonded polymerisation of the kafirin (Byaruhanga et al., 2005) contained in the sorghum BSG and of the hordein in the barley BSG. A higher degree of disulphide cross-linking of a prolamin would lower its solubility and in turn its extraction yields (Muhiwa et al., 2017). The extent of disulphide cross-linking within a prolamin greatly determines its film functional properties (Byaruhanga et al., 2005; Xia et al., 2011). Therefore, this makes it difficult to truly compare the functionality of BSG prolamin films. An ideal experiment to determine whether there are fundamental differences in the properties of hordein and kafirin extracted from BSG would be one that used similar brewing and drying conditions. It is known that 70% aqueous ethanol is not the ideal solvent for barley hordein extraction (Shewry et al., 1980). The most ideal solvent for hordein extraction would have been 50% propan-1-ol. Yet, in this study, 50% propan-1-ol was not used. The use of different extraction solvents for the different prolamin types would have probably affected the film functional properties. This would have introduced another variable into the experiment which would have made the results more difficult to explain.

In the study, SDS-PAGE was used to characterise the prolamin preparations. SDS-PAGE separates individual proteins in a mixture by subjecting them to an electric field and compares their apparent molecular weight to protein standards of known molecular weight (Michalski and Shiell, 1999). The procedure firstly involves the denaturation of the component proteins with an anionic detergent (sodium dodecyl sulphate) that binds to the proteins (Nowakowski, Wobig and Petering, 2014). The binding of the detergent to the proteins imparts a negative charge that is proportional to the molecular mass of the proteins. Finally, the protein components are run through an acrylamide gel matrix which acts as a molecular sieve so that they are separated on the basis of their apparent molecular mass. SDS-PAGE can be run under reducing and non-reducing conditions. With prolamins, non-reducing conditions reveal the presence of the prolamin monomers, dimers and oligomers in the mixture (El Nour et al., 1998). Under reducing conditions, the dimers and oligomers of the prolamins are reduced to their monomeric form; α-, β- and γ- subclasses for kafirin (Muhiwa et al., 2017) and A, B and C-subclasses for hordein (Baxter, 1981). The reduction is done using 2-mercaptoethanol, a reducing agent that breaks disulphide bonds (Hamaker et al., 1995). However, SDS-PAGE is disadvantageous in that there is need for post-separation staining to visualise the bands and it is not a quantitative method. When compared to other protein analytical methods such as isoelectric focusing and reversed-phase high-performance liquid chromatography, SDS-PAGE has low resolution power in separating kafirin subclasses (Blackwell and Bean, 2012). Therefore, for example, α-kafirin subclasses with very narrow molecular weight range (approx. 2-3 kDa) cannot be fully characterised. A major advantage of SDS-PAGE, however, is that it is very widely used to study cereal prolamin proteins and hence results from different researchers can be easily compared. It is also a relatively low cost and high throughput method (Bean and Lookhart, 2000).

The film buffer uptake analysis was determined as described by Muhiwa et al. (2017). To determine their buffer uptake, the films were soaked in sodium phosphate buffer (pH 6.8) for 12 hours at 39°C. Thereafter, the difference in weights of the film before and after soaking was used to calculate the buffer uptake. Buffer uptake analysis was carried out to determine how water resistant the bioplastic films were on immersion in buffer. It was important to know whether the films remained whole on immersion and whether they absorbed buffer and if so, how much. This would give an indication of how suitable the films would be for specific applications that require water resistant properties such as packaging and coating. The films that were used for this analysis were made without plasticiser, hence they were brittle.

Plasticiser was not used because it tends to leach out of the films and this increases their permeability to water (Anyango et al., 2011; Taylor et al., 2013a). In addition, hydrophilic plasticisers such as glycerol increase the movement of water within a film thereby increasing its buffer uptake, whilst hydrophobic plasticisers lower buffer uptake by closing micro-voids within a film (Vieira et al., 2011). The brittleness of the films is due to their polypeptide to polypeptide chain interactions (Krochta, 2002) that reduce their flexibility. As a result, it was found that some of the films cracked during handling and could not be used for the analysis. Furthermore, some films broke after soaking making it difficult to accurately measure their dimensions or blot them prior to measuring their weight.Their brittleness made it impossible to measure their thickness after soaking in buffer, as they would break upon application of pressure from the micrometer. In order to minimise film breakage, the films were handled with extra care.

Measuring the tensile properties of the prolamin films was very difficult. The films were very brittle and broke easily when they were mounted using abrasive paper coated grips on the texture analyser. To reduce the film brittleness, a plasticiser (1:1:1 mixture of polyethylene glycerol, lactic acid and glycerol) was added. When added to a film formulation, plasticisers reduce the hydrogen bonds between polymers by embedding itself in between them (Vieira et al., 2011). This reduces the second-order transition temperature and the elasticity modulus which improves the movement of polymer chains (flexibility). However, the addition of plasticiser is known to reduce the tensile strength and improve the extensibility of bioplastic prolamin films (Krochta, 2002). The extent of such changes is also determined by the choice and amount of plasticiser used (Vieira et al., 2011). In this current research, an external plasticiser was used. Therefore, the plasticiser was not chemically bound to the protein polymers (Niaounakis, 2013). Consequently, some plasticiser might have leached out during handling (Gao, Standing, Wellner, Parker, Noel., Mills and Belton, 2006). This made it difficult to accurately measure the tensile properties of the films as the plasticiser might have greatly affected their functionality.

The plasticised films were conditioned to 50% relative humidity prior to the analysis of their tensile properties. The conditioning was done to ensure that the films had the same relative humidity in order to directly compare their tensile properties. Increasing the relative humidity of films in the presence of a plasticiser causes changes in their mechanical properties (Olivas and Barbosa-Cánovas, 2008). This is because the water has a plasticising effect on films

(Lawton, 2004). When the relative humidity increased, the films became sticky and more flexible, making it difficult to cut them into strips. At times, the films would stretch out as they were being removed from the petri dishes they were placed in during conditioning. This was a drawback as most film strips had to be discarded because they were overstretched prior to analysis. Additionally, this resulted in very variable results such that a large number of films had to be analysed to reduce the large standard deviations.Consequently, films made for tensile properties analyses were more in number than those of buffer uptake analysis. Therefore to improve the efficiency of the formation of tensile properties analyses films, petri dishes, with a larger volume capacity were used instead of silicon trays. To rectify theproblem of overstreching, films could have been conditioned to a lower relative humidity or less plasticiser could have been used.

Table 11: Summary of the effects of the types of prolamin proteins on the functional properties of their films

Prolamin preparation	Effects of prolamin preparations on film buffer uptake	Explanations	Effects of prolamin preparations on tensile properties	Explanations
Standard kafirin	Absorbed the least buffer of all kafirin preparations	Standard kafirin had the highest purity and probably contained the most cysteine residues than the other kafirin preparations. Consequently, this kafirin preparation could form the most disulphide cross-linkages during film formation, resulting in a protein matrix with more resistance to buffer uptake	Standard kafirin films had the highest stress at break values of all prolamin films and had the lowest percentage strain compared to the BSG kafirin type films	Standard kafirin was probably the most disulphide cross-linked kafirin preparation, resulting in films with more resistance to strain and greater tensile strength than films made from other kafirin preparations
Commercial zein	Absorbed the most buffer of all prolamins	This was probably due to its lower hydrophobicity than kafirin. Commercial zein films had a weaker protein matrix than kafirin and BSG hordein films. This is as a result of a lower degree of disulphide cross-linking due to commercial zein having reduced cysteine-rich β- and γ-zein subclasses. The weaker protein matrix would result in films that had less resistance to buffer uptake.	Commercial zein had the highest percentage strain of all prolamins films. The commercial zein films were weaker than the kafirin films	Commercial zein mainly consists of α-zein and largely lacks the cysteine-rich β- and γ-zein subclasses which are responsible for disulphide bonding. Therefore, the films were less disulphide cross-linked and weaker than all the kafirin films. Hence, commercial zein films were more extensible and weaker than any of the kafirin films.

Table 11 continued

Hordein from barley BSG	Absorbed the least buffer of all prolamins	BSG hordein probably had the highest degree of heat-induced disulphide-bonded polymerisation. This resulted in films with a strong protein matrix that had more resistance to buffer uptake than all other prolamins. The impurities present in BSG hordein might have been hydrophobic thus the contributed to the improvement of buffer uptake resistance	Least strong and least extensible of all prolamin films	BSG hordein was the most impure of the prolamin preparations therefore the continuity and homogeneity of the protein matrix in the film was disrupted.
BSG kafirin types	NWD BSG kafirin absorbed the most buffer followed by WHD BSG kafirin and lastly WND BSG kafirin	NWD BSG kafirin films absorbed the most buffer because it contained the lowest amounts of hydrophobic amino acids. WHD BSG kafirin absorbed more buffer than WND BSG kafirin. This was due to the greater number of imperfections, in the form of cracks and flakes in WHD BSG kafirin films, which increased the film surface area for buffer absorption	All these BSG kafirin types had similar tensile properties (percentage strain and stress at break)	All the BSG kafirin types were extracted from sorghum lines with genetically identical parents. Furthermore, the BSG kafirin types were exposed to a similar level of heat-treatment. As a result, films made from these preparations had a similar level of disulphide-bonded polymerisation resulting in similar tensile properties.

Table 11 continued

Commercial zein and kafirin composites	Films absorbed less buffer with increasing kafirin mass ratio contribution	An increase in kafirin content increased the overall film's hydrophobicity	Film tensile stress at break increased whilst % strain decreased with increasing kafirin content	Increase in kafirin content increased the cysteine residues available for disulphide bonding within the films. This resulted in more film cross-linked protein matrices which improved the tensile strength and lowered the extensibility of the films.

7.2 Major research findings

7.2.1 Effect of the brewing process on the functionality of BSG hordein and BSG kafirin films

The various prolamin preparations produced films with a range of film functional properties, as summarised in Table 11. BSG hordein and BSG kafirin films were more resistant to buffer uptake, stronger and less extensible than commercial zein films. However, the BSG hordein and BSG kafirin films absorbed more buffer, were weaker and more extensible than standard kafirin films. Of the films made from heat-treated prolamins (BSG hordein and BSG kafirin types), the BSG hordein films had the most resistance to buffer uptake and were the least strong.

SDS-PAGE showed that standard kafirin, BSG hordein and all the BSG kafirin types were more polymerised than commercial zein. As stated, commercial zein is predominately α-zein (Anderson and Lamsal, 2011) and largely lacks the β- and γ-zein subclasses. The β- and γ-zein subclasses are rich in cysteine, 7 and 15 residues, respectively (Shewry and Tatham, 1990). As shown by SDS-PAGE, BSG hordein contained the B-hordein subclass, standard kafirin and BSG kafirin types contained the β- and γ-kafirin subclasses. These subclasses contain high levels of cysteine residues (Baxter, 1981; Belton et al., 2006). The B-hordein, β-kafirin and the γ-kafirin contain 8, 10 and 15 cysteine residues, respectively (Shewry and Tatham, 1990; Belton et al., 2006). Disulphide cross-links are formed by the oxidative coupling of two cysteine residues whereby an oxidant accepts hydrogen atoms from the thiol groups of the cysteine residues (Gerrard, 2002). Therefore, the more cysteine residues contained by a prolamin the greater its ability to cross-link. As illustrated in the model to explain the effects of disulphide bonding and the presence of impurities on the formation of a prolamin film matrix (Fig. 18), BSG hordein, BSG kafirin types and standard kafirin, which contain cysteine-rich subclasses, would form protein matrices that are more cross-linked than those of commercial zein. The lower degree of disulphide cross-linking within commercial zein films resulted in weaker protein matrices. This was indicated by the lower stress at beak values and high strain values.

Similar reasoning can be used to explain the relatively higher buffer uptake of the commercial zein films than all other films. A weaker protein matrix within the commercial zein films would have provided less resistance to buffer uptake. Furthermore, commercial zein is less hydrophobic than kafirin (Duodu et al., 2003). This can be attributed to the differences in the degree of hydrophobicity of kafirin and zein based on the free energy of hydration values calculated from their amino acid composition (Taylor et al., 2013a). Despite almost similar hydrophobicity levels of the α-zein (-139.78 kcal/mol) and α-kafirin (-140.36 kcal/mol), there is a difference in the hydrophobicity of the γ-prolamins (Duodu et al., 2003). The γ-kafirin (-113,63 kcal/mol) is more hydrophobic than γ-zein (-124.52 kcal/mol), thus overall kafirin is considered to be more hydrophobic than zein (Duodu et al., 2003; Taylor et al., 2013a).

During the mashing stage of the brewing process, BSG hordein and BSG kafirin were exposed to moist heat in the range of 60-85^{o}C. Such conditions would induce disulphide-bonded polymerisation within the prolamins (Byaruhanga et al., 2005; Celus et al., 2006) and result in the formation of a more cross-linked protein matrix, referred to as a "strong web-like microstructure" by Zhao et al. (2008). In addition to the mashing process, the sorghum BSG types were dried at 50^{o}C, whilst barley BSG was probably dried using temperatures as high as 180^{o}C if superheated steam was used (Tang et al., 2005). Drying also further induces disulphide-bonded polymerisation within the prolamins (Byaruhanga et al., 2005; Celus et al., 2006). However, the degree of polymerisation was different. As evidenced by SDS-PAGE, BSG hordein was more polymerised than all the BSG kafirin types. This implies that BSG hordein formed a stronger film protein matrix than the BSG kafirin. However, surprisingly, the BSG hordein films were weaker and more extensible than all the BSG kafirin films. The difference in the tensile property results from the expected, was possibly due to the presence of more impurities within the BSG hordein, which probably disrupts the structural cohesion of prolamin films (Buffo et al., 1997). Lack of uniformity within a protein matrix negatively affects its functionality (Taylor et al., 2013b). In this current study, BSG hordein films were prepared from a less pure protein preparation (71%) than that of the BSG kafirin types (81-89%) (Section 6.1, Table 4). Hence, as illustrated in the model (Fig. 18), BSG hordein films would have more interruptions in their polypeptide to polypeptide disulphide cross-linkages, which would weaken their protein matrix. The weakened protein matrices of the BSG hordein films would cause them to have lower tensile strength and greater extensibility than BSG kafirin. However, the presence of these impurities did not negatively affect the moisture barrier properties of the BSG hordein films (Section 6.5.1, Table 7), probably because they were lipids. This is despite the fact that the prolamins were deffated after extraction. The extraction process might have not completely removed all the lipids present in the prolamins. Lipids are hydrophobic in nature and are known to improve the moisture barrier properties of prolamin films (Krochta, 2002).

Based on the work of Byaruhanga et al. (2005) and Muhiwa et al. (2017), it was assumed that standard kafirin films would be less strong, more extensible and absorb more buffer than the films of the BSG kafirin types. These researchers concluded that heat-treating kafirin would increase its film tensile strength, lower its percentage strain and decrease its buffer uptake owing to heat-induced disulphide cross-linking. However, in this present study, standard kafirin films had greater tensile strength, were less extensible and more resistant to buffer uptake compared to BSG kafirin films. The BSG kafirin types were extracted from sorghum lines that had genetically identical parents with waxy and high protein digestibility traits (Elhassan et al., 2018), which differed from the sorghum variety from which standard kafirin was extracted. Therefore, the standard kafirin may have contained more cysteine residues than the BSG kafirin types, allowing it to be more disulphide cross-linked. As previously mentioned, a higher degree of disulphide-bonded polymerisation in a protein matrix, in this case, the standard kafirin, would reduce its film extensibility, increase its stress resistance and improve its moisture barrier properties.

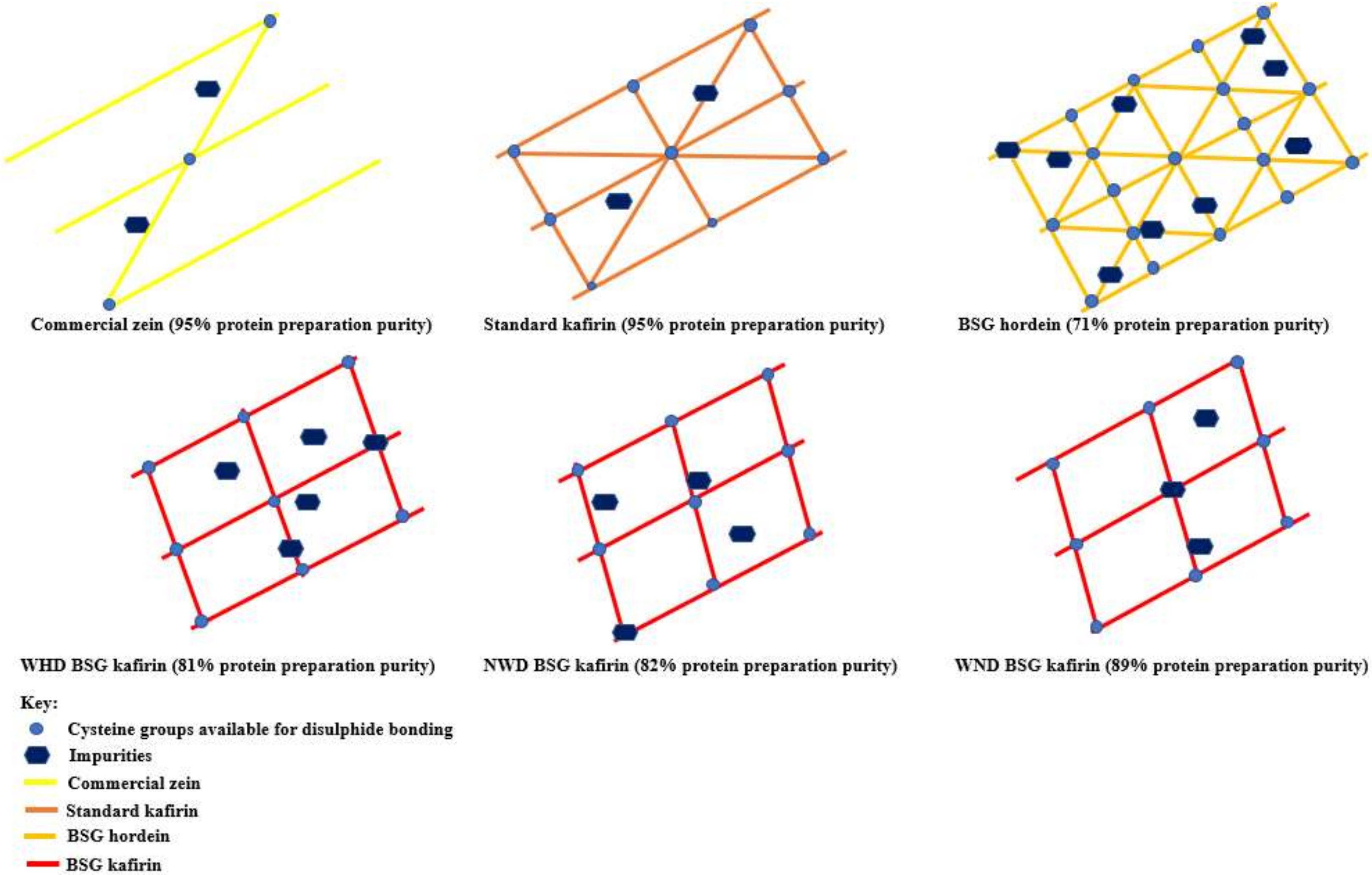

Figure 18: Schematic model to show the effect of disulphide bonding and the presence of impurities on the formation of a prolamin film matrix. The number of lines drawn on each film matrix represents the degree of disulphide cross-linking within the film

7.2.2 Waxy (high amylopectin) and high protein digestibility sorghum lines

The amounts of protein extracted from the sorghum BSG types were significantly different from each other (Table 4). The WND, NWD and WHD BSG contained 44.6 g, 42.2 g and 40.1 g of protein per 100 g (db), respectively. This was expected as the protein content of their initial respective sorghum flours contained significantly different protein contents, which ranked in the same order. SDS-PAGE (Fig. 9) showed that kafirin extracted from WHD, WND and NWD BSG sorghum lines had essentially identical patterns. According to Elhassan et al. (2018), β-kafirin synthesis was suppressed in WHD kafirin and SDS-PAGE showed no bands corresponding it. However, in this study, a faint band in the kafirin extracted from BSG from WHD was observed by SDS-PAGE, which corresponded to β-kafirin.

In this current research, it was assumed that the suppression of cysteine-rich β-kafirin would imply that WHD BSG kafirin would be the least disulphide cross-linked BSG kafirin type. Therefore, films prepared from WHD BSG kafirin would be the least resistant to buffer uptake, most extensible and the least strong amongst the BSG kafirins. However, NWD BSG kafirin films absorbed the most buffer. This was probably because it was the least hydrophobic kafirin since it contained the lowest amount of hydrophobic amino acids (isoleucine, leucine, phenylalanine, valine, cysteine, glycine, alanine, proline and tyrosine) (Section 6.4, Table 6). WHD BSG kafirin films absorbed more buffer than films made from WND BSG kafirins. This was probably because WHD BSG kafirin films developed cracks and flaked during soaking as evidenced by SEM (Fig. 12). WHD BSG kafirin was the least pure prolamin (81%), consequently, the high level of impurities would have disrupted the continuity of the film's protein network, as proposed by Buffo et al. (1997). These disruptions would weaken the film protein matrix causing the film to flake and crack. This resulted in an increase of film surface area of the WHD BSG films allowing more buffer to be absorbed than WND BSG kafirin films.

WHD, WND and NWD BSG kafirins had similar tensile properties (Table 8). When analysed by SDS-PAGE the WHD, WND and NWD BSG kafirin types had similar band intensities for all the kafirin sub-classes and their polymers. This suggested a similar level of heat-induced disulphide-bonded polymerisation. Additionally, as previously mentioned, the BSG kafirins were extracted from sorghum lines that have genetically identical parents (Elhassan et al., 2018). Therefore, it can be assumed that they contained similar amounts of cysteine residues and consequently in a similar level of disulphide cross-linking.

7.2.3 Effects of compositing zein and kafirin on the buffer uptake, tensile strength and extensibility of their films

In this study, commercial zein and kafirin were composited at different ratios to form composite films. Blending kafirin and commercial zein resulted in the formation of films that possessed characteristics of both kafirin and commercial zein. In appearance, the films were homogenous. This was attributed to the fact that the prolamins have a similar chemical composition (DeRose et al., 1989). As mentioned above, commercial zein is essentially α-zein (Anderson and Lamsal, 2011) and that the standard kafirin used contained all subclasses including the cysteine-rich β- and γ-kafirin (Belton et al., 2006). These cysteine residues are involved in the formation of disulphide bonds within protein networks. The β-kafirin acts as a chain extender that links together oligomers of γ- and α_1-kafirin by disulphide bridges (Duodu et al., 2003). The formation of disulphide cross-links has the ability to change the structure of proteins and therefore, the functional properties of the resultant film (Gerrard, 2002). Disulphide cross-linking decreases polypeptide chain mobility (Emmambux et al., 2004) and results in a strong protein network (Da Silva and Taylor, 2005).

Apart from kafirin containing all subclasses, it is more hydrophobic than commercial zein (Oom, Pettersson, Taylor and Stading, 2008). As previously mentioned, the difference in the hydrophobicity of these prolamins is mainly based on the free energies of hydration of their γ-prolamins. As per these calculations, γ-kafirin is more hydrophobic than the γ-zein giving kafirin an overall greater hydrophobicity (Duodu et al., 2003). Based on this, in this current study, it was hypothesised that the inclusion of kafirin in the films would increase the film's tensile strength, moisture resistance and reduce its extensibility. The results were as expected. Commercial zein-kafirin composite films that contained a higher proportion of kafirin were stronger, better moisture barriers and less extensible than those containing a higher proportion of zein (Table 9 and Table 10). Figure 19 is a model that illustrates how the addition of kafirin (with more cysteine residues) to commercial zein (with fewer cysteine residues) increases the degree of disulphide bonding within a film to form a strong protein network, resulting in the above-mentioned properties. It is evident that film functionality can be improved by compositing different prolamins with the desired characteristics. The combination ratios can be adjusted depending on the end-use of the bioplastic film. For example, less gas permeable prolamin bioplastics would be required for fruit coatings to reduce their respiration rate, to give a longer shelf-life (Taylor, Muller and Minnaar, 2015). On the contrary, more permeable prolamin bioplastics would be appropriate for use as edible sponges which can be used by the food industry in products as controlled release systems for flavour and bioactive compounds (Wang, Yin and Padua, 2008).

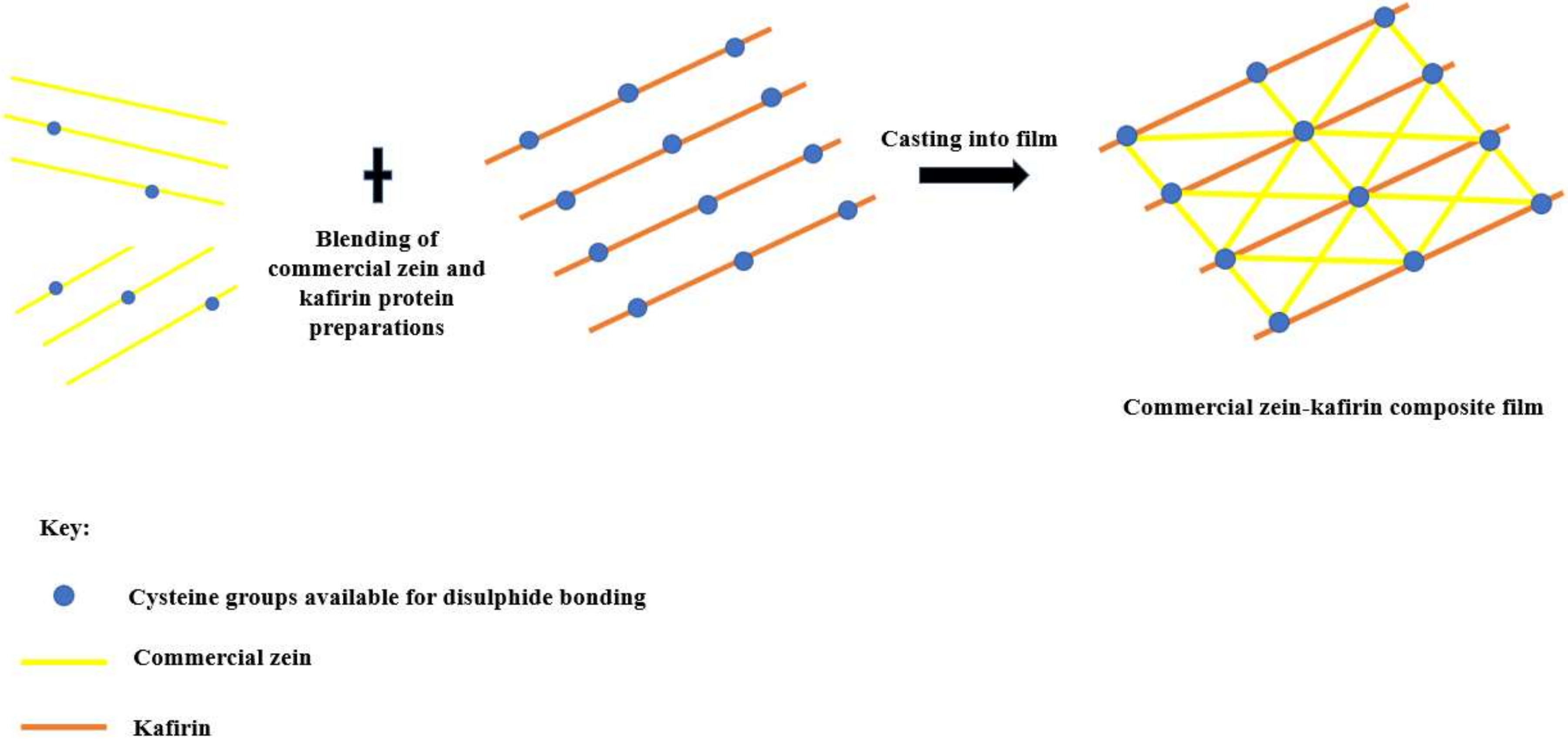

Figure 19: Schematic model to explain the formation of commercial zein and kafirin composite films

Way forward

This study has revealed that hordein and kafirin extracted from BSG have potentially better bioplastic functionality than commercial zein in terms of moisture resistance and tensile strength. Furthermore, bioplastic functional properties can be modified by blending prolamins to form a composite bioplastic. Prolamin bioplastic films have potential for use in biomedical applications as scaffolds in tissue repair, food coating and as encapsulation vehicles (Anyango, Taylor and Taylor, 2013). Developing specific applications for prolamin bioplastics such as those mentioned above will not completely replace synthetic plastics but will help reduce their use. This would help to address environmental pollution since prolamin bioplastics are biodegradable whilst most synthetic plastics are not. Future work should investigate whether BSG kafirin-BSG hordein composite bioplastics have better moisture barrier properties than their individual prolamins. Further research should also be done on the safety of these BSG and kafirin-commercial zein composite bioplastics, as was done on kafirin microparticles and film scaffolds by Taylor, Anyango, Potgieter, Kallymeyer, Naidoo Pepper and Taylor (2014). These researchers assessed the safety and biocompatibility of kafirin microparticles and film scaffolds using an animal model (rodents). They found that kafirin microparticles triggered a chronic inflammatory response within the rodents whilst the films did not. For food coating applications more research should be carried out to determine how these bioplastics might affect the sensory attributes of foods including texture, colour and odour.

This research has also resulted in a promising solution to the environmental pollution concerns caused by agri-wastes such as BSG and DDGS. BSG and DDGS are generated in large quantities by the brewing industry and are currently used as animal feed (Taylor and Taylor, 2018) and landfill (Buffington, 2014). Kafirin (Muhiwa et al., 2017) and hordein are not produced commercially, but zein is. This is probably because commercial zein is extracted from corn gluten, a protein-rich maize wet-milling co-product (Lawton, 2002). Its extraction from a prolamin-rich co-product rather than whole grains would reduce its production cost. DDGS (Muhiwa et al., 2017) and BSG can be used as raw materials for bioplastic fabrication. Sorghum and barley grain protein content is in the range of 7% to 15% (Gupta et al., 2010; Serna-Saldivar and Espinosa-Ramíres, 2019). The removal of starch during brewing increases the concentration of all other components of the BSG including proteins. As a result, BSG has a protein content of 19-30% (Lynch et al., 2016) similar to that of DDGS which contains 28-33% protein (Lim, Li and Klesius, 2011). This makes BSG and DDGS less expensive raw materials for kafirin and hordein extraction. However, research is still required to improve BSG prolamin extraction efficiency in order to further reduce cost and to improve extraction yields and prolamin purity.

8. CONCLUSIONS AND RECOMMENDATIONS

Barley and sorghum BSG contain much higher levels of hordein and kafirin than their respective whole cereal grains. This is because, during the brewing process, starch is hydrolysed into fermentable sugars, leaving the other components in considerably higher concentrations including proteins. These prolamins can be extracted from BSG. Once isolated, the prolamins can be used to make bioplastics as shown by a cast bioplastic film model system.

Exposure of prolamins to heat during the brewing process induces disulphide-bonded polymerisation, more so in hordein than kafirin. This affects the amount of protein that can be extracted and its functional properties. Films made from these prolamins are stronger and better moisture barriers than those made from commercial zein-type, which currently is the only prolamin commercially available. This makes BSG prolamin films better suited for food packaging and as coating materials. However, kafirins extracted from BSG from waxy (high amylopectin) and high protein digestibility sorghum grain form films that are less strong but more extensible than standard kafirin films. BSG hordein films are weaker and less extensible than standard kafirin films. Films made from BSG hordein have better moisture barrier properties than the films made from standard kafirin and BSG kafirin types. Currently, prolamin bioplastics are inferior to synthetic plastics. However, the use of heat treatment and the formation of composites can be used to modify their functional properties for a specific application. Composite films with intermediate properties can be achieved with a 1:1 ratio zein to kafirin. Compared to commercial zein alone, these composite films (ratio 1:1) are 221% stronger, 26% less extensible and 40% more resistant to water uptake. Depending on the intended end-use of the composite films, a reduction or increase in tensile strength may be desirable. Therefore, the ratio of zein to kafirin can be manipulated to achieve the desired tensile properties.

This research has demonstrated the potential of prolamins extracted from brewer's spent grain to produce bioplastics. It has also shown the benefit of compositing in the modification of bioplastic film properties. Going forward, research is needed into further improving the properties of prolamin bioplastics to increase their competitiveness with synthetics plastics. For example, BSG kafirin-BSG hordein composites could be studied since their individual prolamins have good tensile strength and moisture barrier properties, respectively, which are desirable characteristics.

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