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Extraction of kafirin and zein and their film properties

by

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DECLARATION

I, Peter Jackson Muhiwa, declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria, is my own work and has not previously been submitted for a degree at any other university or institution of higher education.

Signature: _____

Date: _____

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ABSTRACT

Extraction of kafirin and zein and their film properties

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Degree: MSc Food Science

Kafirin and zein are alcohol-soluble prolamin-type proteins that can be used to make biodegradable, environmental-friendly bioplastic films and coatings. Despite this potential, there is no commercial process for kafirin extraction. Zein, though commercially produced, is expensive.

Factors affecting kafirin and zein extraction and bioplastic quality were studied. Specifically, the effects of sorghum and maize grain particle size, inclusion of glacial acetic acid and sodium hydroxide in the aqueous ethanol extractant on extract filtration time, protein preparation purity and yield; as well as effect of heating kafirin and zein films on water uptake and film digestibility were investigated.

Sorghum meal with particle size >1 mm gave more rapid extract filtration time than sorghum flour with particle size <250 µm, due to larger inter-particle spaces. Sorghum flour yielded a kafirin preparation with lower purity (50%) than sorghum meal (58%). Kafirin extracted from distillers dried grains with solubles (DDGS) had a higher purity (78%) than kafirin from sorghum meal (68%) and zein (69%). Washing decorticated sorghum and maize meals to remove starch prior to protein extraction substantially increased protein preparation purity. Inclusion of sodium hydroxide resulted in a slightly higher protein preparation purity and yield than inclusion of acetic acid.

SDS-PAGE under non-reducing conditions revealed more dimers in kafirin than zein, while kafirin from DDGS contained polymers, indicating polymerisation due to cross-linking occurring during DDGS drying.

All films absorbed water but remained whole on soaking. Heat treated films prepared from kafirin extracted from washed sorghum meal absorbed less water (approximately 42%), compared to zein films (approximately 47%) due to its greater hydrophobicity and heat

induced intermolecular disulphide cross-linking. Heating had no effect on DDGS kafirin film water uptake because it was highly cross-linked.

In vitro pepsin and total digestion were less for kafirin films than zein films, probably due to the greater hydrophobicity of kafirin. Heating the films resulted in reduced digestibility, probably due to heat induced disulphide bonding.

Extraction of kafirin and zein from coarse meal enables more rapid filtration than from fine flour. Acetic acid-ethanol method appears to be a simpler extraction procedure than NaOH-ethanol method as it does not require protein neutralisation. Washing decorticated sorghum and maize meals prior to protein extraction improves protein preparation purity. Sorghum DDGS shows potential as a starting material for kafirin extraction for use in film making, based on high purity and low film water uptake. Heating the films decreases water uptake properties and reduces film digestibility.

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1 INTRODUCTION

Environmental problems caused by the use of synthetic plastics (Vroman and Tighzert, 2009) have led to increased interest in the use of biodegradable, more environmental-friendly bioplastics from natural renewable resources such as proteins, lipids and carbohydrates (Coma, 2008). Proteins from agricultural sources, both plant and animal have been given considerable attention because of their film-forming abilities and their potential in food applications (reviewed by Gennadios et al., 1994; Bourtoom, 2008).

Prolamins are the storage proteins of cereal grains. They are characterised by high glutamine and proline contents and are generally soluble only in aqueous alcohol solutions (Bean et al., 2006). These proteins can form bioplastic films and coatings intended for use as bioplastic materials in food related and non-food applications (Taylor et al., 2013). Using kafirin (sorghum prolamin) and zein (maize prolamin) to fabricate bioplastic materials seems technologically advantageous as they are amenable to various modifications (reviewed by Rezwan et al., 2006). This is necessary to improve the end-use functionality of bioplastics made from them.

Zein bioplastic films and coatings have been investigated for preservation of fresh, frozen and intermediate moisture foods (Taylor, 2003). Kafirin can form bioplastic films and coatings with superior gas and water vapour barrier properties compared to other prolamins, including zein (Taylor et al., 2005a). This is because kafirin has a more hydrophobic nature and is more cross-linked (Taylor et al., 2015) and is less digestible (Duodu et al., 2003) than zein.

Kafirin and zein exist in concentrated form in low value grain processing by-products such as distillers dried grains with solubles (DDGS), the main co-product from grain-based bioethanol production (reviewed by Wang et al., 2009), brewers spent grains (Mussatto et al., 2006) and corn gluten meal, a by-product of the maize wet milling industry (Lawton, 2002).

Despite the availability of raw materials from which kafirin and zein can be isolated, there is no commercial process for the extraction of kafirin (Belton et al., 2006) and zein, though commercially produced, is expensive (Taylor et al., 2013) due to the high operating costs involved in both extraction and purification (Shukla and Cheryan, 2001; Lawton, 2002).

Therefore, this Master's study was undertaken to investigate raw materials for kafirin and zein extraction, the efficiency of modified percolation extraction method and the quality of kafirin and zein extracted for use as bioplastic films with desirable functional properties.

2 LITERATURE REVIEW

This review explores the potential usage of sorghum and maize prolamin proteins, kafirin and zein respectively, as bioplastics. It focuses on the chemistry, structure, raw materials and extraction methods for kafirin and zein. Investigations into the functionality of these proteins as bioplastics are also reviewed.

2.1 Structure and composition of sorghum and maize kernels

Sorghum and maize kernels are classified botanically as caryopses, i.e. single seeded fruits, in which the fruit coat (pericarp) does not separate naturally from the seed (Koehler and Wieser, 2013). Sorghum and maize kernels consist of three distinctive anatomical components (approximate proportions): pericarp (8%), germ (10%) and the endosperm (82%) (Hoseney, 1994). A precise knowledge of the structure and composition of maize and sorghum grains is necessary for understanding how these cereals can be processed and efficiently utilised. Figure 2.1 shows a diagrammatic section through a sorghum kernel.

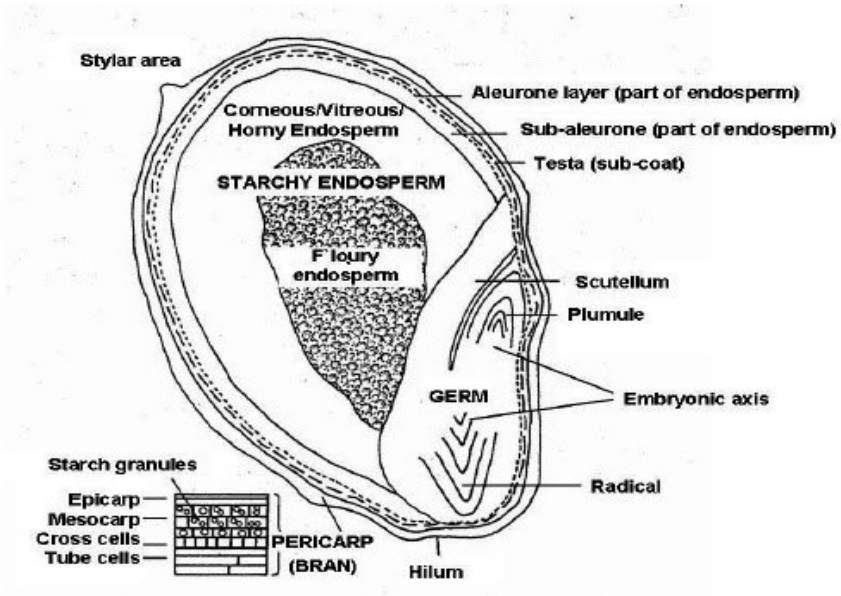

Figure 2.1: Cross section of sorghum grain (Taylor and Belton, 2002)

2.2 Prolamin proteins of maize and sorghum

The prolamins are the major proteins in sorghum and maize kernels and are called kafirin in sorghum and zein in maize. They are synthesised in the rough endoplasmic reticulum of the amyloplast (starch producing organelle) envelope (Buchanan et al., 2000). These proteins are deposited in discrete protein bodies within the starchy endosperm (Hamaker and Bugusu, 2003), as shown in Figure 2.2. Kafirin and zein are aqueous alcohol soluble and generally account for

about half of the proteins (40-55%) in mature grains (Chandrashekar and Mazhar, 1999). They are nitrogen reserves for the plant (Shewry, 2002). Kafirin and zein cannot be completely extracted by aqueous alcohol alone, as some prolamins are cross-linked by disulphide bonding and thus require a reducing agent to cleave these cross-links and increase the extraction efficiency (El Nour et al., 1998). Uncross-linked prolamins (prolamin I) can be extracted with aqueous alcohol alone and the cross-linked prolamins (prolamin II) requires a reducing agent such as sodium metabisulphite in addition to aqueous alcohol. The combination of these two fractions make up the total prolamins.

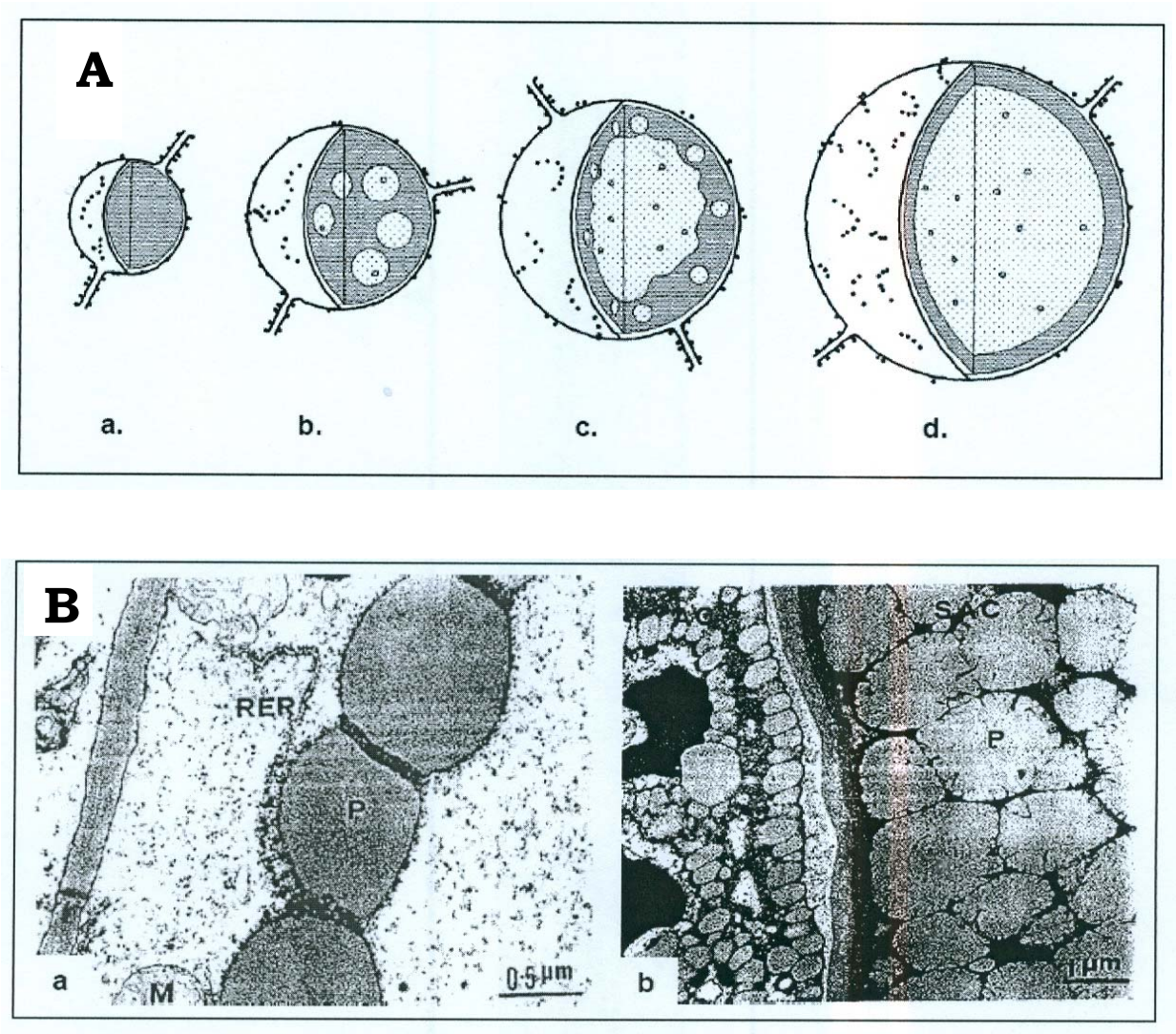

Figure 2.2: Protein body development in maize and sorghum endosperm

A) The heavily stippled regions correspond to regions that are rich in β- and γ-zeins, and the lightly stippled regions correspond to regions rich in α-zein. The protein body is surrounded by rough endoplasmic reticulum (dark dots represent ribosomes). Some β- and γ-zeins are found within the regions that consist primarily of α-zein (heavily stippled inclusions). Localisation of different zein proteins was done using immunolocalisation techniques (Lending and Larkins, 1989).

B) Electron micrograph of sorghum endosperm at soft dough stage of seed development. (a) **M**–mitochondria; **P**–protein body; **RER**–rough endoplasmic reticulum. (b) Sorghum aleurone and sub-aleurone cells at the late hard dough stage of seed development. **AC** – aleurone cell; **M**–matrix protein; **P**–protein body; **SAC**–sub-aleurone cell (Taylor et al., 1985).

2.2.1 Kafirin and zein sub-classes and structure

Kafirin and zein are heterogeneous proteins classified into different sub-classes (α-, β- γ- and δ-) of different proportions (Belton et al., 2006). The sub-classes are characterised based on differences in their solubility, amino acid composition, electrophoretic banding patterns, immunochemical cross-reactions and DNA sequencing (Shull et al., 1991; Mahzar et al., 1993; Belton et al., 2006). All these sub-classes are relatively high in glutamine, proline, asparagine, leucine and alanine. However, they are low in lysine (as shown in Table 2.2) (reviewed by Shewry and Halford, 2002). Proline is a highly hydrophobic amino acid capable of complex folding and thus proteins with high proline contents develop tertiary structures that are intensely hydrophobic and are soluble in aqueous alcohol solutions (Momany et al., 2006). These sub-classes differ mostly in the amounts of methionine (high in β-kafirins and β- and γ-zeins) and cysteine (high in γ-zeins and β- and γ-kafirins) (Shewry, 2002). As a result of the latter, these proteins exhibit extensive cross-linking by disulphide bonds (Belton et al., 2006). The γ-prolamins are stabilised by inter-chain disulphide bonds and are readily soluble in water in their monomeric form (Evans et al., 1987). As compared to gluten, which has a β-sheet structure, kafirin and zein have more α-helical structures (Taylor and Belton, 2002). Both zein and kafirin are more hydrophobic than gluten (Oom et al., 2006). This is because α-zeins and α-kafirins are high in hydrophobic amino acids such as leucine and proline (Belton et al., 2006).

Kafirin is different from zein in that it contains higher proportions of cross-linked polypeptides (Hamaker and Bugusu, 2003). This suggest higher propensity towards intermolecular disulphide cross-linking among kafirins than occurs with zeins. This is despite a high degree of sequence homology between the storage proteins of the two cereals.

Table 2.1 summarizes the major properties of the different kafirin and zein sub-classes with regard to their molecular weight and sequence features.

Table 2.1: Characterisation of the protein sub-classes of zein and kafirin (from Fombang, 2005)

Name	Molecular weight (kDa)	Optimum solvent for extraction	Consensus repeat motifs/sequence features	% total prolamin protein
Maize				
α-zein	21-25	50-95% propan-2-ol	Alanine, Glutamine, Phenylalanine, Proline, Leucine	75-85
β-zein	17,18	30-85% propan-2-ol + 2-mercaptoethanol	Methionine-rich	10-15
γ-zein	27	Water plus reducing agent and up to 80% propan-2-ol plus reducing agent	8 repeats of Proline, Valine, Histidine, Leucine; Cysteine-rich	5-10
δ-zein	10	Water plus reducing agent and up to 80% propan-2-ol plus reducing agent	No repeats	No data
Sorghum				
α-kafirin	23,25	40-90% t-butanol + 2-mercaptoethanol	Alanine, Leucine, Glutamine, Phenylalanine, Proline, Asparagine, Serine, Tyrosine	80
β-kafirin	20,18,16	10-60% t-butanol + 2-mercaptoethanol	Rich in Methionine and Cysteine, two Tryptophan	7-8
γ-kafirin	28	Water plus reducing agent or 10-80% t-butanol plus reducing agent	4 repeats of Proline, Valine, Histidine, Leucine; Cysteine-rich C- terminal domain	9-12
δ-kafirin	15	No data	Methionine-rich	No data

Table 2.2: Amino acid content (mole % of amino acid) of the different kafirin and zein sub-classes (from Fombang, 2005)

Amino acid	Total zein	α-zein	β-zein	γ-zein	Total kafirin	α-kafirin	β-kafirin	γ-kafirin
Asparagine	4.1	5.3	2.5	0	4.8	6.0	3.3	0
Aspartic acid	No data	0	No data	0	No data	0.4	No data	0
Threonine	3.1	2.8	2.5	4.4	2.8	4.0	4.6	4.7
Serine	6.5	6.9	5.0	3.9	4.7	6.0	4.6	4.7
Glutamine	16.4	20.7	18.1	14.7	20.0	24.6	17.8	11.9
Glutamic acid	No data	0.8	No data	1.0	No data	0.4	No data	1.0
Proline	10.7	8.9	8.8	25	11.2	7.7	9.7	23.3
Glycine	2.5	0.8	8.8	6.4	2.7	1.6	6.8	8.8
Alanine	14.5	13.8	13.8	4.9	15.6	14.9	13.4	5.7
Cysteine	1.0	0.4	4.4	7.4	0.7	0.4	4.9	7.8
Valine	4.0	6.9	1.9	7.4	5.6	4.4	5.2	6.2
Methionine	1.5	2.0	11.3	0.5	1.7	0.8	5.7	1.0
Isoleucine	3.7	4.5	0.6	2.0	4.1	5.6	2.3	2.6
Leucine	19.4	17.1	10.0	9.3	15.4	15.3	12.0	8.3
Tyrosine	3.6	2.8	8.8	2.0	3.0	2.8	3.0	2.1
Phenylalanine	5.6	3.3	0	1.0	4.7	2.4	1.9	1.6
Histidine	1.1	1.2	0	7.8	1.6	1.2	0.9	7.8
Lysine	0.1	0	0	0	0.2	0	0.5	0
Arginine	1.3	1.6	3.1	2.5	1.2	0.8	2.7	2.1
Tryptophan	No data	0	No data	0	No data	0.4	No data	0

2.2.2 Structure of zein and kafirin

A possible model for the arrangement of α-zein within a plane as well as for the stacking of molecular planes was developed by Argos et al. (1982). This model as shown schematically in Figure 2.3A was constructed through structure prediction based on the results obtained from circular dichroism. Alpha-zein is considered to consist of cylindrical polypeptide capsules, each containing nine adjacent α-helices. Each helix is made up of 20 amino acids, of which a few are polar and several are hydrophobic. At the ends of each capsule, a repeat sequence of glutamine residues can be found and here the capsules are joined together through hydrogen bonding.

Among the polar and hydrophobic amino acids in the different α-helices, intra- and intermolecular hydrogen bonds and Van der Waals forces exist, causing the zein capsules to aggregate. There are no structural models for kafirin. However, the amino acid compositions of kafirin and zein are very similar (Correa de Souza et al., 2015) and detailed sequence analysis has shown extensive homology between them (DeRose et al., 1989). Thus, it is likely that the structure of α-kafirin is similar to α-zein. Therefore, this model can also be taken as a possible description for α-kafirin molecular structure. The α-kafirin proteins are believed to have a tightly coiled α-helical structure resulting mainly from the repetitive sequence which folds up to form a cylindrical shape (Belton et al., 2006). These are linked together by hydrogen bonds formed between glutamine residues in the turn regions promoting packaging within the protein body.

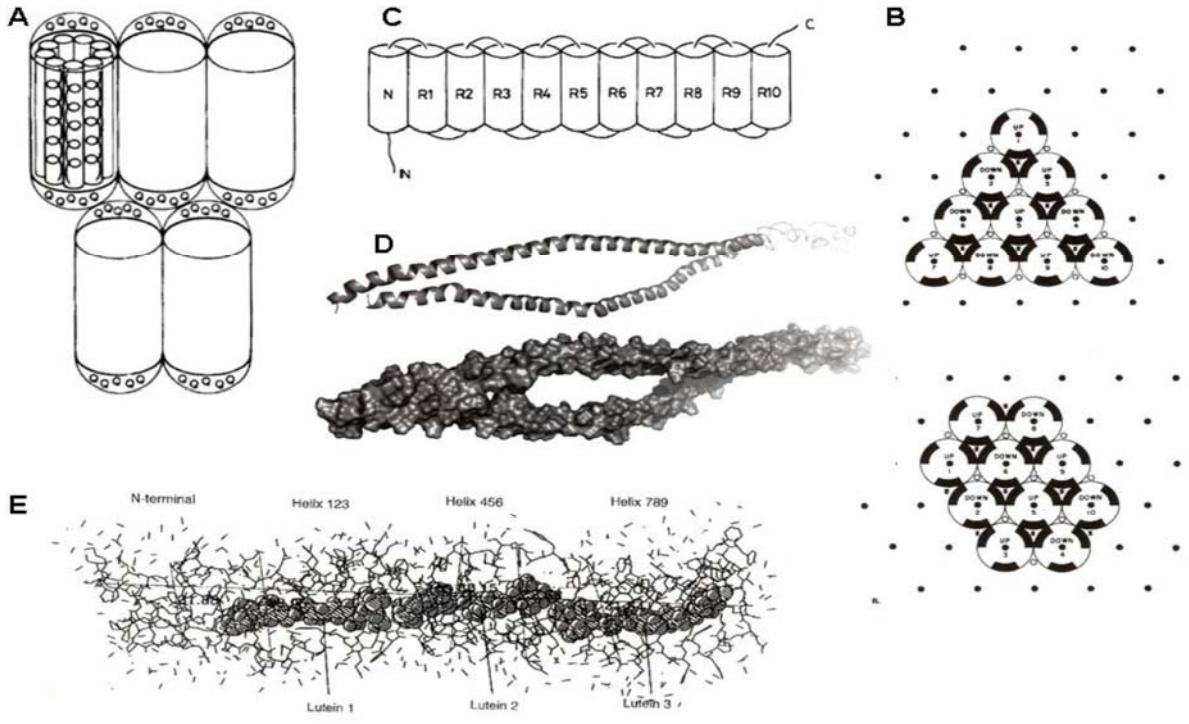

Figure 2.3: Structural models proposed for α-zein. **A**-Argos et al. (1982), **B**-Garratt et al. (1993), **C**-Matsushima et al. (1997), **D**- Bugs et al. (2004), **E**- Momany et al. (2006) (from Taylor, 2008)

Garratt et al. (1993) revised the Argos α-zein model to include all α-prolamins including α-kafirin (Figure 2.3B). This asymmetric structural model was constructed on pairs of the repeat amino acid sequences forming anti-parallel helices arranged in the form of hexagonal wheels with alternating groups of hydrophobic and polar amino acids.

In their research, Tatham et al. (1993) described the Garratt et al. (1993) structural model as having 'asymmetric' particles approximating to prolate ellipsoids or rods. These researchers

further reported that the asymmetric model would have several advantages such as degeneracy of the repeat motif, changes in the number of motifs without disrupting the structure and the ability of the molecules to form films, by orientation of the rods into two- and three-dimensional arrays, stabilized by hydrogen bonding between the side chains of the glutamine, asparagine, threonine, and serine residues.

Further to that, Matsushima et al. (1997) provided physical data that suggested that α-zeins were present in solution as extended structures. These researchers reviewed the Argos et al. (1982) α-zein model, stacking the nine anti- parallel helices in a linear manner (Figure 2.3C) to form a possible structural model for α-zeins (Z22). Each of the tandem repeat units formed by a single α-helix is presented by the cylinder and glutamine-rich 'turns' or loops joining them at the ends. The anti-parallel helices of tandem repeats stack linearly in the direction perpendicular to the helical axis.

Bugs et al. (2004) and Forato et al. (2004) both suggested α-zein structural models based on helical hairpin structures consisting of α-helixes, β-sheets and turns folding back on itself. The Forato et al. (2004) model shows short sections of helices arranged in an extended way connected by loops, turns or sheets. This permits the helices to fold back on themselves or to extend depending on the prevailing environment. The Bugs et al. (2004) model consists of two anti-parallel α-helices formed from coiled- coils to form a superhelical conformation with polar charged and hydrophobic amino acids distributed along the helical surfaces (Figure 2.3D).

The work by Momany et al. (2006) resulted in the development of a new model for the Z19 α-zein. This model comprises of three interacting coiled-coil conformations of helices 1-3 (residues 58-75, 76-98, and 99-113, respectively) with segments positioned end to end (Figure 2.3E). The non polar amino acid side chains form a hydrophobic face inside the triple super helix. This model has also provided room for lutein, a natural carotenoid pigment of zein which is difficult to extract from α-zein, in the core of the triple helical segments and helps to stabilise the configuration.

No secondary structure models for either β-,γ- or δ-zein nor their kafirin homolog have been reported and neither of these zeins contain repeated sequence motifs (Tatham et al., 1993; Taylor et al., 2013).

2.2.3 Raw materials for extraction of kafirin and zein

2.2.3.1 Whole grain flour

Whole grain flour is typically used for extraction of kafirin or zein. However, this results in protein preparations of low purity (approximately 65%) (Lawton, 2002). Dialysing the protein preparation against water as reported by Hojilla-Evangelista et al. (1992) has shown to improve the kafirin and zein preparation purity.

Hamaker et al. (1995) extracted kafirin and zein from whole grain flour using a pH 10 buffer containing aqueous alcohol plus a reducing agent. The authors reported total zein and kafirin preparation purity of 50-56% and 68-73%, respectively from maize and sorghum whole grain flour. El Nour et al. (1998) reported total kafirin preparation purity of 50.8% from sorghum whole grain flour using a sequential extraction with alcohol with and without a reducing agent. Schober et al. (2011) also extracted zein from dry milled maize and obtained a protein preparation purity of 66.8-89.3%.

2.2.3.2 Endosperm flour

The extraction of kafirin and zein can also be carried out using maize and sorghum endosperm flour. Prolamin protein preparation purity from endosperm flour seems to be higher than those from whole grain flour. This is because the removal of the germ entails loss of protein material, mostly albumin and globulins (Fombang, 2005). This would then give a higher prolamin purity in the preparation. Hamaker et al. (1995) extracted kafirin and zein from the endosperm flour. The zein preparation had a protein content of 62-74% and the kafirin preparation had a protein content of 77-82%. Using endosperm flour, Taylor and Schüssler (1986) produced a kafirin preparation with a protein content of 67.3-69.3%. Yau et al. (1999) extracted zein from maize endosperm flour and obtained a zein preparation with a protein content of 52-61%.

2.2.3.3 Sorghum bran

Da Silva and Taylor (2004) investigated the use of sorghum bran as a starting material for the extraction of kafirin. Bran is a co-product from sorghum milling or decortication of sorghum. Pure kafirin was isolated from this bran, but yields were lower than when using flour as the starting material. This was partially because the bran contained high levels of fat, phenolics and other compounds that interfered with protein extraction. In terms of functionality of the extracted kafirin, it was found that bioplastic films made from kafirin extracted from the bran were highly

coloured, less flexible with a less smooth surface texture compared with films from sorghum flour. This was probably due to higher levels of contaminants in the bran kafirin (Da Silva and Taylor, 2005).

2.2.3.4 Corn gluten meal

Corn gluten meal is a by-product of the maize wet milling industry which separates the germ, fibre, protein and starch constituents of the kernel (Neumann and Wall, 1984). It is a low cost raw material which is usually used as an animal feed. Corn gluten meal is high in protein (28%) and 60% moisture if wet, or 62% protein and 10% moisture if the product is dried before further processing (Lawton, 2002). Neumann and Wall (1984) were able to extract proteins from wet corn gluten meal and dried corn gluten meal. The wet corn gluten meal produced zein preparation with 70.4% protein content, while the dried corn gluten meal produced zein preparation with 71.4% protein content.

Parris and Dickey (2001) extracted zein from corn gluten meal and dry milled maize. In addition to α-zein, the authors observed β- and γ-zeins in zein preparation from dry milled maize but only trace amounts of β-zein from corn gluten meal. Zein preparation isolated by aqueous ethanol extraction from dry milled maize contained a mixture of monomers, covalently linked polymers and higher molecular weight aggregates. The preparation contained 80-85% protein. It has been reported that though there are differences in composition of the zein preparations extracted, comparable bioplastic films could be produced from zein preparations extracted from whole grain maize and corn gluten meal as long as the purity of the zein preparations are similar (Parris et al., 1997).

Zein extraction from corn gluten meal using acetic acid by Selling and Woods (2008) resulted in higher amounts of fatty acids and esters, leading to slightly lower protein content (71.7%) than the protein content of commercially obtained zein (88.1%). Zein extraction from corn gluten meal using the modified extraction procedure by Anderson and Lamsal (2011) resulted in zein preparation purity from 63.4-96.1% and yield between 21.6-35.6% when extraction was carried out using 70% 2-propanol and 70% ethanol.

Lawton (2006), extracted zein from corn gluten meal and was able to obtain zein preparation with a protein content of 96%. It was concluded from this work, that though extraction was done with dry corn gluten meal, corn gluten meal with normal moisture contents can be used for extraction.

2.2.3.5 Distillers dried grain and brewers spent grain

During brewing and bioethanol production, the grain starch is solubilised and hydrolysed leaving the co-product known as spent grain or distillers grain (Taylor and Belton, 2002; Xiros and Christakopoulos, 2009), which is a considerably enriched source of protein, fibre and lipids (Fărcaş et al., 2014). The spent grains have a protein content in the range of 20-25% (dry basis) (Mussatto et al., 2006; Aliyu and Bala, 2011). Kafirin and zein can be extracted from these materials (Wolf and Lawton, 1997). Taylor (2003) stated that this protein is however, very difficult to extract in maize and even more difficult in sorghum. The poor solubility of these proteins is believed to be due to protein denaturation during the fermentation and distillation process (Wolf and Lawton, 1997) and because of its complex protein composition (Bandara et al., 2011).

Research on isolation and characterization of zein from maize distillers dried grains with solubles (DDGS) has been done by Wolf and Lawton (1997). The authors obtained a zein preparation purity of 35-57%. This indicates that lipids and pigments were co-extracted with the ethanol.

Xu et al. (2007) extracted zein from DDGS with a protein content of 90.4% and yield of 44%. This was compared to commercial zein with a protein content of 92.4%. The authors concluded that carbohydrates and lipid complex moieties probably accounted for the remaining 10%.

Extraction of kafirin proteins from sorghum DDGS has been conducted by Wang et al. (2009). The researchers characterised the composition as well as chemical and physical properties of kafirin proteins extracted from sorghum DDGS using acetic acid, HCl-ethanol and NaOH-ethanol under reducing conditions. They obtained a kafirin preparation with a protein content of 42.3-98.9% and yield between 24.2-56.8% depending on the method used. A recent study on the extraction of kafirin from DDGS by Lau et al. (2015) resulted in a kafirin preparation with a protein content of 82.3%. The higher protein content of the kafirin was as a result of washing the distillers dried grains three times with five to six volumes of hot water (50^{o}C) to remove most of the water soluble substances.

2.3 Particle size effect on kafirin and zein extraction

Studies have shown that the extraction of zein depends on the particle size of the raw material (Russell and Tsao, 1982; Shukla et al., 2000). Particle size has typically been related to the available surface area for reactions such as solubilisation (Mahasukhonthachat et al., 2010). Cookman and Glatz (2008) observed that the extraction of zein from maize DDGS flour gave higher yield than extraction from coarse material. They attributed this to an increased surface area of the flour for greater contact with the solvent. Erasmus and Taylor (2003) also reported that the extraction of zein can be greatly improved by reducing the particle size.

Russell and Tsao (1982) reported that the extraction of zein depends on the diffusion of ethanol into the material. Because of the rate-limiting effect of diffusion, smaller particles are needed to quickly extract zein. However, research by Dickey et al. (1998) showed that during extraction, vigorous mixing along with raising the solvent temperature to 50^{o}C, disintegrated the endosperm, thereby diminishing diffusion limitations on extraction rates. These authors decorticated the maize, thus reducing the amount of cell wall material. Shukla et al. (2000) reported that high yields of zein (60% of the zein present in maize) could be extracted from ground maize with a particle size of 200-800 µm in 30 to 40 minutes when extracted at 50°C with 70% aqueous ethanol.

Jameson et al. (2001) reported that filterability of the solvent through the grain material is affected by particle size of the material used. Larger inter-particle space of coarse grain material makes it easier for the solvent to filter through faster than fine flour (Palmer, 1999). Sieve clogging when using milled sorghum grains with particle sizes of 0.3 mm have been reported by Barcelos et al. (2011).

2.4 Methods of kafirin and zein extraction

2.4.1 Extraction of kafirin and zein

Prolamin extraction involves its solubilisation, separation and isolation from the cellular constituents with which the proteins are intimately connected chemically and physically. Landry and Moureaux (1970) developed a procedure to isolate five protein fractions from maize grain through sequential extraction. This fractionation procedure, using 2-mercaptoethanol (2-ME) in combination with aqueous alcohol, salt and sodium dodecyl sulphate (SDS) as a detergent, made

it possible to isolate zein as well as cross-linked zein and other proteins with amino acid compositions close to that of zein.

Disulphide bonding of kafirin proteins result in the formation of large polymeric kafirin proteins, which are insoluble in the aqueous alcohols (Hamaker et al., 1995). Cysteine residues also contribute to intra- and inter-molecular disulphide bonding of kafirins (Bean et al., 2011). Thus, the optimum solvent for kafirin is 60% aqueous tert-butanol, due to its higher hydrophobicity compared to other aqueous alcohol solvents, such as ethanol and propanol which are commonly used (Belton et al., 2006).

Jones and Beckwith (1970) found extraction of kafirin with aqueous tert-butanol superior to that with ethanol. Kafirin was soluble in 60% tert-butanol at room temperature as well as at 60°C. They reported that when kafirin is extracted with 60% ethanol, the temperature must be increased to 60°C. Extraction of zein and kafirin from maize and sorghum grains according to the Landry and Moureaux method leads to two prolamin fractions (Taylor et al., 1984). Upon extraction with aqueous alcohol alone, uncross-linked zein is extracted from maize and uncross-linked kafirin from sorghum. When extracting with aqueous alcohol together with a reducing agent, the zein-2 and cross-linked kafirin is extracted (Taylor et al., 1984). These prolamins exist in the endosperm in the form of high molecular weight disulphide (SS)-linked oligomers (El Nour et al., 1998). Emmambux and Taylor (2009) found these oligomers, to be present in greater quantities in kafirin than in zein. Therefore, a reducing agent such as sodium metabisulphite is needed for their extraction.

For zein extraction on a commercial scale, a counter current extractor was developed in the 1940s for continuous zein extraction from maize gluten meal with aqueous propan-2-ol instead of aqueous ethanol (Lawton, 2002). Propan-2-ol has the advantage of not needing a government license for use (Taylor et al., 2005b). Over time, the extraction process has been modified. An added alkali treatment has been included which increases zein stability against gelation (Lawton, 2002). The current method most often used for zein extraction is the method patented by Carter and Reck in 1970 (Lawton, 2002). According to this process, zein is extracted with 60-90% aqueous propan-2-ol or ethanol, containing sodium hydroxide at temperatures between 55°C and 70°C. Though kafirin resembles zein in various properties (Shull et al., 1991), as indicated, it is less soluble in 70% ethanol than zein (Wall and Paulis, 1978). The method of Carter and Reck

(1970) has, however, successfully been used for kafirin extraction from sorghum flour (Da Silva, 2003).

Wallace et al. (1990) developed a novel method for the extraction of zein by extracting all the endosperm proteins in an alkaline buffer containing SDS and 2-ME with the subsequent precipitation of non-zein proteins by addition of ethanol to 70%. This method overcomes the drawbacks of sequential extraction and results in a better quantitative analysis. However, this has not been applied at a large scale.

Hamaker et al. (1995) further investigated this procedure by extracting kafirin and zein from whole grain sorghum and maize flour using a pH 10 buffer containing 1% SDS and 2% 2-ME. Ethanol or tert-butanol was added to the maize and sorghum extracts at 70 or 60%, respectively to precipitate non-prolamin proteins. The authors reported total zein and kafirin preparation purity of 50-56% and 68-73%, respectively.

Parris and Dickey (2001) extracted zein from corn gluten meal and dry milled maize by treating the materials with 0.55% lactic (w/w) acid and 0.2% (w/w) sulphur dioxide at 50ºC for 6 hours before ethanol extraction. This resulted in a 50% increase in zein isolate yield with high solubility (98%). The authors suggested that the pre-treatment cleaved disulphide linkages of the β- and γ-zeins and significantly reduced insoluble aggregates in zein isolates.

Another solvent used to extract kafirin and zein is glacial acetic acid. This is a novel method developed by Taylor et al. (2005b) to extract kafirin. Glacial acetic acid is desirable because of its low dielectric constant, non-inflammable property and acceptability to certain religions and because it is a food compatible solvent. The researchers hypothesized that the low dielectric constant of glacial acetic acid (6.1) enables it to dissolve highly hydrophobic proteins such as kafirin. In their study, aqueous alcohol extractants (70% ethanol at 70°C and 55% isopropanol at 40°C), each containing sodium metabisulphite and sodium hydroxide, were compared against extractants containing glacial acetic acid with and without sodium metabisulphite at 25°C. The authors found that aqueous ethanol at 70ºC and aqueous isopropanol-based metabisulphite containing solutions at 40ºC were effective extractants for kafirin with a recovery of 75-80%. Glacial acetic acid at ambient temperature, with added metabisulphite pre-soak was found to be effective with kafirin yields between 52.8-61.0% being obtained. However, the kafirin extracted with glacial acetic acid with metabisulphite pre-soak was much less pure (42.8%) than that extracted with aqueous ethanol (74.6%) or aqueous isopropanol-based solvents (73.1%). The

purity was increased to 68.0% after washing (desalting) the glacial acetic acid preparation. They concluded that glacial acetic acid in combination with a pre-soaking step using a reducing agent such as sodium metabisulphite can be effective for extracting kafirin and probably zein.

Wang et al. (2009) characterised the composition, chemical and physical properties of kafirin proteins in sorghum DDGS. They extracted kafirin using three different methods: Glacial acetic acid method (Taylor et al., 2005b), HCl-ethanol method (Xu et al., 2007) and NaOH-ethanol method (Emmambux and Taylor, 2003). Using glacial acetic acid resulted in highly purified protein preparation (99%) and the extraction yield was between 24-57% depending on the solvent used. The secondary structure of kafirin preparations by the three methods showed a predominance of an α-helical conformation with smaller proportions of β-sheet conformation.

Gao et al. (2005) conducted a study to find a kafirin extraction method that could be applied industrially for production of bioplastic films with desirable characteristics. A procedure using 70% (w/w) ethanol with 0.5% (w/w) sodium metabisulphite and 0.35% (w/w) sodium hydroxide at 70^{o}C resulted in protein preparations with properties most favourable in terms of yield, film forming properties and food compatibility. Protein aggregation was minimized by maximizing extraction of kafirin with native like α-helical structure and this resulted in films with more desirable properties.

Anderson and Lamsal (2011) developed a modified procedure for the extraction of α-zein from corn gluten meal and compared it against a commercial extraction method. The modification involved raising the concentration of alcohol (2-propanol) in the solvent from 55% (w/w) to 88% (w/w). Five organic solvent mixtures were compared using the modified extraction procedure developed along with the reductant sodium metabisulphite and sodium hydroxide. The three best solvents that were observed included 70% (w/w) 2-propanol, 55% (w/w) 2-propanol, and 70% (w/w) ethanol.

Lau et al. (2015) extracted kafirin from the distillers dried grains using 70% (w/v) ethanol, 0.35% (w/w) sodium hydroxide and 0.5% (w/w) sodium metabisulphite at 70^{o}C for 1 hour. These researchers washed the distillers dried grains three times with five to six volumes of hot water (50^{o}C) to remove most of the water soluble substances. This resulted in a kafirin preparation with a protein content of 82.3%.

Table 2.3: Summary of methods of kafirin extraction (adapted from De Mesa-Stonestreet et al., 2010)

Method	Reagent	Function/interactions broken	Proteins obtained
Sequential extraction procedure (Hamaker et al., 1995)	60% tert-butanol	Weakens hydrophobic interactions and act as a solvent	Prolamins (kafirin-1)
	60% tert-butanol + 0.5% 2-ME	2-mercaptoethanol is a reducing agent that breaks covalent disulphide bonds	Prolamins (kafirin-2/cross-linked kafirins)
Sonication with ethanol (Bean et al., 2006)	70% ethanol (+ sonication)[a]	Ethanol breaks hydrophobic interactions and solubilizes prolamins; sonication breaks cross-links that hold large protein aggregates	Kafirin monomers, cross-linked kafirins
	Dilution of ethanol to 60% with water	Precipitates lipids	Kafirin monomers and cross-linked kafirins (supernatant)
	Further dilution of ethanol (ranging from 50% to 30%)[b]	Precipitates prolamins	Kafirin monomers and cross-linked kafirins
Glacial acetic acid extraction of kafirins (Taylor et al., 2005b)	Pre-soak for 16 h in 0.5% sodium metabisulphite (SMS) at 25ºC	Breaks disulphide bonds	Kafirins
	Glacial acetic acid	Breaks hydrophobic interactions	
	NaOH for adjusting pH to 5	Kafirin precipitation	

Acidic-ethanol method (Wang et al., 2009)	70% ethanol, pH 2 + SMS	Weakens hydrophobic interactions, breaks disulphide bonds and act as a solvent	Kafirins
Alkaline-ethanol method (Emmambux and Taylor, 2003; Wang et al., 2009)	70% ethanol + 0.35% NaOH + 0.5% SMS	Weakens hydrophobic interactions, breaks disulphide bonds and act as a solvent	Kafirins

[a] In some experiments, a reducing agent (sodium metabisulphite or glutathione or cysteine) was added. Sodium metabisulphite and glutathione extracted the most protein. The addition of reducing agents to ethanol was more effective in extracting proteins than sonication
[b] Sodium Chloride may be added and pH may be lowered to 2.5

2.4.2 Safety in the use of solvents for kafirin and zein extraction

The nature of the solvents used to dissolve and extract zein and kafirin should be given considerable attention as many of them may pose health hazards (through the exposure routes such as inhalation, ingestion, skin and eye contact) as well as fire hazards. Some of these chemicals e.g. mercaptoethanol are also not food compatible. As the intended use of the kafirin and zein is for bioplastic film production often for use with food products, safety of the chemicals used is of considerable importance. Only aqueous ethanol and aqueous isopropanol have been used extensively for extraction of zein and kafirin. This is possibly due to their availability and easy recovery. Furthermore, ethanol and isopropanol are food compatible, and can thus be used for extracting kafirin and zein intended for food applications (Da Silva, 2003). There is a problem with aqueous ethanol, which has a flash point of approximately 12^{o}C (Merck, 2012) since at elevated temperatures it is highly inflammable and not acceptable to certain religions (Taylor, 2003). Propanol is also highly inflammable (Merck, 2012).

The other solvents used for kafirin and zein extraction include glacial acetic acid and aqueous tert-butanol. Acetic acid has advantages because of its volatility and no requirement for government licence nor religious concerns (Taylor et al., 2005b). Tert-butanol and glacial acetic acid are classified as not completely harmless (Merck, 2012) as they are flammable and may also pose health risks.

Mercaptoethanol and sodium metabisulphite reducing agents are additives often used in the extraction process (Table 2.3). However, mercaptoethanol is not food compatible (Merck, 2012)

and sodium metabisulphite is classified as a strong poison but is permitted as a food additive up to a given concentration as dictated in South Africa by the South African government legislation (Department of Health Act, 2014).

Commercially, sodium hydroxide is used to improve resistance to gelation of zein and to increase the yield of the zein extracted (reviewed by Shukla and Cheryan, 2001). However, it is also classified as a strong poison (Merck, 2012) but is permitted as a food additive up to given concentration as dictated in South Africa by the South African government legislation (Department of Health Act, 2014).

2.5 Kafirin and zein functionality

2.5.1 Protein films and coatings

Environmental challenges caused by the use of synthetic packaging materials have opened avenues for extensive studies on edible and biodegradable films and coatings from food grade biopolymers such as proteins, lipids, and carbohydrates (Coma, 2008). Films and coatings are applied to food surfaces in thin layers either by wrapping, immersing, brushing or spraying, with the intention of increasing the product quality and shelf-life (Gennadios and Weller, 1990). Films differ from coatings in that they are free-standing, thin sheets usually formed separately and applied on a product, whereas coatings are applied directly to the product surface where it is formed (Dhall, 2013). The film or coating acts as a barrier which may prevent moisture, oxygen, aroma and/or oil from migrating to and from the food (Krochta, 2002).

Proteins from agricultural sources, both plant and animal have been investigated for their film-forming abilities and their use in many food applications (reviewed by Gennadios et al., 1994; Bourtoom, 2008). Protein-based films and coatings have shown potential in enhancing the shelf life of fresh and minimally processed fruits and vegetables (Olivas and Gustavo-Cánovas, 2005; Eça et al., 2014), as carriers of antimicrobial agents (Baldwin et al., 1995) and decreasing oil absorption for deep fat fried foods (Rayner et al., 2000).

Prolamin proteins such as zein, gluten, hordein and kafirin can be used to fabricate coatings and films intended for use as materials in food related and non-food applications (Taylor et al., 2013). Zein in particular has been commercially promoted as an edible film or coating (Gennadios and Weller, 1990). Gluten allergies are the main reason responsible for the lack of commercialisation of gluten films (reviewed by Gennadios et al., 1994). Research carried out on kafirin has shown that it can produce bioplastic films of good tensile and water vapour barrier

properties (Buffo et al., 1997; Byaruhanga et al., 2005). This is probably because it is more hydrophobic and has more disulphide cross-links than zein, enabling it to make stronger films with better water barrier properties (Taylor et al., 2005a; Belton et al., 2006; Byaruhanga et al., 2007).

The method of kafirin and zein extraction affects the composition of the prolamins, which in turn influences the functional properties of the bioplastics made from them (Schober et al., 2011). Gao et al. (2005) studied the effects of kafirin extraction method on film preparation and related the protein secondary structure to the film forming properties. It was observed that methods which maintained the greatest proportion of α-helical structures gave the best films in terms of sensory properties (clarity, colour, flexibility and surface texture), higher tensile strength, higher strain and low water vapour permeability. The authors suggested that the packing of the kafirin molecules in the film might have played a vital role since compact α-helical structures may pack more uniformly than aggregated β-sheet structures. Taylor et al. (2005a) reported that kafirin films with all the kafirin classes present were stronger but less extensible than zein films consisting mainly of α-zein.

2.5.1.1 Principles of protein film formation

Protein film or coating formation has been described as a three-step process which involves denaturation, aggregation and gelation of the protein to form a three dimensional protein matrix (reviewed by Cuq et al., 1998). It is postulated that in order to make a film, at least one component with a high molecular weight is necessary to yield a film matrix with cohesive strength (Banker, 1966). Cohesion is the ability of a material to form strong bonds on a molecular level within itself, which will prevent separation at the point of contact. Proteins are ideal for this purpose, being natural polymers, based on amino acids that are linked together by peptide bonds. Proteins are able to form three-dimensional structures which are mainly stabilised through non-covalent interactions (Cuq et al., 1998), for example Van der Waals forces, hydrogen bonding, electrostatic, hydrophobic or disulphide cross-link interactions between the amino acid units (Krochta, 2002).

In general, to form films proteins are solubilised/dispersed in a suitable solvent, for example aqueous alcohol, with or without plasticizers by mixing at elevated temperatures. Increased temperature and shear results in partial or complete protein denaturation (Da Silva, 2003). According to Wang and Damodaran (1991), partial protein denaturation, usually by heat, shear and/or extreme pH, weakens the tertiary and quaternary (three-dimensional) protein structure,

exposing previously hidden functional groups such as carbonyl (C=O), amide (N-H) and disulphide (S-S) bonds, as well as side chain amine and hydrophobic groups. These functional groups become available for intermolecular interactions and result in the formation of a three-dimensional protein network, entrapping film components during protein aggregation and drying (Gontard et al., 1993).

Yamada et al. (1995), hypothesised that there can be a number of zein protein arrangements in a film (Figure 2.4) depending on the solvent. They suggested an arrangement of zein proteins in a film where the zein molecules will gather forming an aggregated pile. The aggregated pile will expose its hydrophobic or hydrophilic region towards the outer surface depending on the solvent used. They suggested that these aggregates will gather together to form the film during drying. The ellipse in Figure 2.4 corresponds to the zein molecule and the shadowing on the ellipse shows the hydrophobic or hydrophilic region of zein. As stated, the solvent used for the formation of the zein film will affect the arrangement depending on its hydrophobic or hydrophilic nature. In an acetone and water solution, for example, the zein molecules will be aligned to form tubular piles where the hydrophilic solvent (water) will be entrapped, stabilising the hydrophilic region. The outer surface of the pile will be stabilised through hydrophobic interactions with the other more hydrophobic solvent (for example, acetone). However, alcohol with water as the solvent, will have weaker hydrophobicity than acetone with water. According to the authors when this solvent is used, the opposite arrangement will be formed. Zein molecules will be aligned to form the pile structure by entrapping the small amount of alcohol from the solvent in order to stabilize its hydrophobic region and exposing its hydrophilic region. Shi et al. (2009) suggested that zein films prepared from aqueous ethanol solutions are hydrophobic due to the higher amount of polar functional groups at the film surface.

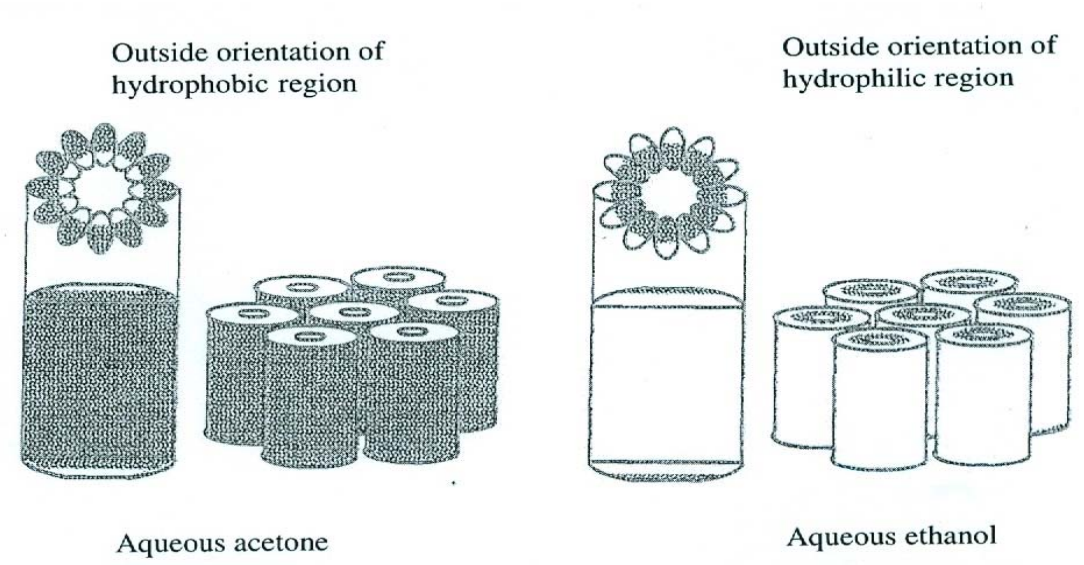

Figure 2.4: Possible arrangement of zein molecules in a film (Yamada et al., 1995)

Wang and Padua (2012) postulated a mechanism for zein self-assembly from single molecules to nanospheres. The α-helical structure of α-zein initially unfolds and transforms into β-sheets (Figure 2.5A and 2.5B) which pack in opposite directions side by side (Figure 2.5C and 2.5D) into a long ribbon stabilised by hydrophobic interactions (Figure 2.5E). The authors extended the model to include the formation of nanotubes by end to end linking of β-sheets via glutamine bridges and then coiling into three-dimensional columns (Figure 2.5F). The cysteine rich β- and γ-prolamins have the ability to form covalent bonds of disulphide linkage between or within the polypeptides which serve to stabilise bioplastics once formed through disulphide bonding (Taylor et al., 2013).

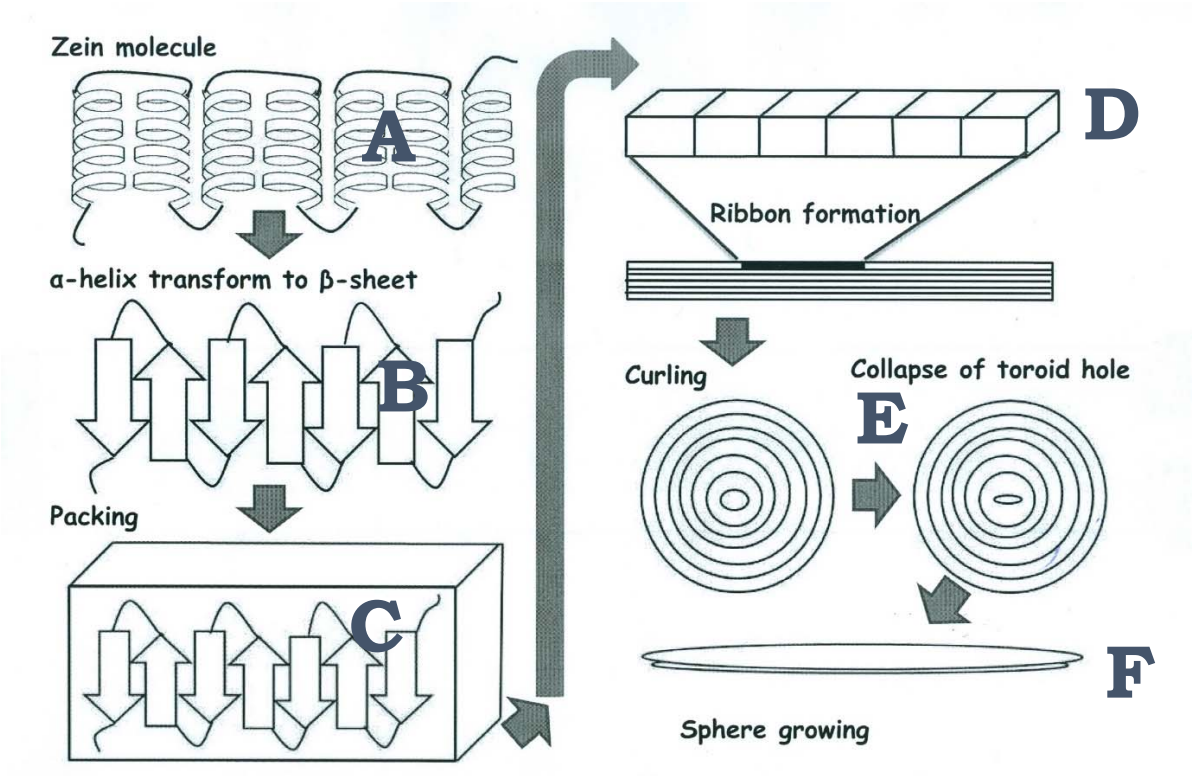

Figure 2.5: Possible mechanism for zein self-assembly from single molecules to nanospheres, according to Wang and Padua (2012) (redrawn by Taylor et al., 2013).

2.5.1.2 Kafirin and zein film making

Proteins can be made into free-standing films by the use of two processes, wet or dry, as illustrated in Figure 2.6. The wet process is also known as the casting method. This process is based on the solubilisation of the proteins or dispersion of proteins in solvents, commonly water or aqueous alcohol (Krochta, 2002). Temperature is elevated in order to dissolve the protein, e.g. kafirin dissolves in 70% ethanol only at 60°C (Hoseney, 1994). The film is formed when the prepared formulation is poured or applied to the casting surface and the solvent is allowed to evaporate (Krochta, 2002). Proteins are separated from the solvent through precipitation or phase changes (Cuq et al., 1998). When the solvent is removed, the film will form due to an increase in the polymer concentration which induces bond formation leading to the formation of a three-dimensional structure.

The dry process for kafirin and zein film making involves thermal processes under low-moisture conditions. The protein is mixed with high shear whilst the temperature is raised above glass transition temperature of the protein. The protein is transformed from a glassy state into a

rubbery state with a general increase in disorder, free volume and mobility of the protein molecules (Cuq et al., 1998). The melted protein-based material is then shaped by extrusion, roller milling or thermo moulding (Cuq et al., 1998). This process was used by Lai, Padua and Wei (1997) to prepare zein resin films by melting zein in a microwave, followed by kneading and rolling to form zein sheets. Oliviero et al. (2010) prepared zein films from film blowing technology using thermoplasticised zein without the solubilisation step. The thermoplasticised zein material prepared by mixing zein and polyethylene glycol 400 in a twin counter rotating internal mixer at 70°C, 50 rpm and 10 minutes, was then compressed to obtain a 1 mm slab. The film was then formed by extruding the thermoplasticised zein material through a twin screw extruder connected with blowing head.

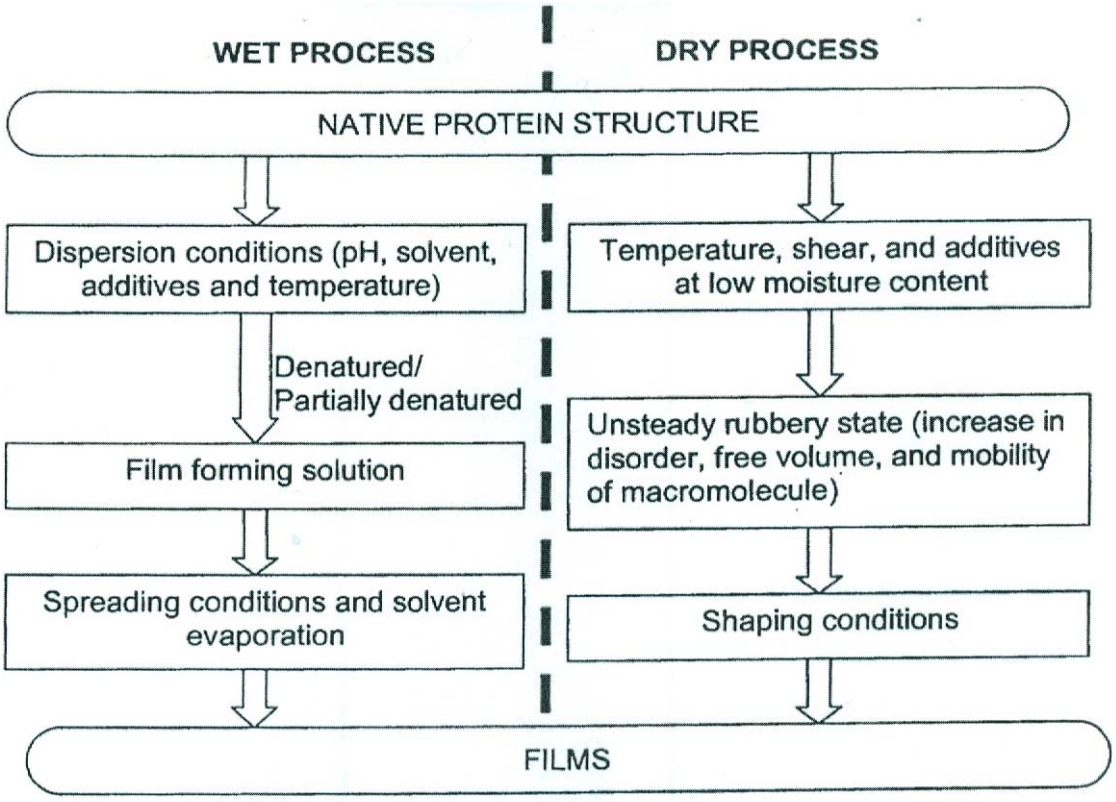

Figure 2.6: Schematic representation of protein film formation by the wet and dry processes (from Emmambux, 2004).

Although prolamin proteins form films, the diverse food packaging requirements necessitate modification of the functional properties of these films (Krochta, 2002). The most promising way to improve cohesion, rigidity, mechanical strength and water barrier properties of the films is through cross-linking (Tulamandi and Rizvi, 2011; Wittaya, 2012). This is possible because of the presence of different functional groups in proteins, which have the ability to interact with a

wide range of active compounds (Wittaya, 2012). Modification methods that have been used to improve functional properties of prolamin films are categorised as; chemical, physical, enzymatic and plasticisation (Taylor et al., 2013). Heat as an example of physical modification is described below.

2.5.1.3 Thermal modification of film properties

Physical treatments have been used to modify functional properties of cast protein films (Ali et al., 1997; Byaruhanga et al., 2007). Microwave heating results in increased tensile strength, tensile strength at break, decreased strain and decreased water vapour permeability of kafirin films (Byaruhanga et al., 2005; Byaruhanga et al., 2007). Such modifications in film properties have been attributed to intermolecular cross-linking within the protein film network through the formation of disulphide and hydrophobic bonds (Pelosi, 1997; Taylor et al., 2013). Pelosi (1997) developed a patent for cross-linking zein. The method involves heating zein in the presence of water to a temperature of at least 130^{o}C which results in the cross-linking of the protein.

2.6 Conclusions

This literature review provides evidence that progress has been made in kafirin and zein extraction and their utilisation as bioplastic films and coatings. Of particular interest is the fact that kafirin has the ability to form bioplastic films and coatings with better water barrier properties than zein. This is due to its more hydrophobic nature as a result of intermolecular cross-linking within the protein film network through the formation of disulphide and hydrophobic bonds. However, exploring the best raw materials and the best extraction methods for kafirin and zein is necessary in order to reduce production costs whilst maintaining the protein functional properties.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Hypothesis 1

Extraction of kafirin and zein from coarse grain material will have a more rapid extract filtration time than fine flour.

Larger inter-particle spaces of coarse grain material makes it easier for the solvent to filter through faster than in fine flour (Palmer, 1999; Jaques et al., 2003).

Hypothesis 2

Inclusion of glacial acetic acid and sodium hydroxide in aqueous ethanol extractant will improve kafirin and zein protein extraction yield.

Inclusion of sodium hydroxide (Gao et al., 2005) and glacial acetic acid (Wang et al., 2009) in aqueous ethanol extractant has been shown to result in a significant increase in kafirin yield. Similarly, inclusion of sodium hydroxide (Anderson and Lamsal, 2011) and glacial acetic acid (Selling and Woods, 2008) in aqueous ethanol extractant has also shown to result in a significant increase in zein yield. These chemicals deamidate kafirin and zein asparagine and glutamine amino acid residues to form aspartic and glutamic acids (Avanti, 2012). This results in protein conformational changes leading to the unfolding of the protein, exposure of hydrophobic regions of the protein to solvation, disruption of hydrophobic interactions, breaking of hydrogen bonds and increased amount of electrostatic repulsions (Cooper, 1999) which makes kafirin (Gao et al., 2005) and zein (Cabra et al., 2007) more soluble.

Hypothesis 3

Heat treatment of kafirin and zein films will reduce film water uptake.

Heating of kafirin powder and kafirin films (Byaruhanga et al., 2006; Byaruhanga et al., 2007) and zein films (Guo et al., 2012) has been shown to greatly increase tensile strength and substantially reduce tensile strain and water vapour permeability of cast films. This is due to heat induced intermolecular disulphide cross-linking of the protein (Pelosi, 1997; Taylor et al., 2006). Also, subjecting kafirin (Gao et al., 2005) and zein (Cabra et al., 2006) to elevated temperature increases the proportion of intermolecular β-sheet structure (Gao et al., 2005).

3.2 Objectives

Objective 1

To determine the effect of sorghum flour/meal particle size during kafirin extraction on extract filtration time, protein yield and protein preparation purity with the aim of minimising extraction time and improving kafirin preparation purity.

Objective 2

To determine the effects of inclusion of glacial acetic acid and sodium hydroxide in aqueous ethanol as extractants on kafirin and zein yield and protein preparation purity with the aim of improving kafirin and zein extraction efficiency.

Objective 3

To determine the effect of heating kafirin and zein films on water barrier properties with the aim of improving their functional properties as bioplastics.

4 RESEARCH

The following research is written in the style of the Journal Cereal Chemistry. Figure 4.1 is a flow diagram of the experimental design.

4.1 EXPERIMENTAL DESIGN

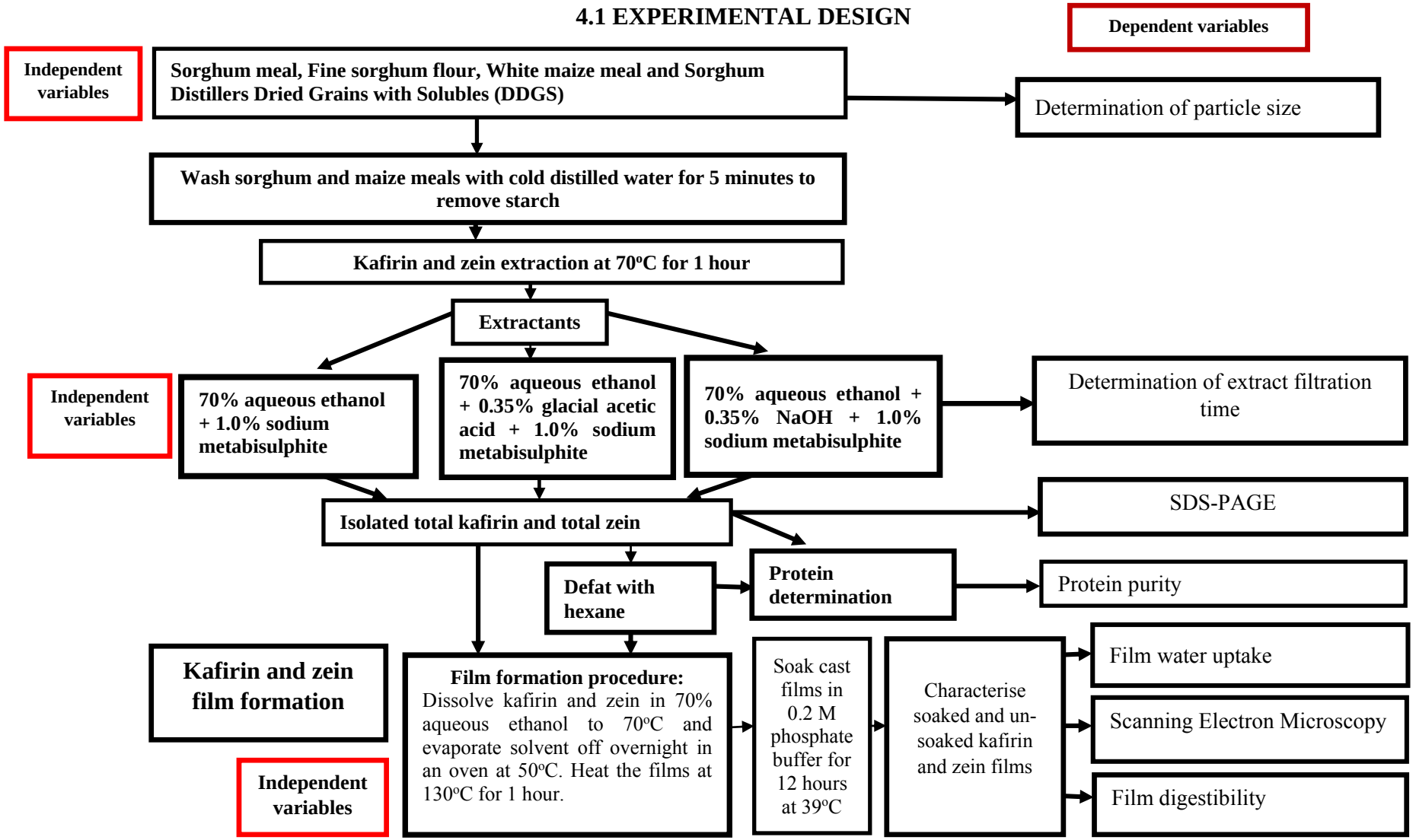

Figure 4.1: Experimental design to determine the effects of sorghum flour/meal particle size, inclusion of sodium hydroxide or glacial acetic acid in aqueous ethanol extractant on extract filtration time, kafirin and zein preparation purity and yield. Also the effect of heat on preformed kafirin and zein film functionality.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The following prolamin protein preparations were used in this investigation: total kafirin, total zein, α-zein and Zein Gold. Commercial zein (α-zein) from yellow maize was obtained from Sigma-Aldrich, Johannesburg, South Africa (Sigma product code Z3625) and Zein Gold was obtained from Zein Protein Products, Marina, CA, USA. Total kafirin was extracted from washed and non-washed coarse decorticated sorghum meal and fine sorghum flour (King Korn Mabele, Tiger Brands, Bryanston, South Africa). Fine sorghum flour was obtained by milling the decorticated sorghum meal using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) fitted with a screen having a mesh opening of 250 µm. Total zein was extracted from washed and non-washed coarse, decorticated, refined, white maize meal (SPAR Super grade maize meal, Pride Milling, Vorsterskroon, South Africa). Kafirin was also extracted from washed and non-washed sorghum distillers dried grains with solubles (DDGS) obtained from a grain bioethanol plant in Texas, USA.

4.2.2 Methods

4.2.2.1 Laboratory safety measures

During the research work, priority was given to safety to ensure a safe working environment. This was achieved by: planning all the investigations in advance and carrying them out after approval by the supervisor. A knowledge of laboratory emergency actions and the location of safety equipment, wearing protective attire (long pants, acid proof laboratory coat, safety shoes, latex gloves, solvent proof face mask, long heat resistant gauntlets) and proper labelling and storage of chemical containers and sample boxes was essential. Care was taken when heating aqueous ethanol, which has a flash point of approximately 12ºC (Merck, 2012) and is highly flammable. Closed glassware and a temperature not exceeding 70ºC was used. Other precautions of not working in the laboratory alone, not eating, drinking or smoking in the laboratory, switching off all electrical appliances after use, proper disposal of wastes and keeping the laboratory clean and tidy after experiments were adhered to. During kafirin and zein extraction, sodium metabisulphite (1.0%, w/w) was used as part of aqueous ethanol extractant, despite its classification as a strong poison associated with health hazards such as irritations of respiratory tract, gastrointestinal tract, eyes and the skin as well as allergic reactions in susceptible individuals (Merck, 2012).

4.2.2.2 Kafirin and zein extraction

Kafirin and zein preparations were extracted using the methods as described by Emmambux and Taylor (2003) and Taylor et al. (2005b). Extraction was carried out with 70% (w/w) aqueous ethanol containing 1.0% (w/w) sodium metabisulphite and 0.35% (w/w) sodium hydroxide or glacial acetic acid at 70°C for 1 hour by modified percolation apparatus as shown in Figure 4.2. The percolation vessel was made of glass and jacketed to enable circulation of water at 70ºC so as to heat the extractant and keep it at 70ºC during the extraction period. Within it was a 250 µm opening mesh screen. The mesh allowed only the liquid extract to be collected without contamination with the grain material. The extractant was percolated twice through the meals and flours. Samples (100 g as is basis) were extracted with the given extractants (250 g) with agitation every 5 minutes. Agitation was carried out by shaking the stainless steel mesh screen using the handle attached to it. The filtration time of the extractant through the sorghum flour, coarse sorghum meal, coarse maize meal and sorghum DDGS meal was measured. The alcohol solvent was allowed to evaporate from the solute overnight at ambient temperature (25ºC) from shallow open trays placed in a fume hood. Later on, cold distilled water (200 ml) (8-10ºC) was used to precipitate kafirin and zein protein preparations.

When sodium hydroxide was used as part of the extractant, the protein suspension was acidified to approximately pH 5.0 with 1 M HCl to precipitate out the protein. The precipitated wet prolamin protein concentrate was then recovered by vacuum filtration using 2 layers of Whatman No. 4 filter paper in a Buchner funnel. The protein preparations were then air dried in a fume hood overnight at ambient temperature (25ºC).

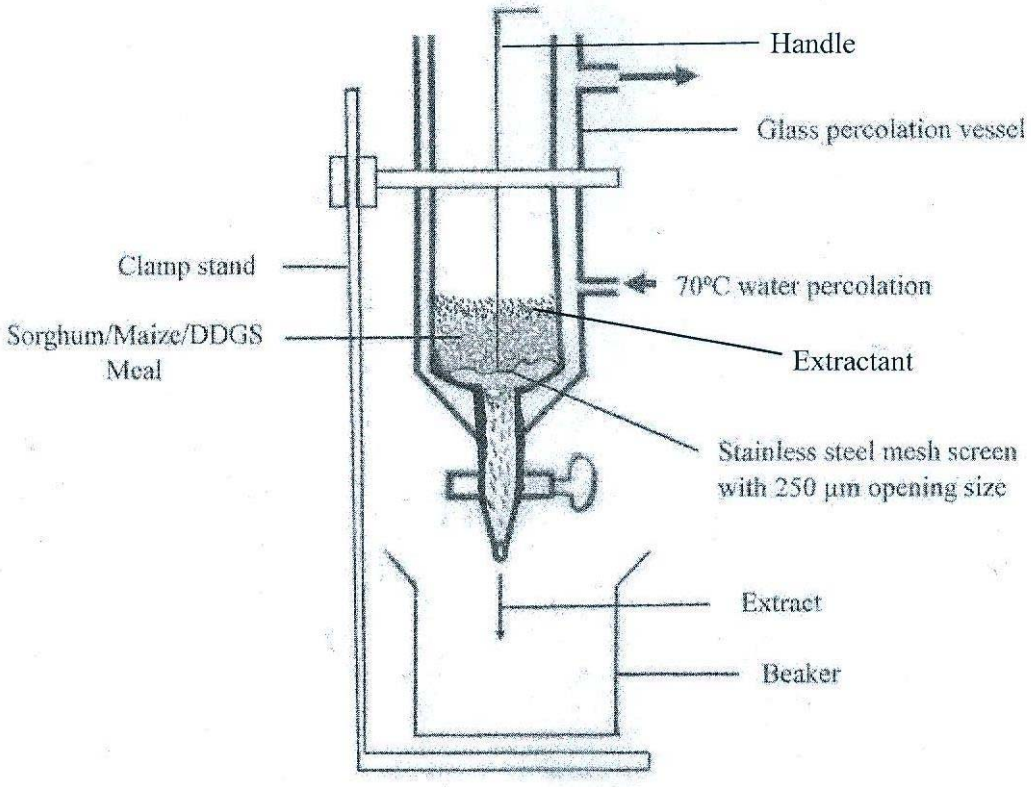

Figure 4.2: Diagrammatic representation of percolation apparatus used to extract kafirin and zein

Total kafirin, DDGS kafirin and total zein preparations were defatted three times at one-hour intervals with n-hexane, at a ratio of one part protein to ten parts n-hexane (w/w), at ambient temperature (25°C), and then dried overnight in the fume hood at ambient temperature (25°C).

4.2.2.3 Analyses

4.2.2.3.1 Moisture

The moisture contents of the raw materials and protein preparations were determined by Approved Method 44-15A Air oven method (AACC, 2000).

4.2.2.3.2 Protein

Protein contents (N x 6.25) of the raw materials and protein preparations were determined by a Dumas total combustion method, Approved Method 46-30 (AACC, 2000). Percentage protein yield for the extractions was calculated as weight of total recovered protein divided by the total amount of protein present in the starting material (dry basis), multiplied by 100.

4.2.2.3.3 Fat

The fat contents of the raw materials and protein preparations were determined using the Soxhlet method, AOAC Approved Method 920-39 (AOAC International, 2000).

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

The kafirin and zein protein preparations were characterised by SDS-PAGE under both reducing and non-reducing conditions, essentially as described by Taylor (2003). A precast 4-12% Bis-Tris polyacrylamide gradient gel (NuPAGE® Novex Gels, Invitrogen, Life Technologies, Carlsbad, CA) 8 cm x 8 cm and 1.0 mm thick with 15 wells was used with a XCell Sure Lock™ Mini-Cell vertical electrophoresis unit (Invitrogen, Life Technologies). Invitrogen Mark 12 unstained molecular weight standards were used as reference. Electrophoresis was carried out at a constant voltage of 200 V, 80 mA and 10 Watts for 1 hour. Approximately 10 µg protein was loaded per sample well. The gel was stained with Coomassie Brilliant Blue, R-250 overnight. After de-staining with 3% (w/w) acetic acid, the gel was scanned on a flatbed scanner.

4.2.2.3.5 Kafirin and zein film preparation

Films were cast based on the procedure described by Taylor et al. (2005a). Films were cast from defatted and un-defatted total kafirin, DDGS kafirin and total zein, α-zein and Zein Gold preparations by dissolving the protein powders in aqueous ethanol without a plasticiser. The kafirin and zein powders [equivalent of 1.2 g pure (100%) protein basis] were weighed into 100 ml Erlenmeyer flasks and then aqueous ethanol, 8.8 g (70% w/w) was added, (in certain cases the amount of aqueous ethanol was increased to 9.8 g, as the protein preparations became viscous and difficult to be spread out on the silicone baking trays). The total weight of the Erlenmeyer flasks and their contents was recorded. The flasks were closed with aluminium foil before being heated to 70°C. The protein solutions were held at 70°C for 10 minutes with continuous, rapid stirring to facilitate dispersion and solubilisation of the protein into the ethanol. After 10 minutes had elapsed, absolute ethanol was added to the flasks until the original weight was obtained. The protein solutions were poured into rectangular silicone baking trays (28 mm x 69 mm) or circular silicone baking trays (55 mm in diameter). The baking trays were swirled around very gently to distribute the contents

evenly on the bottom and were later placed on a level surface (confirmed with a spirit level) and dried in an oven (not forced draft) at 50°C overnight.

A second set of films was prepared from total kafirin, DDGS kafirin and total zein as described and then subjected to heating at 130°C in an oven (not forced draft) for 1 hour. Films were then weighed, placed into petri dishes and scanned with a flatbed scanner. Five different thickness measurements were taken for each film using a micrometer and the mean film thickness calculated. The dimensions were measured using a ruler. The weight of each film was divided by its volume to calculate the density (mg/cm^3).

4.2.2.3.6 Kafirin and zein film water uptake

Films (28 x 69 mm or 55 mm in diameter) immersed in 0.2 M sodium phosphate buffer (pH 6.8) were incubated either in an oven at 39°C or with gentle agitation (30 rpm) in a water bath at 39ºC for 12 hours. After 12 hours, the films were removed, cooled for 10 minutes and scanned using a flatbed scanner while they were still in sodium phosphate buffer to record how film integrity was maintained during incubation. Films were removed from the buffer and the surface water carefully blotted with a paper towel before weighing. Percentage water uptake of the films was calculated as follows:

$$\% \text{ water uptake} = \frac{\text{Mass of films after immersion in buffer (mg)} - \text{initial dry mass (mg)}}{\text{Initial dry mass (mg)}} \times 100$$

4.2.2.3.7 Scanning Electron Microscopy (SEM)

The kafirin and zein films were examined by mounting the dried kafirin and zein film preparations on a stub with double-sided tape and sputter coated with gold. SEM preparations were viewed with a Joel JSM-5800 LV scanning electron microscope (Tokyo, Japan).

4.2.2.3.8 Simulated three phase digestion of the kafirin and zein films

The simulated three phase *in vitro* digestibility of the kafirin and zein films was carried out, as described by Gargallo et al. (2006) with modifications as described below.

Approximately 250 mg of each film sample (bigger films cut into smaller pieces) was accurately weighed and placed into centrifuge tubes. McDougalls artificial rumen buffer (pH

6.8), pepsin (1.0 g/L, Sigma P-7000) was added to 2 L 0.1 M HCl to obtain a pH of 1.9), and 0.5 M, KH_2PO_4, phosphate buffer, pH 7.75, containing 3 g/L pancreatin (Sigma P-7545) were used to digest the samples. The chemicals were preheated to 39°C before being added to the centrifuge tubes containing the samples. During each digestion phase, 35 ml of the buffer, appropriate for that phase was added to each centrifuge tube containing the samples. After which, the centrifuge tubes were incubated at 39ºC with constant rotation in a shaking water bath. The incubation periods for pre-digestion soak, pepsin and pancreatin digestion were 8 hours, 1 hour and 12 hours, respectively. After each digestion period, the centrifuge tubes were centrifuged and the liquid carefully removed with a pipette. Distilled water (10 ml) was added to each centrifuge tube to wash the films. Tubes were centrifuged and water removed. The washing step was repeated twice more. Samples were dried in a forced draft oven at 105ºC for 24 hours. The dried samples were then reweighed to determine the weight of residual material after digestion and the material was carefully scraped out of the centrifuge tubes, photographed and stored. Samples from the previous digestion phase were used for the subsequent digestion phase.

Calculation:

Percentage weight loss after pre-digestion soak = $100 - (W_3-W_1) \times 100/ (W_2 \times$ Film dry matter)

Percentage weight loss after pepsin digestion = $100 - (W_4-W_1) \times 100/ (W_2 \times$ Film dry matter) – (pre-digestion soak)

Percentage weight loss after pancreatin digestion = $100 - (W_5-W_1) \times 100) / (W_2 \times$ Film dry matter) – (pre-digestion soak + pepsin digestion)

Percentage *in vitro* total digestibility (IVTD) = pre-digestion soak + pepsin digestion + pancreatin digestion.

Where:

W_1= Weight of centrifuge tubes (mg); W_2= Initial weight of the films (mg); W_3= Weight of centrifuge tubes and films after pre-digestion soak (mg); W_4= Weight of centrifuge tubes and films after pepsin digestion (mg); W_5= Weight of centrifuge tubes and films after pancreatin digestion (mg).

4.2.2.3.9 Statistical analysis

All the experiments were carried out at least twice. Both one-way and two-way analyses of variance (ANOVA) were performed using SPSS statistics software, version 23 (IBM Corp. NY, USA). Least significant difference test (LSD) at 95% level of confidence was used to determine significant differences.

4.3 RESULTS AND DISCUSSION

4.3.1 Effects of sorghum flour/meal particle size and solvent on prolamin yield

Table 4.1 shows that a high proportion of coarse maize and sorghum meals (76-89%) were retained on the 1000 µm sieve. In contrast, only 25% of the sorghum DDGS meal was retained on the sieve, thus sorghum DDGS meal was much finer than coarse sorghum and maize meals.

Table 4.1: Effect of sieving coarse sorghum meal, coarse maize meal and sorghum DDGS meal using a 1000 µm aperture sieve on the proportion of the meals retained on the sieve

Treatments	Initial weight (g)	>1000 µm (%)	<1000 µm (%)
Coarse sorghum meal	2000	$88.6^{c}\pm1.4$[1]	$11.4^{a}\pm0.9$
Coarse (Super grade) maize meal	2000	$76.1^{b}\pm0.8$	$23.9^{b}\pm1.1$
Sorghum Distillers Dried Grains with Solubles (DDGS)	841	$25.1^{a}\pm0.4$	$74.9^{c}\pm1.5$

[1]Means±standard deviations, n=2

Mean values on the same column but with different superscripts are significantly different ($p<0.05$)

Table 4.2: Particle size distribution, protein and moisture contents of screened sorghum and maize meals, sorghum DDGS meal and sorghum flour (g/100 g)

	Particle size					Protein content (N x 6.25) (% db)	Moisture content (%)
	>2000 µm	<2000 µm - >1000 µm	<1000 µm - >500 µm	<500 µm - >250 µm	<250 µm		
Screened sorghum meal	$0.3^{b}\pm0.2$[1]	$88.6^{d}\pm0.2$	$9.6^{b}\pm0.3$	$0.9^{a}\pm0.2$	$0.5^{a}\pm0.0$	$10.0^{b}\pm0.2$	$10.0^{a}\pm0.0$
Screened DDGS meal	$0.4^{c}\pm0.2$	$25.1^{b}\pm0.4$	$64.9^{c}\pm0.2$	$5.9^{c}\pm0.3$	$2.6^{b}\pm0.3$	$28.2^{c}\pm0.0$	$10.3^{a}\pm0.9$
Screened maize meal	$0.3^{b}\pm0.3$	$86.2^{c}\pm0.4$	$10.4^{b}\pm0.2$	$0.9^{a}\pm0.3$	$0.4^{a}\pm0.3$	$7.5^{a}\pm0.2$	$10.6^{a}\pm0.4$
Sorghum flour	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$3.9^{b}\pm0.2$	$95.8^{c}\pm0.4$	$10.0^{b}\pm0.2$	$10.2^{a}\pm0.2$

db=dry basis
[1]Means ±standard deviation, n=2
Mean values on the same column but with different superscripts are significantly different ($p<0.05$)

Table 4.2 shows that 86-89% of screened maize and sorghum meals had a particle size greater than 1000 µm. In contrast, only 65% of the sorghum DDGS meal had a particle size of >500 µm, while 96% of the fine sorghum flour had a particle size of <250 µm.

Sorghum, maize and sorghum DDGS meals had protein contents of 10.0, 7.5 and 28.2%, respectively. The protein contents were in agreement with those described in the literature. For example, 9-14% for sorghum (Rooney and Serna-Saldivar, 2003), 8-11% for maize (Mesfin and Shimelis, 2013) and 27-30% for sorghum DDGS meal (Tokach et al., 2010). There was no significant difference in the moisture contents of the samples ($p \geq 0.05$), as all of them had moisture content in the range of 10.0 to 10.6%. Moisture contents of less than 14% are recommended for long term storage of cereals to prevent development of moulds (Ullrich, 2010).

Table 4.3: Effects of extractants, sorghum flour/meal particle size and washing of the coarse sorghum meal on extract filtration time, kafirin preparation purity and protein yield

Extractants	Filtration time (sec)	Kafirin preparation extracted (g/100 g sorghum) (db)	Kafirin preparation purity (g protein/100 g preparation (db)	Protein yield[2] (g protein/100 g protein in raw material)	Protein yield/100 g raw material (db)
Coarse sorghum meal					
70% aqueous ethanol + 1.0% KMS + 0.35% acetic acid	$270^{e}\pm2$[1]	$4.7^{d}\pm0.4$	$57.2^{c}\pm0.8$	$26.8^{ab}\pm0.8$	$29.8^{ab}\pm0.2$
70% aqueous ethanol + 1.0% KMS+ 0.35% NaOH	$258^{d}\pm2$	$5.3^{e}\pm0.0$	$58.9^{c}\pm0.2$	$31.1^{ef}\pm0.2$	$34.6^{d}\pm0.6$
70% aqueous ethanol + 1.0% KMS + 0.35% NaOH neutralized	$256^{cd}\pm2$	$4.6^{d}\pm0.3$	$58.7^{c}\pm0.6$	$27.1^{ab}\pm1.2$	$30.1^{ab}\pm0.3$
Mean	**261**	**4.9**	**58.3**	**28.3**	**31.5**
Washed coarse sorghum meal					
70% aqueous ethanol + 1.0% KMS+ 0.35% acetic acid	$240^{a}\pm1$	$3.6^{b}\pm0.2$	$73.7^{e}\pm0.7$	$26.7^{ab}\pm0.3$	$29.6^{ab}\pm0.6$
70% aqueous ethanol + 1.0% KMS+ 0.35% NaOH	$252^{bc}\pm1$	$4.2^{c}\pm0.2$	$70.7^{d}\pm0.9$	$29.3^{de}\pm0.5$	$32.6^{c}\pm0.4$
70% aqueous ethanol + 1.0% KMS+ 0.35% NaOH neutralized	$249^{b}\pm1$	$3.2^{a}\pm0.1$	$71.5^{d}\pm0.7$	$23.0^{a}\pm0.7$	$25.6^{a}\pm0.6$
Mean	**247**	**3.7**	**72.0**	**26.3**	**29.3**
Sorghum flour					
70% aqueous ethanol + 1.0% KMS+ 0.35% acetic acid	$570^{f}\pm2$	$5.8^{f}\pm0.1$	$51.3^{b}\pm1.1$	$29.6^{de}\pm0.4$	$32.9^{c}\pm0.4$
70% aqueous ethanol + 1.0% KMS+ 0.35% NaOH	$570^{f}\pm1$	$5.9^{f}\pm0.2$	$46.9^{a}\pm0.3$	$27.9^{bc}\pm0.3$	$31.0^{b}\pm0.1$
70% aqueous ethanol + 1.0% KMS+ 0.35% NaOH neutralized	$572^{f}\pm2$	$6.3^{f}\pm0.1$	$50.9^{b}\pm0.7$	$32.1^{ef}\pm0.7$	$35.7^{d}\pm1.1$
Mean	**571**	**6.0**	**49.7**	**29.9**	**33.2**

[1]Means ± standard deviations, n=4, KMS = sodium metabisulphite, db = dry basis
Mean values on the same column but with different superscripts are significantly different ($p<0.05$)
[2]The mean protein content of the sorghum meal and sorghum flour was 10.0% (db) (N x 6.25)

Table 4.4: ANOVA for extract filtration time, kafirin preparation extracted, kafirin preparation purity and protein yield

Extract filtration time

Source of variation	Mean square	DF	F	P
Washing	5281.45	1	4.19	0.049
Particle size	750908.66	1	595.74	0.000
Washing*particle size	0.00	0	-	-
Residual	1261.52	32	-	-

Kafirin preparation extracted

Source of variation	Mean square	DF	F	P
Washing	9.34	1	73.44	0.000
Particle size	6.41	1	50.39	0.000
Washing*particle size	0.00	0	-	-
Residual	0.127	33	-	-

Kafirin preparation purity

Source of variation	Mean square	DF	F	P
Washing	1151.35	1	440.56	0.000
Particle size	422.86	1	161.80	0.000
Washing*particle size	0.00	0	-	-
Residual	2.61	33	-	-

Protein yield

Source of variation	Mean square	DF	F	P
Washing	0.43	1	0.13	0.726
Particle size	18.39	1	5.40	0.027
Washing*particle size	0.00	0	-	-
Residual	3.41	33	-	-

The coarse sorghum meal either washed or non-washed had a more rapid extract filtration time (mean 254 seconds) than fine sorghum flour (mean 571 seconds) (Table 4.3). The difference in extract filtration time can be attributed to larger inter-particle spaces between coarse sorghum meal than fine sorghum flour. Larger inter-particle spaces of coarse grains have been linked with rapid filtration of the solvent (Palmer, 1999; Jaques et al., 2003).

The washed sorghum meal produced a kafirin preparation with a higher purity (71-74%) compared to the purity of kafirin preparation from the non-washed sorghum meal (57-59%) and fine sorghum flour (47-51%), depending on the extractant used. Washing of the decorticated sorghum meal to remove starch prior to kafirin extraction was responsible for the increase in kafirin preparation purity. White milky/cloudy wash water was observed in the washed sorghum supernatant and iodine test indicated the presence of starch. The yield of kafirin ranged from 23% to 32% (g protein/100 g protein in raw material, as is basis), but there appeared to be little difference in kafirin preparation purity and yield regardless of the extractant used.

El Nour et al. (1998) found that kafirin extraction from sorghum whole grain flour using a sequential extraction procedure with aqueous alcohol and with or without a reducing agent resulted in a total kafirin preparation purity of approximately 51%. Wu (1978) extracted kafirin from whole ground sorghum by using an alkaline extraction process and obtained a kafirin preparation purity from 48% to 60%. The kafirin preparation purity obtained from fine sorghum flour in this current work is in agreement with the kafirin preparation purity results by Wu (1978) and El Nour et al. (1998) despite differences in extraction procedures and sorghum varieties used.

The results in Table 4.4 show that particle size and washing of the sorghum meal affected extract filtration time, kafirin preparation extracted and kafirin preparation purity. However, only particle size of the material used had an effect on protein yield. The mean squares and F ratios for washing*particle size interaction could not be obtained as the experiment was not a complete design since the comparison was made between the meals and flours whereby washing was only done for the meals and not for the flours.

Table 4.5: Effects of inclusion of glacial acetic acid and NaOH in aqueous ethanol as extractants on extract filtration time, kafirin and zein preparation purity and protein yield from non-washed and washed sorghum and maize meals and sorghum DDGS meal

Extractants	Non-Washed	Washed	Mean effect of extractants	Non-Washed	Washed	Mean effect of extractants	Non-Washed	Washed	Mean effect of extractants	Non-Washed	Washed	Mean effect of extractants
	Extract filtration time (sec)			Kafirin and zein extracted (g/100 g sorghum, DDGS, Maize meal) (db)			Kafirin and zein preparation purity (g protein/100 g preparation) (db)			Kafirin and zein yield* (g protein/100 g in the raw material)		
SORGHUM MEAL												
EtOH + KMS	257±1[1]	256±2	257^{a}±2	7.9±0.4	5.9±0.3	6.9^{a}±1.1	66.9±0.3	79.6±0.5	73.2^{a}±6.8	53.1±0.6	46.7±0.2	49.9^{a}±3.8
EtOH + KMS+ AA	259±3	255±0	257^{a}±3	8.8±0.6	6.9±0.1	7.9^{b}±1.2	68.1±0.2	76.5±0.1	72.3^{a}±4.5	59.9±0.4	52.8±0.4	58.0^{b}±4.8
EtOH + KMS+ NaOH	255±2	259±2	256^{a}±2	8.9±0.1	6.8±0.1	7.8^{b}±1.1	68.9±0.4	85.1±0.1	76.8^{b}±6.2	61.3±0.2	57.8±1.5	59.6^{c}±2.7
Mean effect of washing	257^{a}±3	257^{a}±1		8.5^{b}±0.5	6.5^{a}±0.5		68.0^{a}±1.3	80.4^{b}±1.5		58.1^{b}±3.6	52.9^{a}±3.5	
Extractants	**Non-Washed**	**Washed**	**Mean effect of extractants**	**Non-Washed**	**Washed**	**Mean effect of extractants**	**Non-Washed**	**Washed**	**Mean effect of extractants**	**Non-Washed**	**Washed**	**Mean effect of extractants**
MAIZE MEAL												
EtOH + KMS	255±1	259±2	257^{a}±3	6.6±0.1	4.6±0.1	5.6^{a}±1.0	66.0±0.3	77.4±0.2	71.7^{b}±6.1	59.0±1.2	47.5±0.1	53.3^{a}±5.6
EtOH + KMS+ AA	260±2	259±1	259^{b}±2	7.5±0.0	5.9±0.3	6.7^{b}±0.9	68.3±1.4	73.0±0.3	70.5^{a}±2.9	68.3±0.9	55.6±2.4	67.2^{b}±6.2
EtOH + KMS+ NaOH	260±1	257±1	259^{b}±1	7.8±0.4	6.6±0.2	7.2^{c}±0.6	71.8±0.1	80.3±0.9	76.0^{c}±4.6	71.7±2.0	70.1±1.3	69.4^{c}±2.9
Mean effect of washing	258^{a}±3	259^{a}±1		7.3^{b}±0.6	5.7^{a}±0.9		68.7^{a}±2.6	76.9^{b}±3.2		67.3^{b}±6.6	57.7^{a}±9.0	
Extractant	**Non-Washed**	**Washed**		**Non-Washed**	**Washed**		**Non-Washed**	**Washed**		**Non-Washed**	**Washed**	
SORGHUM DDGS MEAL												
EtOH + KMS+ AA	254±1	254±0		19.5±0.2	17.4±0.6		77.3±0.8	78.5±0.1		53.5±0.5	48.4±1.5	
Mean	254			18.5			77.9			51.0		

[1]Means ± standard deviations, n = 4, EtOH= 70% aqueous ethanol, AA=0.35% Acetic acid, NaOH= 0.35% Sodium hydroxide, KMS= sodium metabisulphite.*The mean protein contents of the sorghum meal, maize meal and sorghum DDGS were 10.0%, 7.5% and 28.2% (db) (N x 6.25), respectively. Mean values in the same column with different superscripts differ significantly ($p<0.05$). Mean values in the same row but with different superscripts are significantly different ($p<0.05$)

Table 4.5 shows that coarse sorghum and maize meals and sorghum DDGS meal (either washed or non-washed) showed similar extract filtration time (mean 257 seconds), irrespective of the extractant. Since all the meals used for extraction had a particle size of greater than 1000 µm, the extract filtration time findings would be expected. As described previously, larger inter-particle spaces of coarse grain material makes it easier for the solvent to filter through faster (Palmer, 1999; Jaques et al., 2003).

The yield of kafirin and zein extracted increased substantially when glacial acetic acid and sodium hydroxide were used as part of the extractant. These chemicals might have improved kafirin and zein solubility by their action of deamidating asparagine and glutamine amino acid residues to form aspartic and glutamic acids (Avanti, 2012). This could have led to protein conformational changes resulting in unfolding of protein, exposure of hydrophobic regions of proteins to solvation, disruption of hydrophobic interactions, breakage of hydrogen bonds and increased amount of electrostatic repulsions (Cooper, 1999). These effects make kafirin (Gao et al., 2005) and zein (Cabra et al., 2007) more soluble in the alkaline/acidic aqueous ethanol extractant. Of most interest was the observation that the amount of kafirin extracted from sorghum DDGS meal was much higher than that from sorghum and maize meals. This is despite challenges such as poor solubility of DDGS zein and kafirin during extraction that have been reported (Taylor, 2003). The poor solubility of these proteins according to literature is believed to be due to protein denaturation occurring during processing conditions such as heating at elevated temperatures, change in pH and fermentation (Wolf and Lawton, 1997), which significantly decreases protein solubility in common extraction solutions (Lagrain et al., 2005). The complex protein composition of DDGS (Bandara, Chen and Wu, 2011) has also been reported as another factor adversely affecting DDGS zein and kafirin solubility. However, Li (2013) reported greater kafirin extraction from DDGS than directly from sorghum flour, possibly due to a strong association between protein and non-protein components in sorghum flour, such as carbohydrates and probably due to the greater starting protein content of sorghum DDGS than sorghum flour.

The kafirin preparation extracted from non-washed sorghum DDGS meal with aqueous ethanol and glacial acetic acid had a higher purity (77%) than the purity of kafirin (67-69%) and zein (66-72%) preparations extracted from non-washed sorghum and maize meals. Washing of sorghum and maize meals to remove starch prior to kafirin and zein extraction substantially increased kafirin preparation purity (77-85%) and zein preparation purity (73-

80%). Washing of DDGS meal prior to kafirin extraction had no significant effect on kafirin preparation purity, probably due to there being little residual starch present in the DDGS meal. Kafirin preparation purity of approximately 82% has been obtained from sorghum DDGS meal (Lau et al., 2015). The reported higher protein purity was probably as a result of the authors washing the distillers dried grains three times with five to six volumes of hot water (50^{o}C) to remove most of the water soluble substances.

The inclusion of sodium hydroxide in the aqueous ethanol extractant resulted in a slight but significant ($p<0.05$) increase in kafirin and zein preparation purity compared to the inclusion of glacial acetic acid. It may be assumed from these results that inclusion of an alkali (sodium hydroxide) was more effective than inclusion of an acid. This agrees with research findings by Cayot et al. (2002) who reported that the pH of the extraction solvent may influence the effectiveness of the reduction reaction. According to these authors the reduction reaction is much more effective in an alkaline environment than in an acidic environment, and that is why the NaOH-ethanol extractant induces more disulphide bond reduction than the acidic-ethanol extractant. Landry and Moureaux (1980) and Coleman and Larkins (1999) observed a decrease in zein preparation purity when sodium hydroxide was used as part of the extraction solvent. The authors attributed their findings to the co-extraction of γ-zein, lipoproteins and non-protein nitrogen. Based on kafirin extraction results of Taylor et al. (2005b) use of glacial acetic acid with sodium metabisulphite pre-soak resulted in a kafirin preparation with lower purity (approximately 43%) than kafirin extracted with the aqueous ethanol or aqueous isopropanol-based solvents (approximately 75%). However, a major drawback with inclusion of sodium hydroxide in the aqueous ethanol extractant is that it introduced another step to the extraction procedure as it had to be neutralised before the protein could be precipitated.

The yield of kafirin from non-washed sorghum DDGS meal was the lowest (54%) when compared with kafirin (60-61%) and zein (68-75%) extracted from non-washed sorghum and maize meals with either glacial acetic acid or sodium hydroxide as part of the extractant. The same trend in yield was also observed with the washed meals. For example, yield of kafirin from washed sorghum DDGS meal was 48%, yield of kafirin from washed sorghum meal was between 53-58% and zein yield from washed maize meal was between 58-67%. But considering the fact that the protein content of the sorghum DDGS starting material was 28.2% as opposed to 10.0% for sorghum and 7.5% for maize, the amount of protein extracted from sorghum DDGS was still much higher. DDGS kafirin yield of 44.2% has been reported

in literature, when extraction was carried out using only acetic acid under reducing conditions (Wang et al., 2009).

Table 4.6: Proximate composition of kafirin and zein preparations and sorghum and maize meals and sorghum DDGS meal

	Dry matter (g/100 g)	Moisture (g/100 g)	Protein content (N x 6.25) (g/100 g)	Fat (g/100 g)
Sorghum meal	90.0	10.0	9.1 (10.0)[1]	2.1 (2.4)
Kafirin preparation from sorghum meal	90.6	9.4	61.2-77.7 (67.5-85.8)	9.9 (10.9)
DDGS meal	89.7	10.3	25.6 (28.2)	4.5 (6.0)
Kafirin preparation from DDGS meal	92.4	7.6	71.6-73.4 (77.5-79.4)	9.4 (10.1)
Maize meal	89.4	10.6	6.8 (7.5)	1.1 (1.3)
Zein preparation from maize meal	93.2	6.8	61.8-74.9 (66.3-80.4)	0.7 (0.7)
Sigma zein	98.3	1.7	94.1 (95.7)	2.4 (2.4)
Zein Gold	98.0	2.0	77.5 (79.1)	4.4 (4.4)

[1]Dry basis in parentheses.

Table 4.6 shows that the commercial Sigma zein and Zein Gold preparations had much lower moisture contents than the kafirin and zein preparations, presumably because they had been dried at elevated temperatures compared to extracted kafirin and zein preparations which were dried at room temperature. Of all the prolamin preparations, Sigma zein had the highest protein content possibly because much of the impurities had been removed during the preparation process. Kafirin preparations from coarse sorghum and sorghum DDGS meals had higher fat contents than the zein preparations. The lower fat content of the maize meal and its corresponding zein preparation may be attributed to the maize milling process which removes much of the fat rich germ (Gwirtz and Garcia-Casal, 2014), since the maize meal used for extraction was highly refined (Super grade).

Table 4.7: Effect of defatting the kafirin and zein preparations with n-hexane on preparation protein content

Prolamin preparation	Kafirin and zein preparation protein content (g protein/100 g preparation (db) before defatting	Kafirin and zein preparation protein content (g protein/100 g preparation (db) after defatting
Kafirin preparation from sorghum meal	$73.5^{b}\pm0.2$[1]	$84.2^{a}\pm0.2$
Zein preparation from maize meal	$70.3^{a}\pm0.6$	$85.0^{a}\pm0.5$
Kafirin preparation from DDGS meal	$78.1^{c}\pm0.4$	$84.3^{a}\pm0.6$
Sigma zein	$95.7^{d}\pm0.2$	Defatting not performed
Zein Gold	$79.0^{c}\pm0.5$	Defatting not performed

db= dry basis
[1]Means± standard deviations, n=2
Means of values in the same column but with different letters are significantly different ($p<0.05$)

Table 4.7 shows that there was no significant difference ($p\geq0.05$) in protein content of the extracted kafirin and zein preparations after defatting regardless of their source. Though defatting was not performed on Sigma zein preparation it still had a higher protein content than the defatted prolamin preparations. Increase in kafirin protein content after defatting has been reported (Taylor, 2003; Taylor et al., 2005b). Similar results have also been reported for zein preparations (Schober et al., 2010; 2011).

4.3.1.1 SDS-PAGE of kafirin and zein preparations under reducing and non-reducing conditions

SDS-PAGE under reducing and non-reducing conditions was used to characterise the kafirin and zein preparations. Under reducing conditions, disulphide bonds are broken down, hence separating kafirin and zein proteins into their monomers (Emmambux and Taylor, 2009). Consequently, kafirin and zein sub-class composition can be distinguished by a number of

bands varying in their apparent molecular weights from about 10 kDa to 28 kDa (Shull et al., 1991). SDS-PAGE of kafirin and zein preparations under both reducing and non-reducing conditions (Figure 4.3) showed bands consistent with monomers. These bands had molecular weights of approximately 13 kDa and 19-26 kDa. The 13 kDa kafirin bands can be assigned to β-kafirin sub-classes (Nunes et al., 2005) and the 13 kDa zein bands can be assigned to β-zein sub-classes (Paraman and Lamsal, 2011), while bands with molecular weights of approximately 19 kDa can be classified as α_2-kafirin (El Nour et al., 1998) or α_2-zein (Paraman and Lamsal, 2011) sub-classes and bands with molecular weights of approximately 24 kDa can be classified as α_1-kafirin (El Nour et al., 1998) or α_1-zein (Paraman and Lamsal, 2011) sub-classes. It was observed that bands with molecular weights of approximately 13 kDa were only clear in zein with SDS-PAGE under reducing conditions (track 1) and fainter in the other tracks. Under non-reducing conditions, bands of approximately 40 kDa and 50 kDa occurred in all prolamin preparations. Bands of approximately 45 kDa to 50 kDa are designated as dimers of kafirin and zein polypeptides (El Nour et al., 1998). They are believed to be due to disulphide cross-linking of the monomeric units α-, β-, and γ-kafirins and zeins. The relative concentration of the dimers was greater in kafirin (extracted from sorghum meal) (track 4) than in zein and DDGS kafirin preparations (tracks 2 and 6, respectively). The lower intensity dimer bands indicated slight polymerisation of the prolamin preparations.

Kafirin extracted from DDGS meal was highly polymerised by disulphide bonds. Under non-reducing conditions (track 6), the bands entering the gel were much fainter than in zein and kafirin preparations (tracks 2 and 4, respectively), indicating the presence of polymers which were so large and that they could not enter the separating gel. This is an indication of polymerisation due to cross-linking occurring during the production of the DDGS.

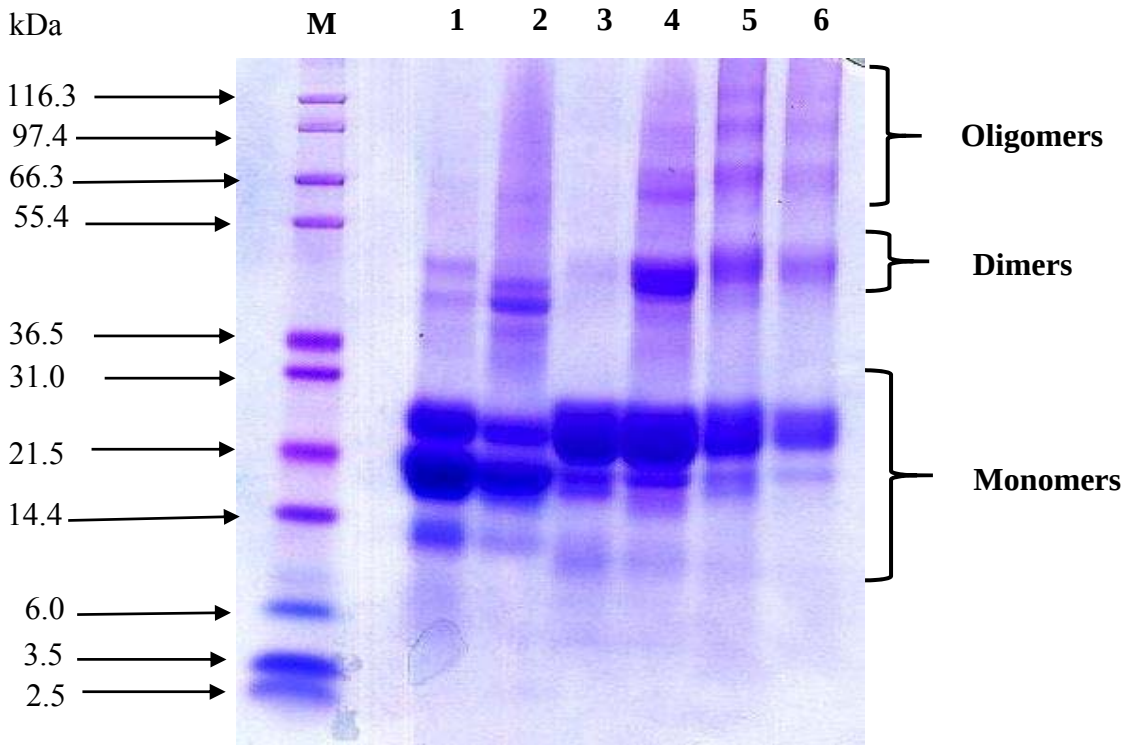

Figure 4.3: SDS-PAGE of zein and kafirin preparations extracted from washed sorghum and maize meals and sorghum DDGS meal with 70% (w/w) aqueous ethanol + 1.0% (w/w) sodium metabisulphite + 0.35% (w/w) glacial acetic acid under reducing and non-reducing conditions: M- molecular weight standards, track 1- zein under reducing conditions, track 2- zein under non-reducing conditions, track 3- kafirin under reducing conditions, track 4- kafirin under non-reducing conditions, track 5- kafirin from DDGS under reducing conditions, track 6- kafirin from DDGS under non-reducing conditions.

4.3.2 Film making quality of the prolamin preparations

The extracted kafirin and zein preparations in addition to Sigma zein and Zein Gold preparations were evaluated for their film making quality. Films were cast by dissolving the prolamin preparations in 70% aqueous ethanol without a plasticiser with heat applied up to 70°C and then evaporating the solvent off at 50°C. All protein preparations including Sigma zein and Zein Gold could form films. All the films were rather brittle due to the absence of added plasticiser. Plasticisers are small molecular weight hydrophilic compounds which may be added to alter the properties of the film or coating such as film flexibility (Donhowe and Fennema, 1994; Krochta, 2002). Plasticisers fill the spaces between the polymer chains

thereby reducing secondary forces and altering the three dimensional structure of the polymer (Vieira et al., 2011; Wittaya, 2012). This reduces the overall energy required to form hydrogen bonds and improves molecular motion thereby improving the flexibility of the coating or film (Paramawati et al., 2001; Kushwaha and Kawtikwar, 2013) by overcoming film brittleness caused by extensive intermolecular forces (Hernandez-Izquierdo and Krochta, 2008). In the current work, a plasticiser was deliberately not added so as to evaluate the intrinsic properties of the prolamin preparations.

It was observed that the kafirin preparation extracted from sorghum meal was difficult to dissolve in aqueous ethanol. The viscous solution was more difficult to spread out on the silicone baking trays. Additional solvent was added to produce films. This was in contrast to DDGS kafirin, zein, Sigma zein and Zein Gold preparations which dissolved with ease when heated to 70^{o}C in aqueous ethanol. Similar observations with kafirin dissolution have also been reported (Da Silva, 2003; Taylor, 2003).

Table 4.8: Effects of washing the sorghum meal prior to kafirin extraction and of heat treatment of the cast kafirin films at 130°C for 1 h on film water uptake

Treatment	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating
	Film weight (mg db[1])			Film area (cm^2)			Film thickness (µm)			Film density (mg/cm^3)		
Heat treated	234.6±2.5[2]	219.8±4.9	227.2[a]±9.1	19.2±0.2	18.7±0.5	18.9[a]±0.4	75.6±1.9	77.5±1.9	76.5[a]±1.9	1619.2±0.3	1479.5±0.3	1549.3[a]±0.3
Non-heat treated	227.9±0.1	229.1±2.3	228.5[a]±1.5	18.7±0.5	18.8±0.7	18.8[a]±0.5	81.2±0.6	79.1±0.1	80.2[a]±1.3	1499.6±0.3	1719.0±0.6	1609.3[b]±2.8
Mean effect of washing	231.2[b]±4.1	224.4[a]±6.2		18.9[a]±0.4	18.8[a]±0.5		78.4[a]±3.5	78.3[a]±1.4		1559.4[a]±0.2	1599.2[a]±3.1	
Treatment	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating
	Film weight after immersion (mg)			Area after immersion (cm2)			Weight difference (mg)			Film water uptake (%)(weight difference/initial film weight*100)		
Heat treated	374.7±4.6	311.4±4.0	343.0[a]±36.7	22.0±1.0	20.8±0.8	21.4[a]±1.0	140.1±2.1	91.6±0.8	115.9[a]±28.0	59.7±0.3	41.7±1.3	50.7[a]±10.4
Non-heat treated	378.0±0.8	355.4±2.4	366.2[b]±12.5	22.5±0.3	20.4±0.2	21.5[a]±1.2	149.1±0.6	126.3±0.1	137.7[b]±13.1	65.4±0.2	55.1±0.5	60.3[b]±5.9
Mean effect of washing	375.8[b]±3.0	333.4[a]±5.5		22.3[b]±0.7	20.6[a]±0.5		144.6[b]±5.3	109.0[a]±20.0		62.6[b]±3.2	48.4[a]±7.8	

[1]db =dry basis
[2]Means± standard deviations, n=4
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

Table 4.9: Effects of washing the maize meal prior to zein extraction and of heat treatment of the cast zein films at 130°C for 1 h on film water uptake

Treatment	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating
	Film weight (mg db[1])			Film area (cm^2)			Film thickness (µm)			Film density (mg/cm^3)		
Heat treated	239.1±1.7[2]	241.0±2.7	240.1[a]±2.1	18.5±0.2	17.1±0.5	17.8[a]±0.9	79.1±0.7	80.7±1.2	79.9[a]±1.2	1637.9±0.4	1747.5±0.7	1692.7[a]±0.5
Non-heat treated	240.5±1.2	239.7±1.6	240.1[a]±1.3	17.8±0.5	17.1±0.5	17.4[a]±0.4	77.7±1.0	79.4±1.7	78.6[a]±1.5	1744.7±0.4	1766.4±0.5	1755.6[b]±0.5
Mean effect of washing	239.8[a]±1.5	240.3[a]±2.0		18.1[b]±0.5	17.1[a]±0.4		78.4[a]±1.1	80.1[a]±1.4		1691.3[a]±0.5	1756.9[b]±0.6	
Treatment	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating
	Film weight after immersion (mg)			Area after immersion (cm2)			Weight difference (mg)			Film water uptake (%)(weight difference/initial film weight*100)		
Heat treated	384.6±1.2	355.0±1.4	369.8[a]±17.3	20.3±0.4	19.0±0.5	19.6[a]±0.8	145.5±0.5	114.0±2.4	129.7[a]±18.2	60.8±0.6	47.3±0.5	54.1[a]±7.8
Non-heat treated	401.1±1.7	380.7±1.4	390.9[b]±11.8	21.2±1.6	19.5±0.2	20.3[a]±1.4	160.5±0.5	141.1±0.2	150.8[b]±11.3	66.7±0.1	58.9±0.5	62.8[b]±4.6
Mean effect of washing	392.8[b]±9.6	367.9[a]±15.1		20.7[a]±1.1	19.2[a]±0.4		153.0[b]±8.7	127.5[a]±15.7		63.8[b]±3.4	53.1[a]±6.7	

[1]db =dry basis
[2]Means± standard deviations, n=4
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

Table 4.10: Effects of washing the sorghum DDGS meal prior to kafirin extraction and of heat treatment of the cast DDGS kafirin films at 130°C for 1 h on film water uptake

Treatment	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating
	Film weight (mg db[1])			**Film area (cm^2)**			**Film thickness (µm)**			**Film density (mg/cm^3)**		
Heat treated	265.3±2.1[2]	268.5±2.4	266.9^b±2.6	17.7±0.4	17.6±0.3	17.6^a±0.3	84.2±1.1	88.0±0.3	86.1^a±2.3	1787.7±0.4	1732.7±0.4	1760.2^b±0.4
Non-heat treated	243.9±0.3	242.1±0.4	243.0^a±1.1	18.4±0.0	18.2±1.0	18.2^a±0.3	86.8±1.5	88.6±0.3	87.7^a±1.4	1534.3±0.2	1517.9±0.3	1526.1^a±0.2
Mean effect of washing	254.6^a±12.4	255.3^a±15.3		18.0^a±0.5	17.8^a±0.4		85.5^a±1.8	88.3^b±0.4		1661.0^a±1.0	1625.3^a±1.1	
Treatment	Non -Washed	Washed	Mean effect of heating	Non-Washed	Washed	Mean effect of heating	Non- Washed	Washed	Mean effect of heating	Non- Washed	Washed	Mean effect of heating
	Film weight after immersion (mg)			**Area after immersion (cm^2)**			**Weight difference (mg)**			**Film water uptake (%)(weight difference/initial film weight*100)**		
Heat treated	375.9±1.8	386.2±2.3	381.0^b±6.2	20.4±0.2	19.2±0.2	19.8^a±0.7	110.6±4.0	117.7±0.2	114.1^b±4.7	41.7±1.8	43.8±0.5	42.8^b±1.6
Non-heat treated	335.0±0.5	326.7±1.2	330.9^a±4.8	19.7±0.5	20.4±0.3	20.1^b±0.5	91.1±0.3	84.6±1.3	87.8^a±3.9	37.4±0.1	35.0±0.1	36.2^a±1.4
Mean effect of washing	356.5^a±34.4	355.4^a±23.6		20.0^a±0.5	19.8^a±0.7		100.8^a±11.5	101.1^a±19.1		39.5^a±2.7	39.4^a±5.1	

[1]db=dry basis
[2]Means± standard deviations, n=4
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

Table 4.11: ANOVA for interaction between washing of the sorghum and maize meals and sorghum DDGS meal prior to kafirin and zein extraction and of heat treatment of the cast kafirin and zein films at 130°C for 1 h on film water uptake

Kafirin films

Source of variation	Mean square	F	DF	P
Washing	0.006836	162.91	4	0.000
Heating	0.003365	332.58	4	0.000
Washing*Heating	0.136099	6.84	4	0.004

Zein films

Source of variation	Mean square	F	DF	P
Washing	0.001526	158.97	4	0.002
Heating	0.001994	321.09	4	0.000
Washing*Heating	0.285918	4.99	4	0.003

DDGS kafirin films

Source of variation	Mean square	F	DF	P
Washing	0.018648	1380.03	4	0.204
Heating	0.000181	12.64	4	0.020
Washing*Heating	0.108385	2.06	4	0.476

Tables 4.8, 4.9 and 4.10 show the effects of washing sorghum and maize meals and sorghum DDGS meal prior to kafirin and zein extraction and of heat treatment of the cast kafirin and zein films on film water uptake. Heat treated kafirin films from kafirin preparation extracted from the washed sorghum meal absorbed less water (approximately 42%) after immersion in pH 6.8 phosphate buffer compared to heat treated kafirin films from kafirin preparation extracted from non-washed sorghum meal (approximately 60%) (Table 4.8). Non-heat treated kafirin films from kafirin preparation extracted from non-washed sorghum meal showed higher water uptake (approximately 65%) compared to non-heat treated kafirin films from kafirin preparation extracted from washed sorghum meal (approximately 55%). A similar trend was also observed with the zein films (Table 4.9). This was in contrast to DDGS kafirin films

where non-heat treated DDGS kafirin films from kafirin preparation extracted from washed and non-washed sorghum DDGS meals showed less water uptake (approximately 35% and 37%, respectively) compared to heat treated DDGS kafirin films from kafirin preparation extracted from washed and non-washed meals (approximately 44% and 42%, respectively) (Table 4.10).

The lower percentage of water absorbed by heat treated kafirin and zein films from kafirin and zein preparations extracted from washed sorghum and maize meals can be attributed to two factors. Firstly, the reduced starch present in the films as a result of washing the sorghum and maize meals prior to kafirin and zein extraction might have led to reduced hydration of starch granules on soaking. Swelling of starch granules when exposed to moist conditions has been reported in literature (Fredriksson et al., 1998). It is further reported that the presence of more disulphide bonds around the starch granules restricts starch granule swelling and starch gelatinisation (Chandrashekar and Kirleis, 1988; Ezeogu et al., 2008).

Secondly, heat treatment of kafirin films has been reported to be responsible for modification in film functional properties such as water uptake (Byaruhanga et al., 2007). This is due to protein cross-linking through the formation of disulphide and hydrophobic bonds (Zavodszky et al., 2001). The cross-linking of polypeptide chains through heat treatment can cause a decrease of hydrophilic functional groups and therefore lower water binding capacity of the films leading to a decrease in film water uptake (Soliman et al., 2009; Peters et al., 2015).

As stated, heating had no effect on the water uptake of the DDGS kafirin films. The lack of an effect can be attributed to the conditions that the DDGS had undergone during production, including heating at elevated temperatures, pH adjustments and fermentation (Cromwell et al., 1993). These conditions could have resulted in more disulphide cross-linking of the kafirin. Overall, non-heat treated DDGS kafirin films had the lowest water uptake of all the films tested.

A significant interaction was found between washing of the sorghum and maize meals prior to kafirin and zein extraction and heating of the kafirin and zein films on film water uptake ($p<0.05$). There was no significant interaction between washing of the sorghum DDGS meal prior to kafirin extraction and heating of the DDGS kafirin films on film water uptake ($p\geq0.05$) (Table 4.11).

Figure 4.4 also shows the appearance of the cast kafirin and zein films before and after immersion in 0.2 M phosphate buffer (pH 6.8) and incubation at 39^{o}C for 12 hours. It was observed that both the kafirin and zein films absorbed water but remained whole on soaking. Non-heat treated kafirin and zein films from kafirin and zein preparations extracted from non-washed meals showed a higher swelling after soaking compared to heat treated kafirin and zein films from kafirin and zein preparations extracted from washed meals. This was probably as a result of more water being absorbed by the starch granules in non-heat treated films from kafirin and zein preparations from non-washed meals. Fredriksson et al. (1998) reported that when starch granules are put in water, large quantities of water become entrapped within the starch granules and between the solubilised starch molecules through inter-hydrogen bonding and this allows the starch granules to hydrate and swell.

Heating of the films also resulted in reduced film swelling. Schmidt et al. (2012) reported that the degree of swelling of the polymeric networks depend on the interactions between the fluid and the polymer and on the structure and properties of both the solvent and the polymer. According to these authors, a higher degree of cross-linking results in a decrease in the swelling ability of the polymer because as the fluid dissolves the network, the elastic forces of the polymer counteract the swelling.

DDGS kafirin films from kafirin preparation extracted from both washed and non-washed meals had the same level of film swelling after soaking, probably due to the DDGS kafirin preparation purity from both non-washed and washed meals which was almost the same (77% and 79%, respectively).

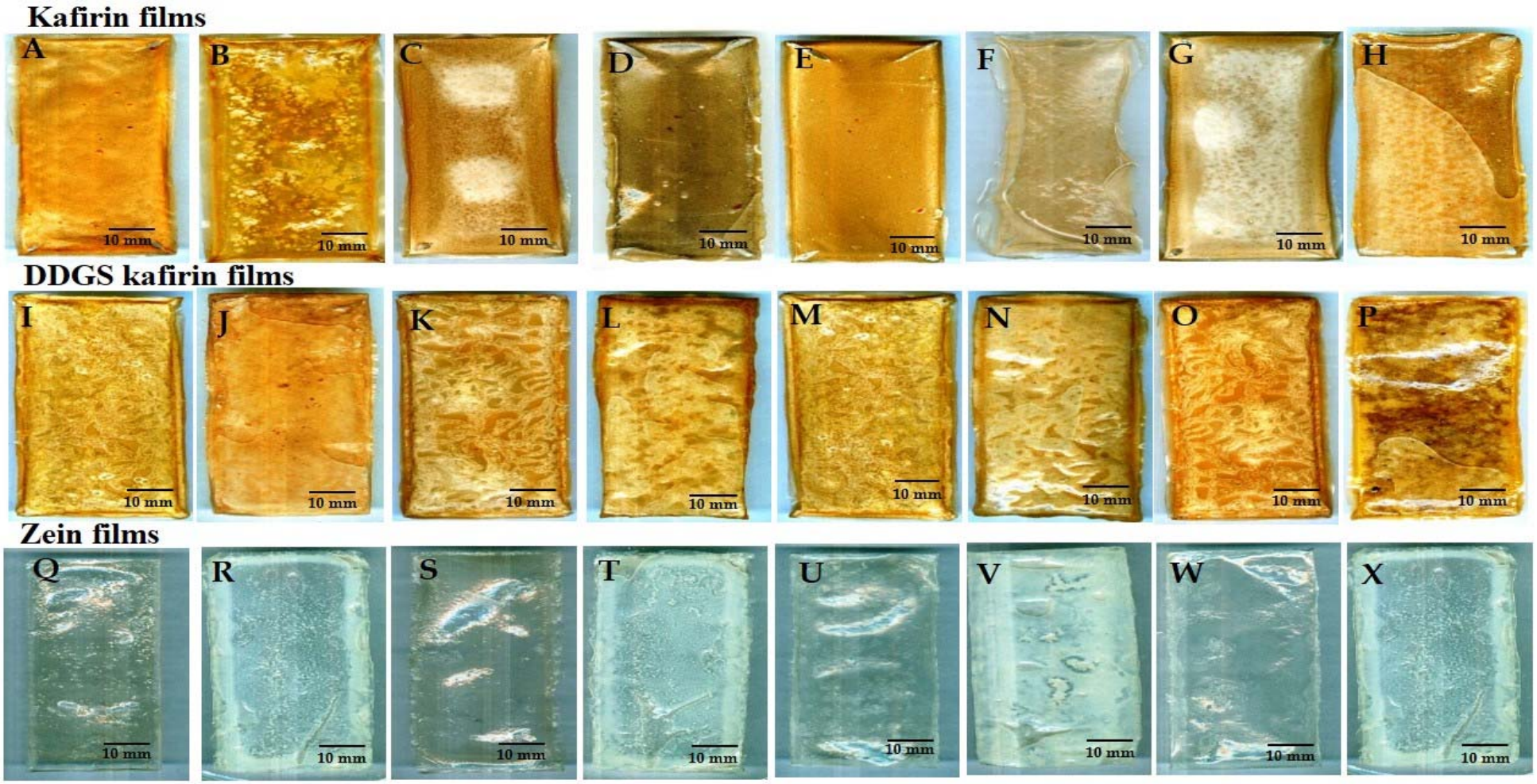

Figure 4.4: Effects of washing sorghum and maize meals and sorghum DDGS meal prior to kafirin and zein extraction and heating of cast films on film water absorption.

(A-B) heat treated kafirin films kafirin extracted from washed meal) before and after immersion; (**C-D**) non-heat treated kafirin films (kafirin extracted from non-washed meal) before and after immersion; (**E-F**) heat treated kafirin films (kafirin extracted from non-washed meal) before and after immersion; (**G-H**) non-heat treated kafirin films (kafirin extracted from washed meal) before and after immersion; (**I-J**) heat treated DDGS films (kafirin extracted from washed DDGS meal) before and after immersion; (**K-L**) non-heat treated DDGS films (kafirin extracted from non-washed DDGS meal) before and after immersion; (**M-N**) heat treated DDGS films (kafirin extracted from non-washed DDGS meal) before and after immersion; (**O-P**) non-heat treated DDGS films (kafirin extracted from washed DDGS meal) before and after immersion; (**Q-R**) heat treated zein films (zein extracted from washed meal) before and after immersion; (**S-T**) non-heat treated zein films (zein extracted from non-washed meal) before and after immersion; (**U-V**) heat treated zein films (zein extracted from non-washed meal) before and after immersion; (**W-X**) non-heat treated zein films (zein extracted from washed meal) before and after immersion.

4.3.2.1 Effects of defatting the kafirin and zein preparations and of heat treatment of the films on film water uptake

Tables 4.12, 4.13 and 4.14 show the effects of defatting the kafirin and zein preparations and of heat treatment of the cast films on film water uptake. The heat treated kafirin films from undefatted kafirin preparations showed lower water uptake (approximately 41%) after immersion in pH 6.8 phosphate buffer compared to heat treated kafirin films from defatted kafirin preparations (approximately 60%). Non-heat treated kafirin films from defatted kafirin preparations showed the highest water uptake (approximately 70%) compared to non-heat treated kafirin films from undefatted kafirin preparations (approximately 53%). A similar trend was found with the zein and DDGS kafirin films (Tables 4.13 and 4.14, respectively). However, the least amount of water (35%) was absorbed by the non-heat treated DDGS kafirin films from undefatted DDGS kafirin preparation.

The higher amount of water absorbed by non-heat treated kafirin and zein films from defatted prolamin preparations compared to non-heat treated kafirin and zein films from undeffated prolamin preparations was presumably due to defatting. A decrease in zein film water absorption with increasing addition of fatty acids has been reported (Lai et al., 1997). These authors attributed their findings to the sealing off of the surface pores in the films by fatty acids which enable films to maintain their structure in aqueous environments. A similar reason could also be responsible for the high amount of water absorbed by heat treated kafirin and zein films from defatted prolamin preparations compared to heat treated kafirin and zein films from undefatted prolamin preparations. Combination of proteins with fatty acids as lipid film components as reported by Taylor (2003) has been found to make the film structure more compact and more hydrophobic which increases film water resistance (Lai and Padua, 1998; Da Silva, 2003).

Furthermore, the lower water absorption shown by the heat-treated kafirin and zein films from the defatted and undefatted prolamin preparations compared to non-heat treated kafirin and zein films from defatted and undefatted prolamin preparations can be attributed to the effect of heat treatment (Byaruhanga et al., 2006; Byaruhanga et al., 2007; Guo et al., 2012). As described, heat treatment of films results in modification in film functional properties such as film water uptake due to intermolecular disulphide bond cross-linking of the protein induced by heat (Pelosi, 1997; Byaruhanga et al., 2005). However, heating had no effect on

DDGS kafirin film water uptake possibly due to the reasons discussed previously regarding the DDGS production process.

Table 4.12: Effects of defatting the kafirin preparations and of heat treatment of the cast kafirin films at 130°C for 1 h on film water uptake

Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight (mg db[1])			**Film area (cm^2)**			**Film thickness (µm)**			**Film density (mg/cm^3)**		
Heat treated	299.3±1.0[2]	298.8±4.9	298.8[a]±0.3	18.2±0.3	18.7±0.5	18.5[a]±0.4	75.4±0.3	77.5±1.9	65.0±17.7	2181.5±0.4	2056.6±0.3	2625.5[a]±4.1
Non-heat treated	299.8±1.8	297.9±2.3	298.9[a]±1.3	19.0±0.4	18.8±0.7	18.9[a]±0.1	76.6±0.7	79.1±0.1	66.6[a]±17.7	2053.4±0.2	1999.3±0.6	2662.1[a]±4.3
Mean effect of defatting	299.6[a]±0.4	298.1[a]±0.2		18.6[a]±0.5	18.8[a]±0.1		76.0[a]±0.8	78.3[a]±1.1		2117.5[a]±0.6	2028.0[a]±3.7	
Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight after immersion (mg)			**Area after immersion (cm^2)**			**Weight difference (mg)**			**Film water uptake (%)(weight difference/initial film weight*100)**		
Heat treated	478.3±1.6	421.4±4.0	449.9[a]±40.0	21.2±1.1	20.8±0.8	21.0[a]±0.3	179.0±0.7	123.2±0.8	151.1[a]±39.5	59.8±0.4	41.3±1.3	50.6[a]±13.1
Non-heat treated	508.5±1.7	455.4±2.4	482.0[b]±37.5	20.3±0.6	20.4±0.2	20.4[a]±0.1	208.7±0.3	157.5±0.1	183.1[b]±36.2	69.6±1.4	52.9±0.5	61.3[b]±11.8
Mean effect of defatting	493.4[b]±21.5	338.4[a]±24.0		20.8[a]±0.6	20.6[a]±0.3		193.9[b]±21.0	140.4[a]±24.3		64.7[b]±6.9	47.1[a]±8.2	

[1]db=dry basis,
[2]Means± standard deviations, n=3
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

Table 4.13: Effects of defatting the zein preparations and of heat treatment of the cast zein films at 130°C for 1 h on film water uptake

Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight (mg db[1])			Film area (cm^2)			Film thickness (µm)			Film density (mg/cm^3)		
Heat treated	367.8±1.6[2]	369.7±2.7	368.8[a]±1.3	18.2±0.2	17.1±0.5	17.7[a]±0.8	78.8±1.1	80.7±1.2	79.8[a]±1.3	2572.0±0.0	2679.0±0.7	2625.5[a]±4.1
Non-heat treated	369.3±0.1	370.4±1.6	369.9[a]±0.8	17.9±0.7	17.1±0.5	17.5[a]±0.6	79.2±1.4	79.4±1.7	79.3[a]±0.1	2600.7±0.0	2723.5±0.5	2662.1[a]±4.3
Mean effect of defatting	368.6[a]±1.1	370.1[a]±0.5		18.1[b]±0.2	17.1[a]±0.0		79.0[a]±0.3	80.1[a]±0.9		2586.4[a]±0.3	2701.3[b]±0.4	
Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight after immersion (mg)			Area after immersion (cm^2)			Weight difference (mg)			Film water uptake (%)(weight difference/initial film weight*100)		
Heat treated	591.2±0.3	543.7±1.4	567.5[a]±33.6	21.0±1.6	19.0±0.5	20.0[a]±1.4	223.4±1.8	174.0±2.4	198.7[a]±34.9	60.7±0.9	47.1±1.4	53.9[a]±9.6
Non-heat treated	641.3±1.6	586.5±1.4	613.9[b]±38.7	21.4±0.3	19.5±0.2	20.5[a]±1.3	272.0±1.4	216.1±0.2	244.1[b]±39.5	73.7±0.4	58.3±0.8	66.0[b]±10.9
Mean effect of defatting	616.3[b]±35.4	565.1[a]±30.3		21.2[a]±0.3	19.3[a]±0.4		247.1[b]±34.4	195.1[a]±29.8		67.2[b]±9.2	52.7[a]±7.9	

[1]db=dry basis
[2]Means± standard deviations, n=3
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

Table 4.14: Effects of defatting the DDGS kafirin preparations and of heat treatment of the cast DDGS kafirin films at 130°C for 1 h on film water uptake

Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight (mg db[1])			Film area (cm^2)			Film thickness (µm)			Film density (mg/cm^3)		
Heat treated	314.7±0.4[2]	316.5±2.4	315.6[a]±1.3	17.0±0.7	17.6±0.3	17.3[a]±0.4	87.1±0.1	88.0±0.3	87.6[a]±0.6	2126.4±1.2	2041.9±0.4	2084.2[a]±1.8
Non-heat treated	318.7±1.0	315.4±0.4	317.1[a]±2.3	18.2±0.9	18.2±1.0	18.2[a]±0.0	85.3±0.4	88.6±0.3	87.0[a]±2.3	2056.1±0.3	1971.3±0.3	2013.7[a]±2.4
Mean effect of defatting	316.7[a]±2.8	316.0[a]±0.8		17.6[a]±0.8	17.9[a]±0.4		86.2[a]±1.3	88.3[a]±0.4		2091.3[a]±0.6	2006.6[a]±1.3	
Treatment	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating	Defatted	Undefatted	Mean effect of heating
	Film weight after immersion (mg)			Area after immersion (cm^2)			Weight difference (mg)			Film water uptake (%)(weight difference/initial film weight*100)		
Heat treated	466.0±1.9	458.6±2.3	462.3[b]±5.2	20.4±0.2	21.0±1.6	20.7[a]±0.4	151.3±2.3	142.1±0.2	146.7[b]±6.5	48.1±0.9	44.9±0.5	46.5[b]±2.3
Non-heat treated	479.6±1.3	432.7±1.2	456.2[a]±33.2	19.7±0.5	18.8±0.9	19.3[a]±0.6	160.9±2.3	117.3±1.3	139.1[a]±30.8	50.5±0.9	35.0±0.1	32.4[a]±9.4
Mean effect of defatting	472.8[b]±9.6	445.7[a]±18.3		20.1[a]±0.5	19.9[a]±1.6		156.1[b]±6.8	129.7[a]±17.5		49.3[b]±1.7	41.1[a]±5.4	

[1]db=dry basis
[2]Means± standard deviations, n=3
Means of values in the same column but with different letters are significantly different ($p<0.05$)
Means of values in the same row but with different letters are significantly different ($p<0.05$)

4.3.2.2 Effects of prolamin preparations and agitation of the kafirin and zein films on film water uptake

The data in Table 4.15 shows that kafirin films that were not agitated (non-agitated) absorbed less water (approximately 55%) after immersion in pH 6.8 phosphate buffer compared to agitated kafirin films (approximately 65%). A similar trend was found with the zein, Sigma zein and Zein Gold films. Higher water absorption was found for both agitated and non-agitated Zein Gold and Sigma zein films compared to kafirin and zein films. The higher water uptake exhibited by agitated kafirin and zein films compared to non-agitated kafirin and zein films can be attributed to the fact that agitation might have allowed more water to penetrate quicker into the films. Secondly, the availability of the three protein subclasses (α-, β- and γ-) (Shewry and Halford, 2002) in total kafirin and total zein preparations might have enabled cross-linking by disulphide bonding between the β- and γ-species. This could be responsible for the lower water uptake of both agitated and non-agitated total kafirin and total zein films compared with Sigma zein and Zein Gold films, since these preparations consist mainly of α-zein sub-class. The α-zein sub-class is reported to be less able to form disulphide linkages because of its lower amount of cysteine residues (Taylor et al., 2007).

Figure 4.5 shows the appearance of the agitated and non-agitated kafirin and zein films. It was observed that after immersion in phosphate buffer, the films from the laboratory prepared total kafirin and total zein, both agitated and non-agitated, maintained film integrity (absorbed water but remained whole on soaking). This was in contrast to the agitated and non-agitated Sigma zein and Zein Gold films, which showed the highest degree of swelling and distortion in film shape. The level of film shape distortion was greater for agitated films than non-agitated films. The area of the agitated Sigma zein and Zein Gold films after incubation could not be calculated due to the disintegration of these films.

Table 4.15: Effects of prolamin preparations and of agitation of the kafirin, zein, Sigma zein and Zein Gold films on film water uptake

Treatment	Film weight (mg db[1])		Film area (cm^2)		Film thickness (µm)		Film density (mg/cm^3)		Film weight after immersion (mg)		Area after immersion (cm^2)		Weight difference (mg)		Film water uptake (%)	
	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated	Agitated	Non-agitated
Kafirin films	177.7[2] ±1.8	176.7 ±0.8	21.3 ±2.5	23.2 ±0.8	62.5 ±1.1	62.0 ±1.6	1348.0 ±1.0	1236.1 ±0.3	292.3 ±2.1	274.5 ±3.0	22.1 ±1.5	23.7 ±0.7	114.5 ±1.8	97.8 ±3.3	64.5^{Ab} ±1.8	55.3^{Aa} ±2.0
Zein films	179.0 ±1.4	179.0 ±2.1	22.3 ±1.2	23.2 ±0.8	64.0 ±0.9	63.0 ±1.3	1257.8 ±0.3	1241.1 ±0.2	327.7 ±1.2	309.5 ±2.4	23.1 ±0.4	23.6 ±0.7	148.7 ±2.3	130.5 ±1.3	83.1^{Bb} ±1.9	72.9^{Ba} ±1.1
Sigma zein films	180.7 ±1.5	178.8 ±1.3	22.9 ±0.7	22.5 ±0.5	63.1 ±1.3	62.7 ±1.1	1252.6 ±0.2	1269.2 ±0.2	379.9 ±0.8	367.8 ±2.6	ND	23.2 ±0.5	199.1 ±1.3	189.0 ±2.1	110.2^{Cb} ±1.6	105.9^{Da} ±1.2
Zein Gold films	173.1 ±0.8	174.5 ±1.3	22.6 ±0.5	22.5 ±1.1	64.1 ±1.3	62.8 ±0.6	1179.3 ±0.2	1235.7 ±0.4	363.5 ±2.5	350.9 ±2.7	ND	23.2 ±0.7	190.4 ±2.1	176.4 ±2.7	110.0^{Cb} ±1.0	102.1^{Ca} ±1.9

[1]db=dry basis
[2]Means± standarad deviaations, n=4
ND= Not Determined
Means of values in the same row but with different lower case superscript letters differ significantly ($p<0.05$)
Means of values in the same column with upper case superscript letters differ significantly ($p<0.05$)

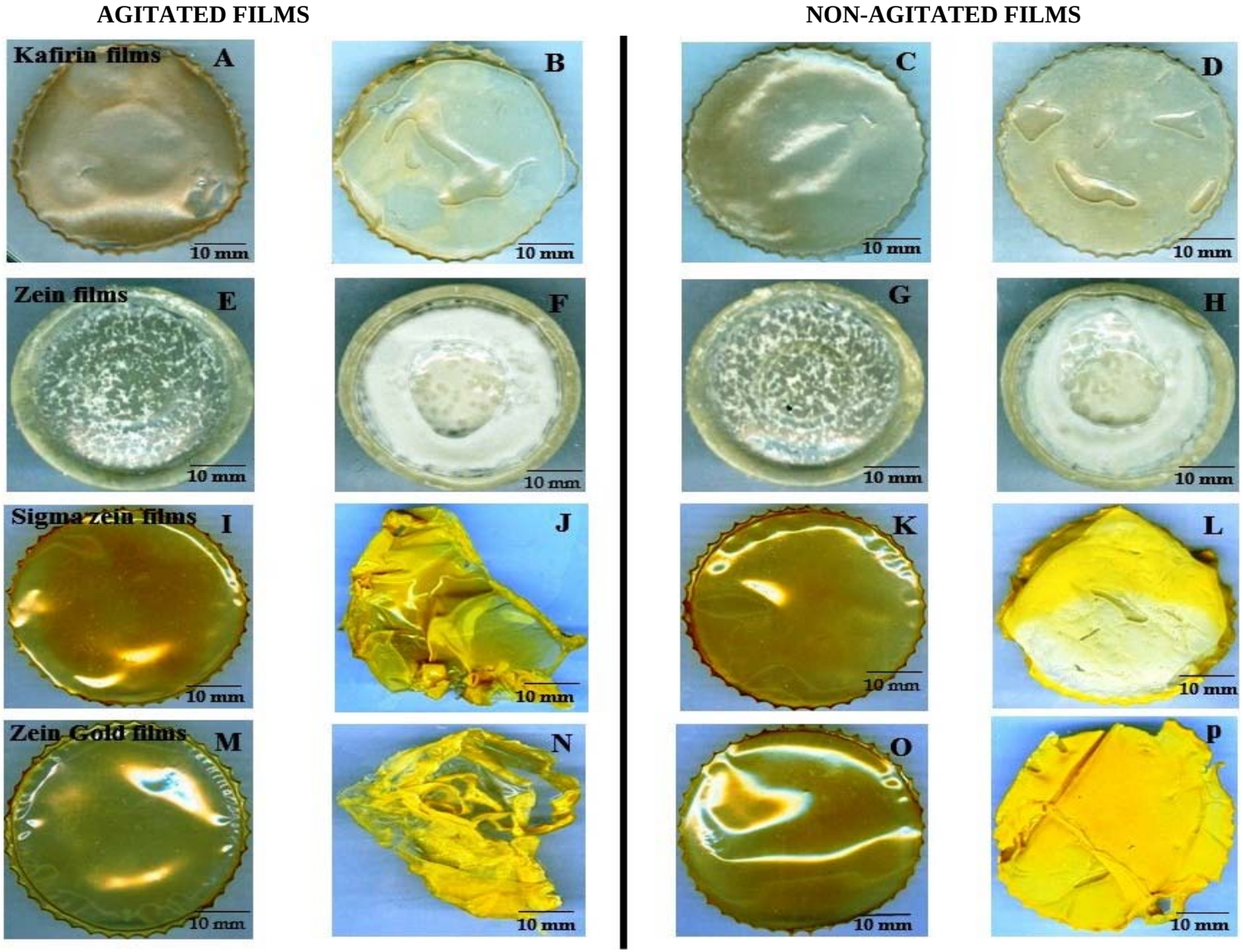

Figure 4.5: Effects of prolamin preparations and of agitation of the kafirin and zein films on water absorption and maintenance of film integrity.

4.3.2.3 Effects of washing the sorghum and maize meals prior to kafirin and zein extraction and of heating of the cast films on film surface morphology

Scanning electron microscopy (SEM) of heat treated kafirin and zein films prepared from kafirin and zein preparations extracted from washed sorghum and maize meals (Fig. 4.6A and 4.6C, respectively) revealed smooth surfaces with very few defects. DDGS kafirin films showed more defects (Fig. 4.6E) before soaking. After soaking, kafirin films (Fig. 4.6B) developed a few small pores which were distributed throughout the films. Zein films (Fig. 4.6D) developed many smaller pores, also distributed throughout the films. On soaking, DDGS kafirin films (Fig. 4.6F) developed pores which were larger in size than those in kafirin and zein films.

SEM of non-heat treated kafirin and zein films prepared from kafirin and zein preparations extracted from non-washed sorghum and maize meals (Fig. 4.7G and 4.7I) showed the presence of starch granules with some pores before soaking. DDGS kafirin films (Fig. 4.7K) showed no starch granules but had small pores distributed throughout the films. After soaking, kafirin films, zein films and DDGS kafirin films (Fig. 4.7H, 4.7J and 4.7L, respectively) all had developed pores and defects. The pores in the zein films were much larger in size than those in kafirin and DDGS kafirin films.

Washing of the sorghum and maize meals prior to kafirin and zein extraction could have led to the reduced amount of starch granules in heat treated films prepared from kafirin and zein extracted from washed meals. Starch granules were present in large numbers in the non-heat treated kafirin and zein films prepared from kafirin and zein extracted from non-washed meals. Starch granules could not be observed in heat treated and non-heat treated DDGS kafirin films prepared from kafirin extracted from washed and non-washed sorghum DDGS meal. This further supports the earlier suggestion that there was little starch present in the DDGS meal. Heating and its subsequent inducement of cross-links in kafirin and zein films could have led to the fewer number of pores present in these films. The decrease in pore size and number has been presumably related to the changes in film functional properties, such as biodegradability, tensile strength and water uptake (Byaruhanga et al., 2005; Byaruhanga et al., 2007; Taylor et al., 2009). The appearance of the heat treated kafirin films in this study were similar to SEM of the surface of kafirin films shown by these authors.

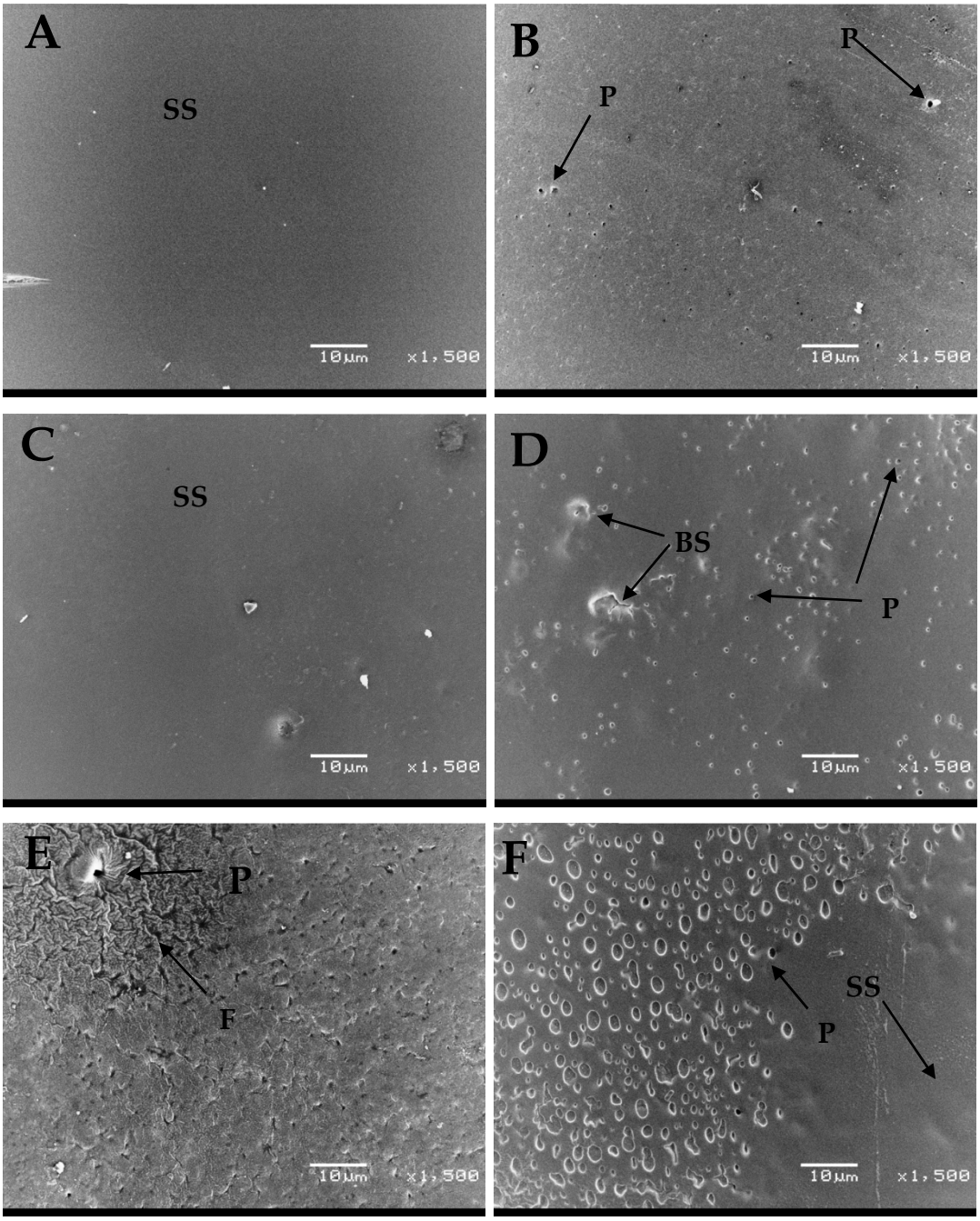

Figure 4.6: SEM showing the effects of washing the sorghum and maize meals prior to kafirin and zein extraction and heating of cast kafirin and zein films on film surface morphology before and after immersion in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39°C: (**A-B**) Kafirin films before and after immersion; (**C-D**) Zein films before and after immersion; (**E-F**) DDGS kafirin films before and after immersion; SS-Smooth surface, P-Pore, F-Folded structure, BS-Broken starch granule.

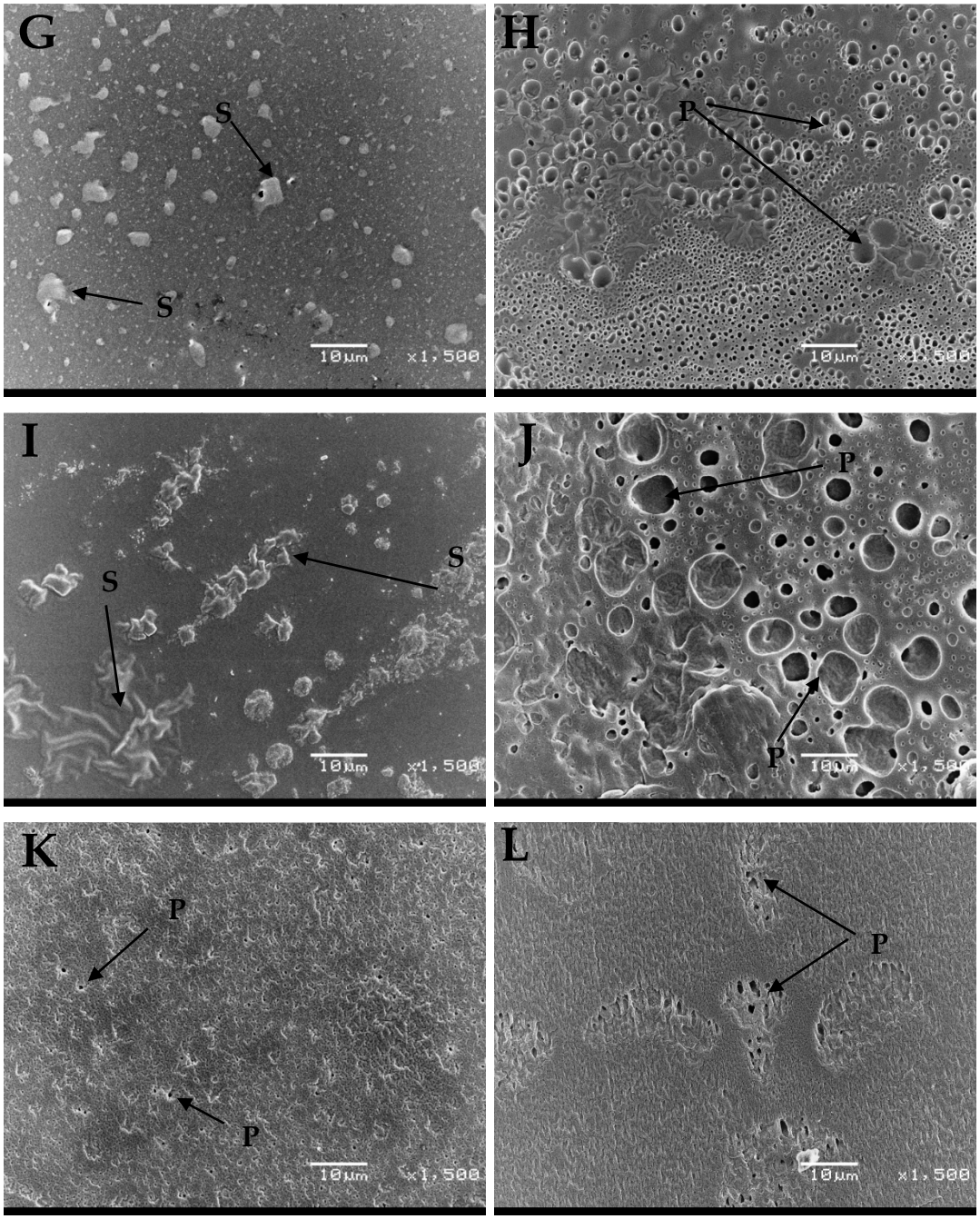

Figure 4.7: SEM showing the effects of not washing of sorghum and maize meals prior to kafirin and zein extraction and no heat treatment of cast kafirin and zein films on film surface morphology before and after immersion in 0.2 M phosphate buffer (pH 6.8) for 12 hours at 39^{o}C: (**G-H**) Kafirin films before and after immersion; (**I-J**) Zein films before and after immersion; **(K-L)** DDGS kafirin films before and after immersion; S- Starch granule, P- Pore.

4.3.3 Simulated three phase digestion of the heat treated and non-heat treated kafirin and zein films

Table 4.16 shows that there was no significant difference ($p>0.05$) in the amount of heat treated and non-heat treated kafirin and zein films that were used for *in vitro* protein digestion. Variations were observed in the thickness of the films used. Similar observations have also been reported (Da Silva, 2003; Taylor et al., 2009). The authors attributed their observations to differences in the purity of the different protein preparations used to cast films. Since different prolamin preparations with different preparation purity were used to cast films in this study, this might have led to the difference in thickness of the resultant films. The heat treated kafirin and zein films used for digestion had a higher dry matter than the non-heat treated kafirin and zein films. This could be due to the effect of heating which removed some of the water from the heat treated films.

The film digestion data in Table 4.17 shows that there was no significant difference ($p\geq0.05$) in the weight loss of both heat treated and non-heat treated kafirin and zein films after the pre-digestion soak. The *in vitro* pepsin and total film digestion were less for kafirin films than zein films. However, higher digestion values were found for Sigma zein and Zein Gold films. There were little variations between the *in vitro* pancreatin digestion of heat treated and non-heat treated kafirin and zein films from the same prolamin preparations (Table 4.17). For example, the *in vitro* pancreatin digestion of heat treated and non-heat treated kafirin films was 21% and 22%, respectively, while that of Sigma zein and Zein Gold films was between 12-14%. This was in contrast to *in vitro* pepsin and total film digestion which had reduced digestibility for heat treated kafirin and zein films.

The hydrophobic nature of kafirin and zein (Duodu et al., 2003) and lack of enzymes in the pre-digestion soak solution could be responsible for the higher amounts of kafirin and zein films that were retained after the pre-digestion soak.

The lower *in vitro* pepsin and total film digestibility of kafirin films than zein films may be due to the greater hydrophobicity of kafirin than zein (Hamaker and Bugusu, 2003). This phenomenon makes the kafirin structure less accessible to pepsin digestion (Taylor et al., 2007) and consequently rendering it less digestible than zein (Duodu et al., 2003).

Heating of the films resulted in reduced *in vitro* pepsin and total film digestion probably due to heat induced disulphide bonding. A reduction in *in vitro* pepsin digestion of cast kafirin films as a result of heat treatment has been reported (Byaruhanga et al., 2005). As pointed out, heating of kafirin films (Byaruhanga et al., 2007) and zein films (Guo et al., 2012) results in modification in film functional properties due to intermolecular disulphide cross-linking induced by heat. It can therefore be assumed that the reduced digestion of the heat treated films was caused by enhanced film cross-linking density that slowed the penetration of digestive enzymes into the film networks.

The little variations observed with the *in vitro* pancreatin digestion between heat treated and non-heat treated films could be attributed to the ability of pancreatin enzymes in cleaving polymeric units linked by intermolecular disulphide bonds (Sharma et al., 2015). This would result in reduction of the polymers into smaller molecules which would be more accessible to proteolytic enzymes (Kuo et al., 2011). Kaur et al. (2010) observed further digestion of the undigested proteins and the peptides formed during gastric digestion when the same samples were digested with pancreatin under simulated small intestinal conditions.

Figure 4.8 shows the appearance of kafirin and zein films after each stage of digestion. It was observed that all the zein films aggregated and formed a dough like structure during the digestion period (starting from pre-digestion soak to pancreatin digestion) when they were incubated at 39^{o}C at different times. This agrees with earlier research findings which have shown that zein in excess of water when heated above its glass transition temperature (T_g) (~29 °C) changes from a glassy state to a viscoelastic rubbery state (Oom et al., 2008; Schober et al., 2011). However, the kafirin films remained intact but they became wrinkled. Zein Gold films became dull yellow in colour while Sigma zein films had an orange-yellow colour. Similar colour observations have been reported for digested Zein Gold and Sigma zein coatings (Pretorius, 2008). The colours were probably as a result of the carotenoid, xanthophylls and other pigments present in the zein (Shukla and Cheryan, 2001).

The relationship between film total digestibility and film water uptake for the kafirin and zein bioplastic films is shown in Figure 4.9. A positive linear relationship was found between film water uptake and film digestibility. As film water uptake increased, film digestibility increased as well. This in essence indicates the usefulness of film water uptake, as a simple

predictor of digestibility of kafirin and zein films when used as encapsulation agents and coatings.

Table 4.16: Weight, thickness and dry matter of kafirin, zein, Sigma zein and Zein Gold films used for three phase simulated digestion

Treatments	Film weight (mg) (db)		Film thickness (µm)		Dry matter of the films (%)	
	Heat treated	Non-heat treated	Heat treated	Non-heat treated	Heat treated	Non-heat treated
Kafirin films cast from kafirin preparations extracted from washed coarse sorghum meal using percolation method	$250.1^{a}\pm0.2$[1]	$250.3^{a}\pm0.2$	$88.2^{c}\pm0.7$	$87.5^{c}\pm1.2$	$90.6^{ab}\pm0.6$	$88.5^{a}\pm1.3$
Kafirin films cast from kafirin preparations extracted from washed coarse DDGS meal using percolation method	$250.2^{a}\pm0.1$	$250.1^{a}\pm0.1$	$85.6^{bc}\pm1.6$	$78.5^{a}\pm0.7$	$92.4^{ab}\pm0.4$	$88.6^{a}\pm1.7$
Zein films cast from zein preparations extracted from washed coarse maize meal using percolation method	$250.2^{a}\pm0.2$	$250.3^{a}\pm0.1$	$78.6^{a}\pm0.6$	$80.3^{ab}\pm1.0$	$93.1^{b}\pm0.2$	$90.0^{ab}\pm0.1$
Sigma zein films	$249.8^{a}\pm0.4$	$250.0^{a}\pm0.4$	$61.8^{a}\pm0.7$	$63.1^{a}\pm0.8$	$92.8^{b}\pm0.2$	$88.6^{a}\pm0.4$
Zein Gold films	$249.9^{a}\pm0.5$	$249.8^{a}\pm0.4$	$62.9^{a}\pm1.4$	$62.2^{a}\pm0.5$	$91.3^{b}\pm0.4$	$87.5^{a}\pm0.3$

db=dry basis
[1]Means ± standard deviations, n=4 for film weight and film thickness and n=2 for film dry matter
Mean values on the same column but with different superscripts are significantly different ($p<0.05$)

Table 4.17: Effects of prolamin preparations and of heat treatment on the *in vitro* digestibility of kafirin and zein films

Treatments	Pre-digestion soak (%)		Pepsin digestion (%)		Pancreatin digestion (%)		Total digestion (%)	
	Heat treated	Non-heat treated	Heat treated	Non-heat treated	Heat treated	Non-heat treated	Heat treated	Non-heat treated
Kafirin films cast from kafirin extracted from washed coarse mabele meal using percolation method	$1.6^{Aa}\pm0.8$[1]	$1.9^{Aa}\pm1.3$	$28.3^{Ab}\pm2.3$	$36.2^{Bc}\pm2.2$	$21.1^{Ac}\pm3.4$	$21.9^{Ac}\pm1.7$	$51.0^{Ab}\pm3.4$	$60.0^{Bc}\pm1.7$
Kafirin films cast from kafirin extracted from washed coarse DDGS meal using percolation method	$1.1^{Aa}\pm0.5$	$1.3^{Aa}\pm0.8$	$20.5^{Aa}\pm1.8$	$39.3^{Bc}\pm3.6$	$17.5^{ABb}\pm3.4$	$14.8^{Aab}\pm1.5$	$39.1^{Aa}\pm3.4$	$55.4^{Bb}\pm1.5$
Zein films cast from zein extracted from washed coarse maize meal using percolation method	$1.6^{Aa}\pm0.8$	$1.9^{Aa}\pm1.1$	$38.2^{Ac}\pm1.9$	$59.1^{Bd}\pm3.9$	$21.5^{Bc}\pm3.2$	$12.2^{Aa}\pm1.7$	$61.3^{Ac}\pm3.2$	$73.2^{Bd}\pm1.7$
Zein Gold films	$1.0^{Aa}\pm0.3$	$1.8^{Aa}\pm0.4$	$74.1^{Aef}\pm0.3$	$75.6^{Ag}\pm0.5$	$13.0^{Aa}\pm2.1$	$12.1^{Aa}\pm0.5$	$88.1^{Aef}\pm2.1$	$89.5^{ABf}\pm0.5$
Sigma zein films	$1.6^{Aa}\pm0.1$	$1.8^{Aa}\pm0.3$	$72.1^{Ae}\pm1.2$	$74.9^{ABg}\pm0.9$	$12.0^{Aa}\pm1.1$	$14.3^{Aab}\pm1.1$	$85.7^{Ae}\pm1.1$	$91.0^{Bf}\pm1.1$

[1]Means ± Standard deviations, n=6
Mean values in the same column (for each digestion phase) with different lower case superscript letters differ significantly ($p< 0.05$)
Mean values in the same row (for each digestion phase) with upper case superscript letters differ significantly ($p< 0.05$)

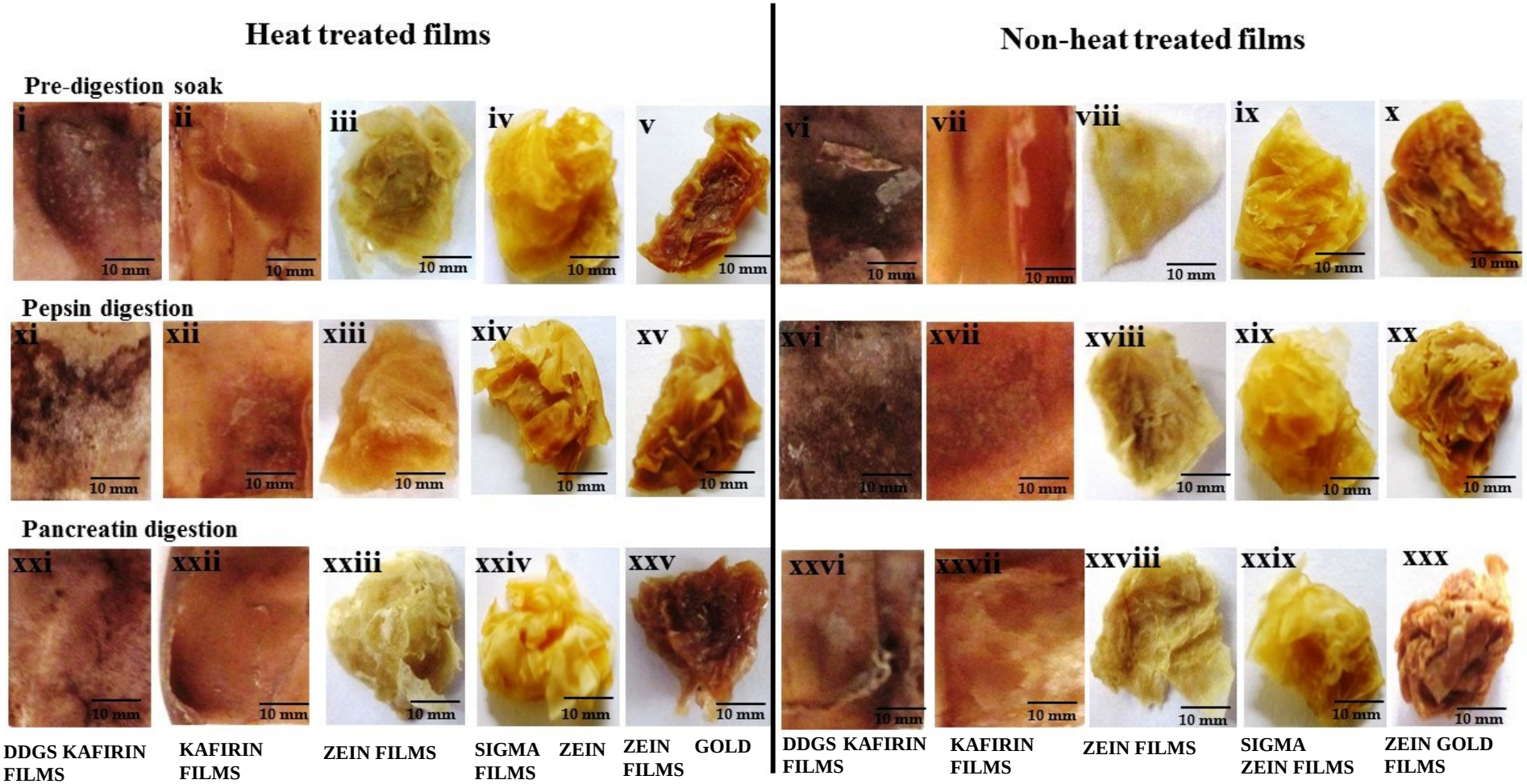

Figure 4.8: Effects of prolamin preparations and three phase digestion (pre-digestion soak, pepsin and pancreatin) on the appearance of kafirin and zein films

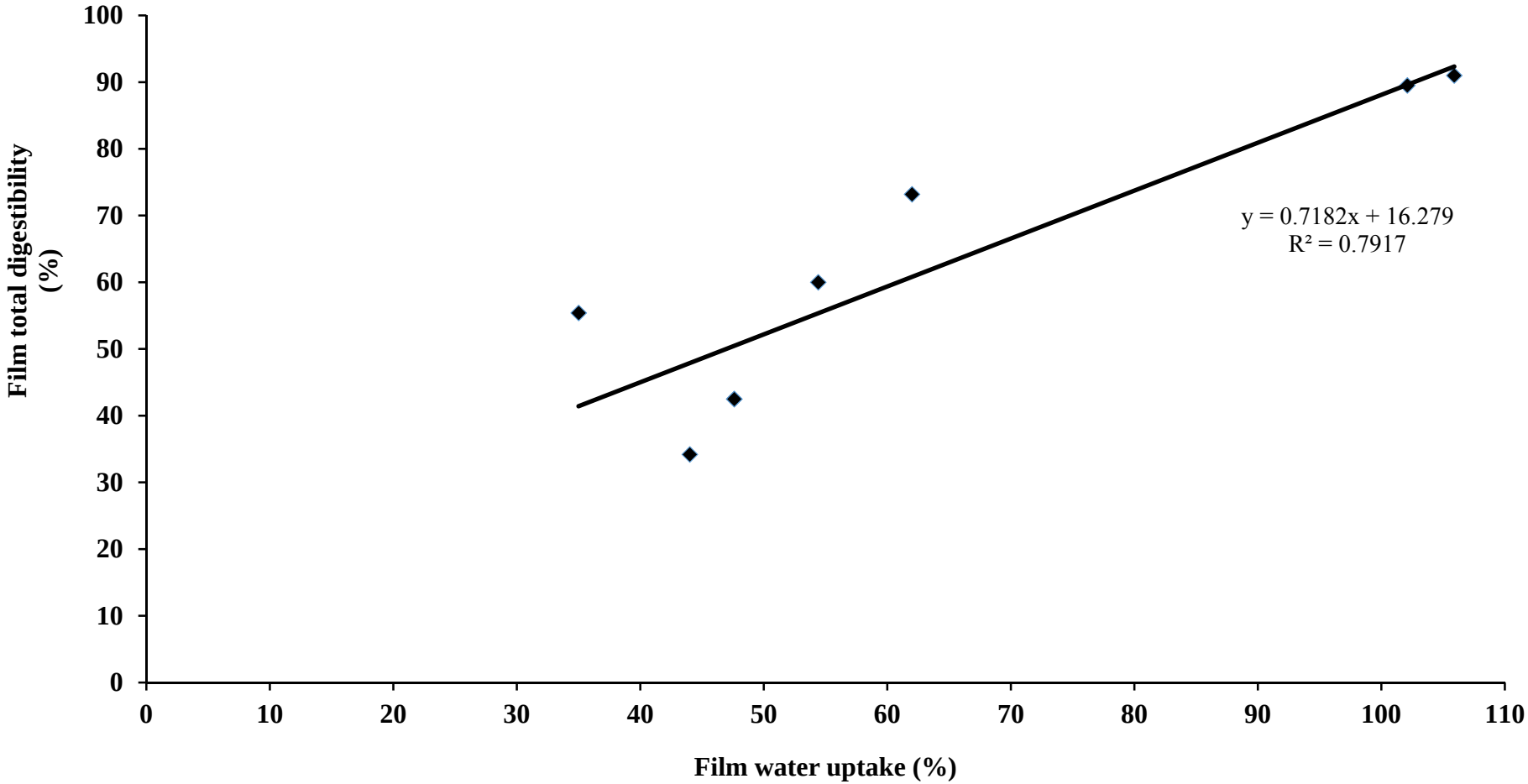

Figure 4.9: Correlation between film total digestibility and film water uptake for kafirin and zein films (p=0.05, r=0.8898, df=7)

4.4 Conclusions

A modified type of percolation extraction method shows to be an efficient procedure for kafirin and zein isolation using either sodium hydroxide or glacial acetic acid in the aqueous ethanol extractant under reducing conditions. Washing of decorticated sorghum and maize meals to remove starch prior to kafirin and zein extraction appears to have great effect in increasing kafirin and zein preparation purity. In addition, the extracted kafirin and zein preparations have shown to possess better film making qualities even without the addition of a plasticiser. Heat treatment of the kafirin and zein films improves water barrier properties and reduces film digestibility probably due to heat induced disulphide bonding. Film water uptake is a good indicator of kafirin and zein film digestibility.

5 GENERAL DISCUSSION

This discussion will first provide a critical review of the percolation method investigated for kafirin and zein extraction. Subsequently, it will also examine the raw materials used for extraction in the study. The implications of the main findings of this research, which include: rapid extract filtration time, increased kafirin and zein preparation yield due to inclusion of glacial acetic acid and NaOH to the aqueous ethanol extractant, improved protein preparation purity as a result of washing the decorticated grains prior to extraction, increased film water uptake as a result of defatting the prolamin preparations, and reduced film water uptake and film digestibility as a result of post heat treatment of the films will be discussed. A possible mechanism responsible for cross-linking of the kafirin and zein proteins and how they relate to film water uptake and film digestibility will also be discussed.

5.1 Critical review of the experimental work

A modified type of percolation method was investigated for its efficiency in extracting kafirin and zein prolamin proteins. The technique used was not true percolation as the meals/flours was immersed in the extraction solvent before letting it drain out through the meals/flours. In this current work, solvent circulation could not be performed on a laboratory scale as no suitable spark proof pump was available. Nonetheless, the equipment allowed the filtration of the solvent through the meals and fine sorghum flours. The sorghum and maize meals and sorghum DDGS meal were retained by the 250 µm opening mesh screen within the percolation vessel. However, some of the fine material in the sorghum flours was not retained by the screen. The percolation extraction technique enabled a rapid extract filtration time for the sorghum and maize meals and sorghum DDGS meal. This could reduce energy costs since it eliminated centrifugation of the extractant. The method also enabled the use of less extractant, 250 g of aqueous ethanol per 100 g meal as opposed to 2500 g of extractant used in a prolamin extraction from flour by conventional method (Emmambux and Taylor, 2003). This could result in further cost reduction. The use of less aqueous ethanol, which has a flash point of approximately 12^{o}C (Merck, 2012), reduced the likelihood of the formation of electrical sparks and so could be considered a safer process. Heating the aqueous ethanol at a temperature of 70^{o}C using an insulated water circulated jacket equipment prevented the formation of vapour which could have triggered ignition of the flammable vapour in the presence of an ignition source.

The relative protein contents of the raw materials used for extraction were: 10% for sorghum meal and fine sorghum flour, 7.5% for maize meal and 28.2% for sorghum DDGS meal (dry basis). Sorghum DDGS meal could be a viable material for kafirin extraction by the modified percolation method due to its much higher protein content. Using whole grain coarse ground maize and sorghum meal as the raw materials for kafirin and zein isolation could be advantageous as these materials offer reduced milling costs than more refined materials. In addition, raw materials which have been decorticated may result in loss of much of the outer endosperm material which is rich in prolamin proteins. In contrast, whole coarse sorghum and maize flour contain the whole endosperm of the grain. However, whole grain has a much higher fat content. This would then lead to increased production costs as the extracted prolamin preparations would have to be defatted with hexane. In this current work, defatting of the prolamin preparations extracted from coarse sorghum and maize meals and sorghum DDGS meal was carried out to determine its effect on water uptake of the cast kafirin and zein films.

The use of coarse sorghum and maize meals in this study enabled pre-washing of the materials to remove starch prior to extraction, a process which considerably increased kafirin and zein preparation purity. It was not possible to wash the fine sorghum flour using running tap water as no separation of starch and non-starch material would take place.

Table 5.1: Summary of effects of sorghum and maize meals, sorghum DDGS meal and sorghum flour particle size, washing of the meals and inclusion of glacial acetic acid and NaOH in aqueous ethanol as extractants on extract filtration time, kafirin and zein preparation purity and kafirin and zein preparation yield.

Raw materials	Particle size of materials used	Effect of particle size on extract filtration time	Effect of washing the meals prior to kafirin and zein extraction on kafirin and zein preparation purity	Effects of inclusion of glacial acetic acid and NaOH in aqueous ethanol as extractants on kafirin and zein preparation yield
Decorticated coarse sorghum meal	>1000 µm	Rapid extract filtration time due to larger inter-particle spaces	Large increase in kafirin preparation purity due to removal of starch	Substantial increase in yield due to improvement in kafirin solubility by NaOH and glacial acetic acid
Decorticated coarse (Super grade) maize meal	>1000 µm	Rapid extract filtration time due to larger inter-particle spaces	Large increase in zein preparation purity due to removal of starch	Substantial increase in yield due to improvement in zein solubility by NaOH and glacial acetic acid
Sorghum distillers dried grains with solubles	>1000 µm	Rapid extract filtration time due to larger inter-particle spaces	Small increase in sorghum DDGS kafirin preparation purity due to much less residual starch being present	Data only available for inclusion of glacial acetic acid in the aqueous ethanol extractant. Resulted in a greater increase in the amount of kafirin preparation extracted and increase in yield due to increased kafirin solubility
Fine sorghum flour	<250 µm	Slow extract filtration time due to smaller inter-particle spaces	Lowest kafirin preparation purity due to co-extraction of starch. Washing was not performed as no separation of starch and non-starch material would take place	Lower kafirin preparation yield due to extraction of starch which reduced the protein content, which was also used to calculate the yield

Table 5.2: Summary of effects of washing the meals prior to kafirin and zein extraction, defatting of prolamin preparations, agitation of the films and heat treatment of the films on film water uptake and *in vitro* film digestibility

Prolamin preparations	**Effects of washing the meals prior to kafirin and zein extraction and post heat treatment of the cast films on film water uptake**	**Effect of defatting of prolamin preparations on film water uptake**	**Effects of prolamin preparations and agitation of the cast films on film water uptake**	**Effect of prolamin preparations on *in vitro* digestibility of the films**	**Effect of heating of the films on *in vitro* film digestibility**
Kafirin from sorghum meal	Washing of the meals and heat treatment of the films greatly reduced kafirin film water uptake due to starch removal and heat induced disulphide cross-links	Defatting increased kafirin film water uptake due to the presence of many film surface pores	Agitation of the films increased kafirin film water uptake due to more water penetrating quickly into the films	Kafirin films were less digestible than zein films	Heating of the kafirin films resulted in a large reduction in film digestion due to disulphide cross-links
Zein from maize meal	Washing of the meals and heat treatment of the films greatly reduced zein film water uptake due to starch removal and heat induced disulphide cross-links	Defatting increased zein film water uptake due to the presence of many film surface pores	Agitation of the films increased zein film water uptake due to more water penetrating quickly into the films	Zein films had a high digestion than kafirin and DDGS kafirin films	Heating of the zein films resulted in a large reduction in film digestion due to disulphide cross-links
Kafirin from sorghum DDGS meal	Washing of the meals and heat treatment of the films slightly increased DDGS kafirin film water uptake due to the kafirin being already cross-linked	Defatting increased DDGS kafirin film water uptake due to the presence of many film surface pores	Agitation with DDGS kafirin films was not performed	DDGS kafirin films were much less digestible than kafirin and zein films	Heating of the DDGS kafirin films resulted in a larger reduction in film digestion due to disulphide cross-links
Sigma zein	Not performed with Sigma zein films	Defatting not performed	Agitation of the films greatly increased film water uptake due to a much higher amount of water penetrating into the films	Sigma zein films had a much higher digestion than kafirin, DDGS kafirin and zein films	Heating of the Sigma zein films resulted in a smaller reduction in film digestion due to very little disulphide cross-links
Zein Gold	Not performed with Zein Gold films	Defatting not performed	Agitation of the films greatly increased film water uptake due to a much higher amount of water penetrating into the films	Zein Gold films had a much higher digestion than kafirin, DDGS kafirin and zein films	Heating of the Zein Gold films resulted in a smaller reduction in film digestion due to very little disulphide cross-links

5.2 Discussion of experimental findings

As shown in Tables 4.2 and 5.1, all the meals used for extraction had a particle size larger than 1000 µm, while the fine sorghum flour had a particle size of less than 250 µm. As discussed, coarse sorghum and maize meals and sorghum DDGS meal, either washed or non-washed, had a much more rapid extract filtration time than fine sorghum flour. This can be attributed to the differences in inter-particle spaces between the meals and fine sorghum flour. The fact that fine sorghum flour produced a kafirin preparation with lower purity shows that more starch was co-extracted with the prolamin preparation. The kafirin preparation purity from fine sorghum flour of this study agrees with values in the literature (Wu, 1978; El Nour et al., 1998). Washing of the sorghum and maize meals to remove starch prior to kafirin and zein extraction increased kafirin preparation purity by 12.4% and zein preparation purity by 8.2%. However, washing of sorghum DDGS meal led to a smaller increase in kafirin preparation purity (1.2%), probably due to much less residual starch being present. In addition, removal of the fat rich germ (Gwirtz and Garcia-Casal, 2014) in the sorghum and maize meals during the decortication process, might have contributed to an increase in kafirin and zein preparation purity due to reduced co-extraction of fat with the prolamin preparations.

Furthermore, the inclusion of either glacial acetic acid or sodium hydroxide in the aqueous ethanol extractant led to a substantial increase in kafirin and zein yield. According to Cooper (1999) glacial acetic acid and sodium hydroxide bring a change in the protein conformational structure, unfolding the protein, exposing the hydrophobic regions of the protein to solvation, disrupting hydrophobic interactions, breaking hydrogen bonds and increasing the amount of electrostatic repulsions. The change in protein conformational structure, makes kafirin (Gao et al., 2005) and zein (Cabra et al., 2007; Nonthanum, 2013) more soluble in the alkaline/acidic aqueous ethanol extractant. It was also found that inclusion of sodium hydroxide in the aqueous ethanol extractant resulted in a slightly greater prolamin preparation purity than inclusion of glacial acetic acid (Table 4.5). This agrees with literature which has shown the effectiveness of an alkaline-ethanol extractant than acidic-ethanol extractant (Cayot et al., 2002). Contrastingly, other researchers have reported of a decrease in zein preparation purity when sodium hydroxide was used as part of the extraction solvent (Landry and Moureaux, 1980; Coleman and Larkins, 1999). According to these authors, this was due to co-extraction

of γ-zein, lipoproteins and non-protein nitrogen by the alkaline conditions. However, these authors did not use glacial acetic acid as part of the extraction solvent in their investigations.

It was also found that coarse decorticated sorghum meal gave the same protein yield as fine sorghum flour (Table 4.3). Agitation of the extractant and the coarse materials and use of a high extraction solvent temperature, as reported by Dickey et al. (1998) could be responsible for this. According to the authors, these factors enable diffusion of the extraction solvent into the endosperm cells and through the starch granules and into the prolamin protein bodies.

As stated in Chapter 4.3.2 and Table 5.2, washing of the meals prior to kafirin and zein extraction and heating of the cast films both led to reduced film swelling and film water uptake after the cast films were incubated in pH 6.8 phosphate buffer for 12 hours. Schmidt et al. (2012) reported that the degree of swelling of the polymeric networks depend on the interactions between the fluid and the polymer and on the structure and properties of both the solvent and the polymer. According to these authors, a higher degree of cross-linking results in a decrease in the polymer's ability to swell because as the fluid dissolves the network, the elastic forces of the polymer counteract the swelling. In this study therefore, the lower degree of swelling and the low water uptake with heat treated kafirin and zein films prepared from kafirin and zein extracted from washed meals can be attributed to removal of starch as a result of washing the meals prior to kafirin and zein extraction, resulting in less starch granules present to take up water during the gelatinisation process. Heat treatment of the films may have contributed to the reduced degree of film swelling and film water uptake due to the formation of disulphide cross-links (Byaruhanga et al., 2007). The fact that non-heat treated DDGS kafirin films showed less swelling and the lowest water uptake indicates that the protein was already cross-linked due to DDGS production conditions such as heating and fermentation (Cromwell et al., 1993). This was supported by SDS-PAGE under non-reducing conditions which showed presence of high molecular weight polymers (Figure 4.1).

Defatting of the prolamin preparations (Chapter 4.3.2.1), resulted in an increase in film water uptake. Lai et al. (1997) reported that fats contribute to the sealing off of the film surface pores. According to these authors, this enables the bioplastic films to maintain their structure in aqueous environments. Consequently, a combination of proteins with fatty acids as lipid film components, as reported by Taylor (2003), can make the film structure more compact and

more hydrophobic. This increases water resistance of the films (Lai and Padua, 1998; Da Silva, 2003).

As discussed in Chapter 4.3.2.2, agitating the films during the incubation period resulted in more water being absorbed. The water uptake of agitated Sigma zein and Zein Gold films was much higher than with total kafirin and total zein films (Fig. 4.3). As these preparations are essentially α-zein and so far less cross-linked than total kafirin and total zein films (Taylor et al., 2007), agitation might have allowed more water to penetrate into the films. However, the presence of all the three protein sub-classes (α-, β- and γ-) (Shewry and Halford, 2002) in the total kafirin and total zein preparations might have aided cross-linking by disulphide bonding involving the β- and γ- cysteine rich sub-classes. This could have been responsible for the lower water uptake of both agitated and non-agitated total kafirin and total zein films than Sigma zein and Zein Gold films. Additionally, the greater water uptake of the zein films than the kafirin films agrees with documented literature (Taylor et al., 2015) which has shown that kafirin is more hydrophobic and more cross-linked than zein.

As discussed (Chapter 4.3.3), the total kafirin films had lower *in vitro* pepsin and *in vitro* total digestion (IVTD) than the total zein films. The greater hydrophobicity of kafirin than zein (Duodu et al., 2003) could be responsible for this. Both heat treated and non-heat treated Sigma zein and Zein Gold films were highly digestible. This could be because these films were cast from protein preparations consisting of protease susceptible α-zeins which have very little disulphide cross-links (Taylor et al., 2007).

Furthermore, the *in vitro* pepsin and total digestibility of heat treated films was lower than that of non-heat treated films (Chapter 4.3.3). Since the digestibility of a protein is dependent on the accessibility of susceptible peptide bonds to specific proteolytic enzymes (Carbonaro et al., 1998), cross-linking of kafirin and zein could have prevented enzyme access to peptide bonds or could have masked sites of enzyme attack. Reduction in protein digestibility of kafirin films (Byaruhanga et al., 2007) and zein films (Matthews et al., 2011) has been observed after heating and has generally been attributed to the formation of disulphide linked protein polymers which are resistant to proteolysis leading to a reduction in digestibility (Byaruhanga et al., 2007; Guo et al., 2012; Becker and Yu, 2013).

Figure 5.1 shows possible mechanisms for cross-linking kafirin and zein (Duodu, 2000). Using this model, it is suggested that the application of heat to kafirin and zein films can break intra-chain hydrogen bonds that would otherwise stabilise the α-helical structure. Kafirin and zein polypeptides forming these helices may thus become unravelled and aligned next to each other to form the intermolecular β-sheet conformation. This would result in dityrosine cross-links (Gerrard, 2002) and disulphide cross-links (Byaruhanga et al., 2007), leading to formation of kafirin and zein polymers and a rigid compact film structure which would restrict water absorption and accessibility of the enzymes to the proteins, thus leading to a lower water uptake and a reduction in protein digestibility, as observed.

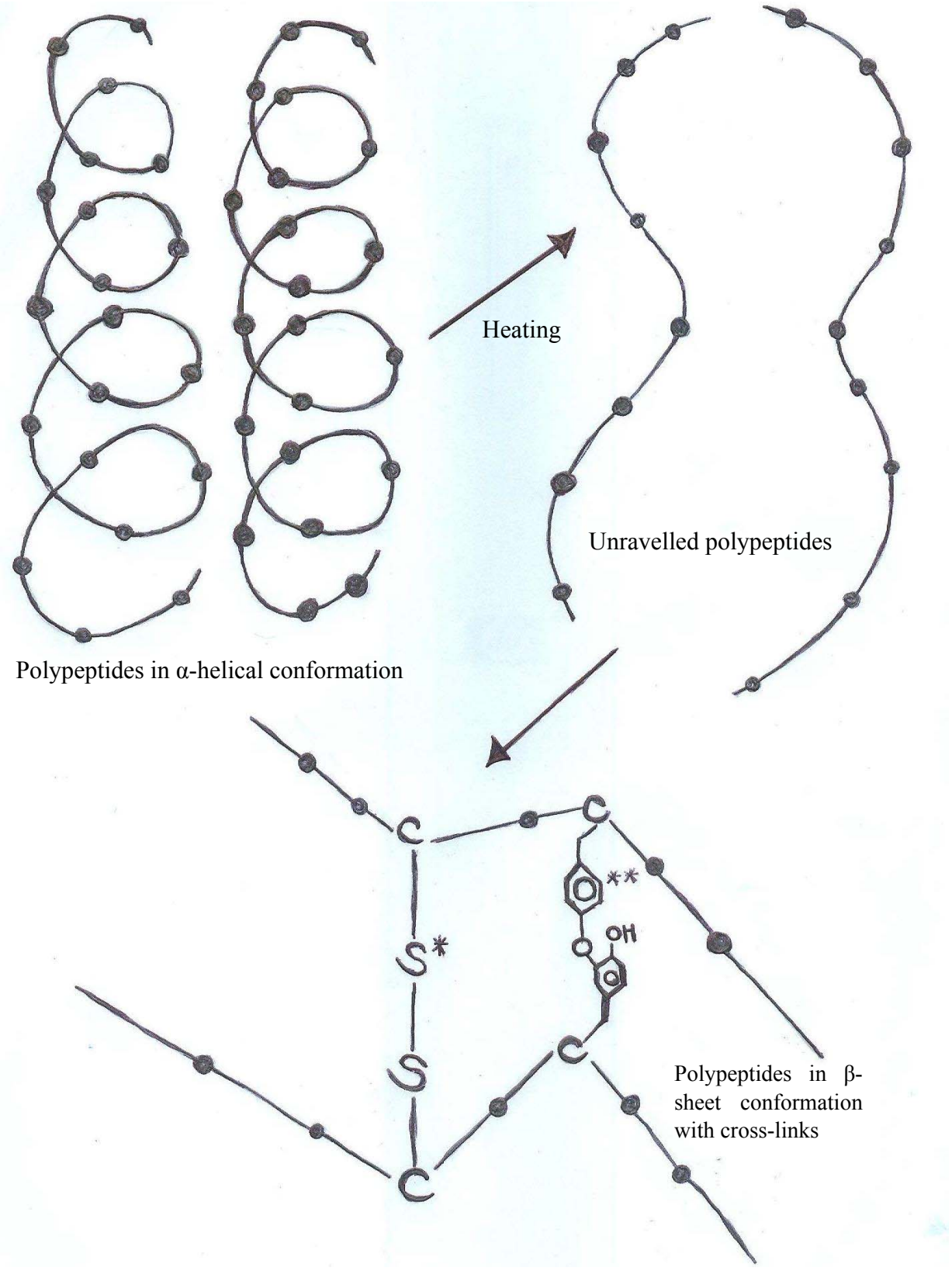

Figure 5.1: Representation of α-helix to β-sheet conformational change on heating followed by cross-link formation between polypeptides chains (adapted from Duodu, 2000).

—●—●—Amino acid residues, * disulphide cross-links, ** dityrosine cross-links

5.3 Way forward

This study has shown that the modified type of percolation method can be effectively used to extract kafirin and zein from different coarse particle raw materials using either sodium hydroxide or glacial acetic acid in the aqueous ethanol extractant. The extracted prolamin preparations were able to form bioplastic films when heated in aqueous ethanol to a temperature of 70^{o}C. Although kafirin and zein extraction was carried out using coarse sorghum and refined white maize meals, sorghum DDGS meal and fine sorghum flour, other raw materials such as brewers spent grains (BSG) and corn (maize) DDGS meal could also be used for the extraction process. These materials could be viable for kafirin and zein isolation due to their much higher protein content compared to sorghum and maize meals/flours. For example, brewers spent grains has a relative protein content of 20% (Mussatto et al., 2006) and corn DDGS meal has a relative protein content of 28-33% (Lim et al., 2011).

This present work has also created interesting avenues for further investigation. A promising route to investigate is the assessment of the extracted prolamin preparations for their potential in dough-based foods (Hamaker and Bugusu, 2003) and as coatings for food products (Krochta, 2002) or as an oral drug delivery system that can delay or target the release of medications to the lower gastro intestinal tract (GIT) (Philip and Philip, 2010).

6 CONCLUSIONS AND RECOMMENDATIONS

A percolation type of extraction method can be used to effectively isolate kafirin and zein proteins from coarse sorghum meal, coarse sorghum distillers dried grains with solubles (DDGS) and coarse maize meal. This method has several advantages over the conventional method whereby prolamins are extracted from flour. A smaller quantity of extraction solvent can be used. It also reduces costs through elimination of centrifugation of the extraction mixture to separate the prolamin solution from the flour.

Another important finding is that coarse decorticated materials can be used for kafirin and zein extraction and can give the same protein yield as fine flour. This is probably due to agitation of the extractant and the coarse materials and employing a high extraction solvent temperature (70^{o}C). These factors presumably enable diffusion of the extraction solvent into the endosperm cells and through the starch and glutelin protein matrix and into the prolamin protein bodies.

Furthermore, kafirin and zein preparations of high purity can be obtained by washing the coarse decorticated meals prior to kafirin and zein extraction. This is primarily due to the removal of starch which would otherwise pass through the mesh filter holding the coarse meals and contaminate the prolamin protein preparations. A smaller increase in prolamin preparation purity is obtained by washing the sorghum and probably the corn (maize) DDGS meals. This is undoubtably a result of less residual starch being present in DDGS. Thus, washing of DDGS meals prior to prolamin extraction using the percolation method is not necessary. However, the particle size of the material should be greater than 1000 µm to prevent it contaminating the extracted prolamin preparations.

An improvement in kafirin and zein solubility and yield can be achieved when glacial acetic acid is included in an aqueous ethanol extractant containing a reducing agent. Similar effects can also be achieved with the inclusion of sodium hydroxide. This is probably as a result of deamidation of the kafirin and zein asparagine and glutamine amino acid residues to form aspartic and glutamic acids. The resulting change in protein conformational structure would break hydrogen bonds between adjacent polypeptide chains and disrupts hydrophobic interactions.

Heat treatment of films produced from the kafirin and zein preparations reduces film water uptake and film digestibility. Hence, heat treatment can be used to improve the barrier properties of kafirin and zein films/coatings. Application of heat to the films promotes protein cross-linking through the formation of disulphide bonds, more so in the kafirin than the zein. Heat treatment did not reduce water uptake of DDGS kafirin films as the protein was already cross-linked as a result of thermal treatment during DDGS production. Heating Sigma zein and Zein Gold films showed little effect on film digestibility. This is probably due to these proteins being predominantly α-zein, and can only undergo limited disulphide cross-linking.

A positive linear relationship exists between film water uptake and film digestibility. This indicates the usefulness of film water uptake, as a simple predictor of digestibility of kafirin and zein films when used as encapsulation agents and coatings.

Going forward, research is needed into the application of the prolamin preparations isolated using the percolation extraction method in dough-based foods, as agents for encapsulation and as coatings.

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