Flour and dough properties of sorghum lines with modified endosperm protein and starch characteristics

By

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Submitted in the partial fulfilment of the requirements for the Degree

PhD Food Science

in the

Department of Food Science
Faculty of Natural and Agricultural Sciences
University of Pretoria
South Africa

June 2016

DECLARTION

I hereby declare that this thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other University or Institution of Higher Education.

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June 2016

ABSTRACT

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Supervisor: Prof J.R.N. Taylor
Co-supervisor: Prof M.N. EmmambuxSorghum is a critically important cereal food crop in Africa because of its drought-tolerance. However, sorghum does not produce good quality flour for dough-based products such as bread. This is on account of the fact that kafirin, its prolamin storage protein does not produce a viscoelastic dough and its starch has a high gelatinisation temperature

Recently, sorghum lines have been developed by genetic modification and by conventional mutation breeding that have altered synthesis of some kafirins, resulting in a high protein digestibility trait, here referred to as GM-HD and HD, respectively. Additionally, lines have been developed with both the conventionally bred HD trait plus the waxy (high amylopectin) trait (WHD).

The aim of this work was to determine the effects of these sorghum types on sorghum flour and dough quality.

The HD lines had altered protein body structure with loosely packed starch granules and a floury endosperm, irrespective of whether they were waxy or non-waxy. WHD lines had higher paste viscosity and formed much softer and less sticky pastes than the non-waxy, normal protein digestibility lines. Flours of the WHD lines also had much higher solubility than the non-waxy-normal protein digestibility lines. At 30^{o}C, the water solubility of WHD lines flour was similar to a commercial wheat bread flour.

At both 30°C and 60°C, GM-HD lines had a significantly higher water soluble fraction compared to their null controls (N). Peak viscosity and holding strength of the GM-HD lines were also significantly higher than the N sorghums. Further, pasting temperature and setback values of GM-

HD were significantly lower than N. Rheological analysis revealed that G′ (storage modulus) and G″ (loss modulus) of GM-HD doughs were higher than N sorghum dough during amplitude sweep and temperature sweep analysis.

SDS-PAGE of the GM-HD sorghums showed that γ-kafirin was missing, unlike their null controls. SDS-PAGE also revealed that the HD had a missing β-kafirin band. 2-D PAGE showed that there were spots of MW about 27 kDa (the apparent molecular weight of γ-kafirin) missing in the GM-HD.

For the first time, viscoelastic doughs were formed from kafirin. This was achieved by first dissolving in glacial acetic acid and then precipitating the dough by rapid cold water addition. These doughs formed fibrils, a characteristic believed to be essential to viscoelasticity. The fibrils in kafirins doughs from GM-HD and HD were more, thinner, and more compact and had a regular (non-ruptured) arrangement compared to their normal sorghums. FTIR did not show differences among the different kafirins in terms of the ratio of α-helical conformation to β-sheet conformation in secondary structure of kafirin (α/β ratio). However, the relative proportion of α-helical conformation increased in kafirin doughs prepared with glacial acetic acid compared to kafirin doughs prepared with dilute acetic acid, i.e. after addition of water.

Suppression of β-kafirin synthesis appeared to be the cause of the floury endosperm trait in the conventionally bred HD lines, which was associated with a less compact protein matrix. Reduction in γ-kafirin in the GM-HD lines seemed to increase sorghum dough elasticity through improving protein-starch interaction. Viscoelastic doughs can be formed from kafirin but only if the kafirin is first dissolved in organic solvent. Although both the combined HDW and GM-HD lines improved the sorghum flour and dough properties, due to the waxy trait the HDW have more potential for improving sorghum end-use quality for making dough-based food products.

DEDICATION

This thesis is dedicated to:

My lovely parents Salaheldin and Sayda,

My lovely siblings Marwa, Israa and Muaaz,

And my sweet niece Amasi

For their love, patience and infinite support

ACKNOWLEDGEMENTS

I would like whole-heartedly to express my sincere gratitude to my supervisor (MY TEACHER), Prof John R. N. Taylor, for the opportunity to work with him and supervise me until I complete my PhD study. Also, for his patience, guidance and endless help at formal and informal levels.

I am also grateful to my co-supervisor, Prof Emmambux M. N. Apart from his supervisory role, thanks to him for the opportunity to learn the advanced rheological aspect of my work and for his nurturing sprit.

I also thank Dr Janet Taylor, for her kind hearted and unlimited help with electrophoresis, microscopy and others. This is really appreciated.

Thanks for University of Pretoria and Howard G. Buffett Foundation through the Norman E. Borlaug Institute for International Agriculture of Texas A & M AgriLife Research for the financial support.

Special thanks from my heart to Prof Lise Korsten at University of Pretoria for her assistance until I formally enrolled as a PhD student at University of Pretoria, it is really appreciated.

I am very grateful to my cousins Amira and Bashir in Pretoria for their encouragement and uncountable favours that they provided me until I got my PhD.

I want to deliver special thanks to my friends Asaad Khairallah and Husam Ali for their great help and valuable advices

I am grateful to the academic staff, support staff and fellow postgraduate students at the Department of Food Science, University of Pretoria, your assistance is unforgettable.

I would like to deliver my thanks to Mrs Rianie van der Linde - Librarian at UP - for her contentious encouragement.

I wish to thank Mr Alan Hall and Mrs Antoinette Buys for their help with microscopy.

Above all, thank you Allah.

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1 INTRODUCTION

There is steady increase in the population of sub-Saharan Africa, where it is expected that the population of the sub-Saharan Africa will reach about 2 billion by 2050 (UN, 2011) compared to 434 million in 1984 (Goliber, 1985). Due to climate change it is estimated that about 70 million people in Africa will be at risk of hunger (Parry et al. 1999). It has been stated that the biggest problem as a result of climate change in Africa is food security (Brown et al. 2007). Drought will be one of the biggest impacts of climate change and sorghum as one of the most drought tolerant crops will be the appropriate crop to be cultivated in Africa (Hattori et al., 2005).

Because the climate in Africa is not generally suitable for wheat cultivation, wheat and wheat flour are imported to Africa in a high cost mainly for bread making. Tadesse and Straziuso (2012) estimated that in 2012 African countries were spending US $12 billion to import 40 million tons of wheat. Thus, utilization of sorghum as a stable food instead of wheat will mitigate the negative effect of wheat importation on the African economy.

It has also been apparent that coeliac disease is much more widespread than previously thought (Fasano and Catassi, 2001). The basic treatment for coeliac disease is the total lifetime avoidance of gluten intake (Kasarda, 2001). Sorghum has been shown not to elicit adverse reactions (Ciacci et al., 2007) and it is often recommended as a suitable food for coeliac patients.

However, there are reasons that retard using sorghum flour especially for bread making. A major cause of the inferior bread-making properties of sorghum is that kafirin, the sorghum prolamin protein, does not exhibit the viscoelastic dough-holding properties of wheat gluten in normal dough systems (Oom et al., 2008). Although some wheat-free breads have been produced commercially using sorghum, their loaf volume were low and they had a compact crumb structure (Arendt et al., 2002). Furthermore, the starch granules in the corneous endosperm of sorghum are surrounded by hydrophobic matrix proteins (Munck, 1995). These hydrophobic matrix proteins can reduce the extent of water absorption and water solubility of sorghum starch. In turn, this may lead to inadequate functionality of sorghum flour because water absorption leads to swelling of starch granules which is related to dough and pasting properties.

Novel combined waxy and high protein digestibility sorghum lines have been developed using conventional breeding by Texas A & M University (Jampala et al., 2012). Also genetically modified sorghums with reduced γ-kafirin and high protein digestibility were developed by the Africa Biofortified Sorghum (ABS) project (Biosorghum (2010). Both the conventionally bred and genetically modified sorghums are hypothesized to induce improvement in the sorghum flour and dough quality. Further, developing a method to form dough from kafirin by using glacial acetic acid which was initiated in form of kafirin microparticles formation by Taylor (2008). In this current study, although this method is targeting the kafirin and not the whole sorghum flour but is assumed that success of forming kafirin dough would indicate the possibility of formation of sorghum dough. This assumption is because gluten protein plays a critical role in dough formation mainly in the viscoelasticity of the dough which is required for bread making (Belton, 1999).

2 LITERATURE REVIEW

2.1 INTRODUCTION

This literature review largely deals with the biochemistry, functional quality and nutritional quality of the storage proteins and starch of conventionally bred and genetically modified sorghums. Also, the chemical composition and functional properties of other major cereals such as wheat and maize will be reviewed. The main goal of this study is to improve the quality of sorghum flour as an alternative source for bread making. As maize is known to have similar properties to sorghum flour especially in terms of protein, it will also be reviewed. The major components of sorghum i.e. starch and sorghum prolamin protein (kafirin) will be the focus because of their importance in flour functional properties. Since the research requires different advanced techniques, the review will discuss the principles and application of these techniques.

2.2 SORGHUM STARCH

The starch of most sorghum types have the normal ratio of amylose to amylopectin. There are, however, so-called waxy mutants which have a higher proportion of amylopectin. The ratio of amylose/amylopectin in sorghum starch differs depending on genetic background and environment (Beta and Corke, 2001). Sorghum starches have been classified as normal (non-waxy) and waxy based on amylose/amylopectin ratio. Normal sorghum starch has 20–35% amylose. Waxy sorghum starch essentially does not contain amylose (Sang et al., 2008), while the heterowaxy and normal sorghum starches contain about 14% and 23.7% amylose by weight, respectively. These authors found that the side chain lengths of amylopectin within the three types of the sorghum starch (waxy, heterowaxy and normal) do not have significant differences in terms of their distribution by area % of the amylopectin. Within the three types of sorghum starch, about 43-45% is of chain length 6 to 15 degree of polymerization (DP). The chain length of 16 to 36 DP exists in the range from 49-50%. The longest chain length of DP ≥ 37 is in the range from 5-6%.

2.2.1 Sorghum starch granules

Sorghum grain has both a corneous (hard) and a floury (soft) starchy endosperm (Figure 2.1), while wheat has single type of endosperm. Starch is present in plants in the form of granules composed of macro (amylose and amylopectin) and minor components (Liu, 2005). Sorghum starch granules

are generally similar to maize starch granules in terms of their shape and size. The size of sorghum starch granules is about 5 to 20 µm. Wheat has two different types of starch granules, the large, lenticular A-type granules and the small, spherical B-type granules (Zeng et al., 2011). Sorghum starch granules normally have spherical shape.

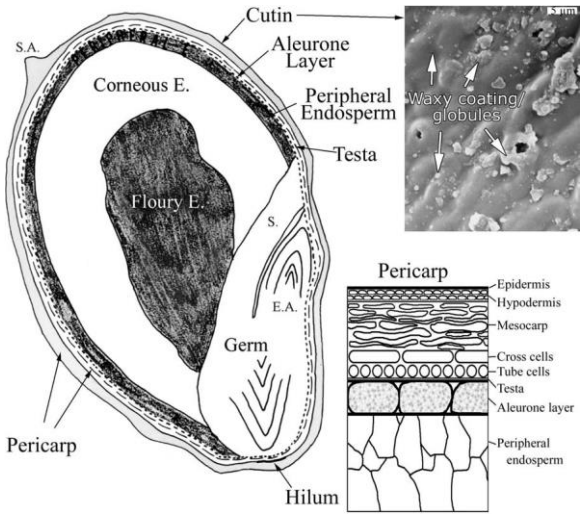

Figure 2.1 Sorghum grain structure, with details of the cuticle from the outside of the grain (Earp et al., 2004)

S.A. = Stylar area; E.A. = Embryonic Axis; S = Scutellum.

2.2.2 Sorghum starch gelatinization

The gelatinization temperature of sorghum starch is higher around (70°C) than wheat starch, which approximately (58°C) (Bewley et al., 2006). This can be attributed to the long side chains of amylopectin of sorghum starch compared to wheat starch (Chung et al., 2008). The approximate weight-average chain length (CLw) of sorghum amylopectin and wheat is 30.1 and 26.8, respectively. There is a positive relation between starch gelatinization temperature and amylopectin side chain length which forms double helixes (Matsuki et al., 2003). Long double helixes increase the stability of the starch and a high temperature is needed to dissociate and separate their hydrogen bonds (Zimm and Bragg, 1959). Sorghum and maize normally have similar

starch properties (Rooney and Waniska, 2000). However, some sorghum starches have higher gelatinization temperature compared to some maize starches (Subramanian et al., 1994). Some sorghum starches have lower onset gelatinization temperature (T_0) approximately 59.3°C than yellow maize starch around 63.0°C (Zainab et al., 2011). These differences in gelatinization temperature can be attributed to the same reasons that make sorghum starch gelatinization temperature higher than wheat (Campbell et al., 1996).

The protein matrix in the sorghum corneous endosperm encloses the starch granules (Taylor et al., 2006). This results in high pasting temperature due to the hydrophobic property of the protein which retards water absorption and reduces expansion of starch granules. Different sorghum starches have been investigated in terms of their pasting properties, and compared with a commercial maize starch (Beta et al., 2000). These authors found that the temperature at initial viscosity rise (T_i) of the sorghum starches is lower (average 69.4°C) than the T_i of maize starch, about 73.6°C. They attributed the differences in the initial swelling temperature (T_i) to the dissimilarity in kernel structure of the grains i.e. the sorghum cultivars had intermediate to floury endosperm. However, they assumed that the lower T_i of sorghum starch may be due to the partial pre-gelatinization of sorghum starch resulting from the alkali treatment during starch isolation.

2.3 SORGHUM PROTEINS

Cereal proteins can be classified into four groups based on extraction and solubility, prolamins e.g. (kafirins) (aqueous alcohol-soluble), albumins (water-soluble), globulins (salt-soluble), and glutelins (detergent + reducing agents + alkaline pH-soluble) (Shewry et al., 1995). Crosslinked kafirins (aqueous alcohol + reducing agent-soluble) may be counted as a separate protein group although they are identical to the kafirins.

2.3.1 Sorghum grain protein distribution

Kafirins are only present in the sorghum grain endosperm (Haikerwal and Mathieson, 1971). The sorghum pericarp is noted to contain some 3% of the sorghum grain protein (Rooney, 1996). Sorghum germ contains around 20% of sorghum protein (Wall and Blessin, 1970). Approximately. 32 to 34% of the germ's proteins are low molecular weight nitrogen and albumins and globulins, which are rich in essential amino acids. The endosperm contains a much lower proportion (1.5 to

2.5% of endosperm proteins) of low molecular weight nitrogen (LMWN) and albumins plus globulins compared to the other parts of grain such as pericarp and germ. The second largest protein group in the endosperm is the G3-glutelin which represents 13.6 to 17.3% of the endosperm proteins, which is poor in glutamine (11.2%) and rich in lysine (6.1%). It is assumed that the G3-glutelins form the glutelin matrix that surrounds the protein bodies in sorghum floury endosperm (Taylor et al., 1984) while kafirin represents around 70% of the endosperm's proteins (Taylor and Schüssler, 1986).

The proportions of protein in the corneous and floury endosperm are different. The amount of the protein in the corneous endosperm varies from 1.5 to 2 times higher than the floury endosperm (Watterson et al.,1993). Also, the percentage of kafirin in the corneous endosperm is higher (5.8-8.5) than the floury endosperm (2-2.4). However, the amount of albumin and globulins is higher in the floury endosperm and the amount of glutelin is similar in both types of endosperm. The major anatomical part in the sorghum grain containing the albumins and globulins is the germ (Taylor and Schüssler, 1986). The endosperm glutelins are high molecular weight proteins and they surround the protein bodies in the protein matrix in the endosperm (Taylor et al., 1984) and exist as glutelin polymers linked by disulphide bonds.

2.3.2 Non-kafirin proteins

The albumin, globulin and glutelin proteins represent the non-kafirin proteins in sorghum. The nutritional value of albumins and globulins in terms of lysine content is higher compared to kafirin. The albumins and globulins are the first fraction of sorghum proteins that can be extracted during the sequential Osborne fractionation (Daiber and Taylor 1982). Both albumin and globulin proteins represent around 23% of the total sorghum protein (Taylor and Belton, 2002). The glutelins are soluble in dilute alkali and can be classified as the residue that is left following the extraction of the albumins and globulins, and kafirin (Wilson, 1983). Glutelins are the second largest protein fractions in sorghum after the prolamins (Taylor and Schüssler, 1986). Glutelins represent 24-29% of the endosperm protein.

2.3.3 Kafirin

Kafirin is a prolamin protein. In sorghum, kafirin is located in endosperm protein bodies (Taylor et al., 1984). Kafirin cannot be extracted totally by using only an alcohol-water mixture. This is due to the disulphide bond cross-linking which bond the kafirins themselves and with other proteins. Because of this crosslinking there is need for a reducing agent to break these bonds to increase the efficiency of the kafirin extraction (El Nour et al., 1998). The sequential kafirin extraction in this regard leads to two fractions of kafirins. These two fractions are uncrosslinked kafirin (kafirin-1) which is extracted by aqueous alcohol and the crosslinked kafirin (kafirin-2) that requires inclusion of a reducing agent. The combination of these two fractions form the total extracted kafirin. It has been found that the kafirin-1 is rich in monomeric α-Kafirin with some oligomeric and polymeric components, while the kafirin-2 is rich in β-, and γ-kafirins (Watterson et al., 1993).

Kafirins are rich in glutamine, proline, alanine and leucine, but are essentially free of lysine (Taylor & Schüssler, 1986). Kafirin proteins are known to be the more hydrophobic compared to the other prolamin proteins such as zein and gliadin (Duodu et al., 2003). The hydrophobicity of the kafirin is due to its higher proportion of non-polar amino acid residues, where alanine and leucine form more than 30% of the kafirin proteins. Therefore, kafirins are most efficiently extracted using 60% aqueous tertiary butanol with reducing agent to cleave the disulphide bonds (Belton et al., 2006). This is due to the higher hydrophobicity of butanol compared to other aqueous alcohol solvents (ethanol and propanol) which are commonly used.

Table 2.1 Amino acid content (mole % of amino acid) of total sorghum kafirin and the main kafirin sub-classes

Amino acid	Total kafirin[1]	α-kafirin[2]	β-kafirin[2]	γ-kafirin[2]
Asparagine	4.8	6.0	3.3[a]	0
Aspartic acid	c	0.4	c	0
Threonine	2.8	4.0	4.6	4.7
Serine	4.7	6.0	4.6	5.2
Glutamine	20.0	24.6	17.19[b]	11.9
Glutamic acid	c	0.4	c	1.0
Proline	11.2	7.7	9.7	23.3
Glycine	2.7	1.6	6.8	8.8
Alanine	15.6	14.9	13.4	5.7
Cysteine	0.7	0.4	4.9	7.8
Valine	5.6	4.4	5.2	6.2
Methionine	1.7	0.8	5.7	1.0
Isoleucine	4.1	5.6	2.3	2.6
Leucine	15.4	15.3	12.0	8.3
Tyrosine	3.0	2.8	3.0	2.1
Phenylanine	4.7	2.4	1.9	1.6
Histidine	1.6	1.2	0.9	7.8
Lysine	0.2	0.0	0.5	0.0
Arginine	1.2	0.8	2.7	2.1
Tryptophan	c	0.4	c	0.0

[a] Asparagine + Aspartic acid expressed as Asparagine [b]

[b]Glutamine + Glutamic acid expressed as Glutamine [c] [c]Not available

[1]Evans et al. (1987)

[2]Taylor and Belton (2002)

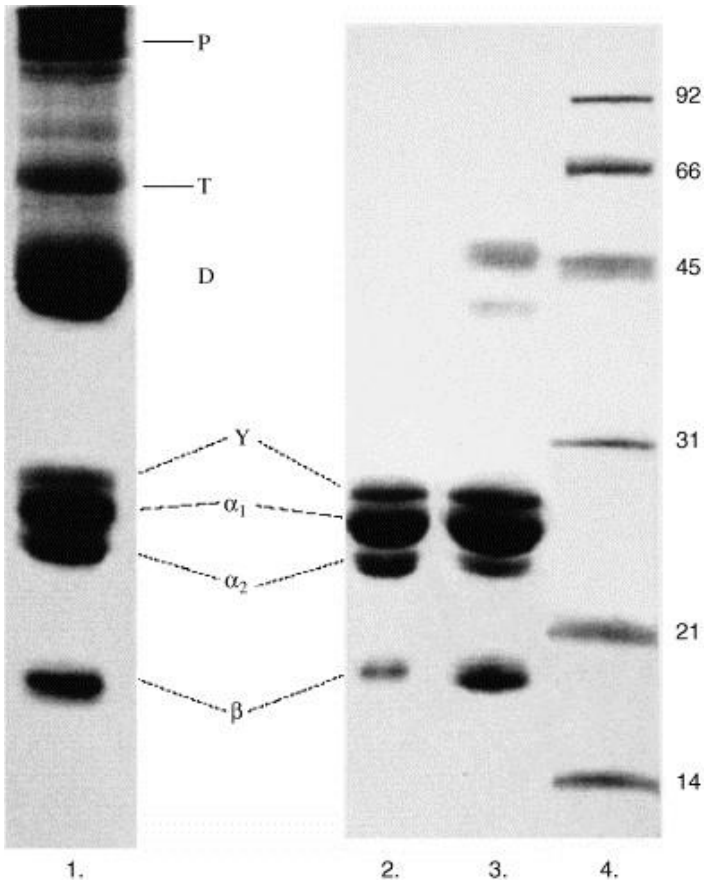

Figure 2.2 Total sorghum kafirin fractioned through SDS-PAGE to its sub-classes α-, β- and γ-kafirin

Lane 1, kafirin-1 under non-reducing conditions; lane 2, kafirin-2 under reducing condition; lane 3, kafirin-2 under reducing condition; lane 4, M_r standard proteins. P, T and D indicate polymers, trimers and dimers. α_1, α_2, β and γ indicate the kafirin sub-classes (El Nour et al., 1998).

2.3.3.1 *Kafirin physicochemical characteristics*

Kafirins not subjected to wet thermal treatment from both normal and mutant sorghums showed a 54–58% α-helical conformation content when they were analysed using FTIR spectroscopy (Duodu et al., 2001). A similar study on native kafirin displayed a 58% α-helical conformation content (Gao et al., 2005). Thus, it can be said that the native secondary structure of kafirin is rich in α-helical conformation. While, if the kafirin is subjected to an external factor such as heat, the secondary structure of kafirin may be become richer in β-sheet conformation as a result of

unfolding (Duodu et al., 2001). As the isoelectric point of kafirin is 6 (Anyango et al., 2012), a manipulation by changing the pH to below this point can result in a change in kafirin secondary structure. Then, due to this change in structure, the kafirin behaviour for dough formation may be changed as well.

2.3.3.2 *Sub-classes of kafirin*

Four kafirin sub-classes have been identified, α-, β-, γ- and δ-kafirins (Belton et al., 2006). The relative proportions of α-, β-, γ- and δ-kafirins vary depending on the endosperm type and are Approximately 66-80%, 7-13%, 9-21% and less than 1%, respectively (Laidlaw et al., 2010).

2.3.3.2.1 *Alpha-kafirin*

Alpha-kafirin is mainly located in the central part of the endosperm protein bodies (Shull et al., 1992). Alpha kafirin represents almost 80% of the total kafirin (Shewry, 2002). Two sub-classes of α-kafirin are identified through SDS-PAGE (Figure 2.2) of 25 and 23 kDa (El Nour et al., 1998). Glutamine, alanine and leucine are the major amino acids of α-kafirin (Table 2.1)

2.3.3.2.2 *Beta kafirin*

Beta kafirins are located mainly at the periphery of the endosperm protein bodies and as inclusions within the protein body (Shull et al., 1992). Beta-kafirin represents 7-8% of total kafirin. In terms of molecular weight, three major bands have been identified by SDS-PAGE of approximate M_r 20, 18 and 16 kDa (Dicko et al., 2006). Glutamine, alanine, leucine and glycine are found in high proportion in β-kafirin. This amino acid composition of β-kafirin makes it tend to be soluble in low concentrations of alcohol (10-60% tert-butanol plus 2-mercaptoethanol) (Shull et al., 1992). Beta-kafirin contains a high amount of the sulphur-containing amino acids methionine and cysteine. The presence of 10 cysteine residues within the β-kafirin polymer results in either inter- or intra-molecular disulphide bonds, which lead to the formation of oligomers and polymers (Belton et al., 2006). This explains why a reducing agent is required in order to extract β-kafirin effectively (Shewry, 2002). Very large polymers can be formed due to the bonding of the oligomers of α-kafirins and γ-kafirins through β-kafirin which acts as a bridge between α- and γ-kafirins (El Nour et al., 1998). These very large polymers are only extractable under reducing condition. In terms of digestibility, β-kafirin is more digestible in the intact protein body compared

to α-kafirin. However, this is only due to the location of the β-kafirin in the periphery of the protein body, where it is more susceptible and exposed to proteolytic enzymes before α-kafirin (Oria et al., 1995).

2.3.3.2.3 *Gamma kafirin*

Like β-kafirin, γ-kafirin is located predominantly at the periphery of the protein bodies and also scattered as inclusions within the protein body (Shull et al., 1992). Gamma-kafirin represents 9-12% of the total kafirin extracted from the corneous endosperm part (Watterson et al., 1993). While in the floury endosperm, γ-kafirin accounts for 19–21%. Due to the high amount of the hydrophilic amino acids glycine and histidine (Table 2.1), γ-kafirin can be extracted effectively in low concentrations of alcohol (Shull et al., 1992). Furthermore, γ-kafirin can be extracted using water containing a reducing agent (Taylor et al., 1989). This could raise confusion as although γ-kafirin is more hydrophobic than α- and β-kafirins as seen from the hydration energies (Shewry, 2002), γ-kafirin is the only water soluble kafirin fraction (Taylor et al., 1989). Belton et al. (2006) tried to explain this contradiction by attributing it to the high level of histidine in γ-kafirin (7.8 mol %). The authors further explained the pKa of histidine is about six, which can generate a high degree of ionisation. The latter can result in electrostatic repulsion and hence water solubility. Gamma-kafirin contains high levels of cysteine and proline and low levels of lysine and aspartic acid (Watterson et al., 1990). This high proportion of disulphide bonding cysteine is the reason for the need of a reducing agent to achieve efficient extraction through dissociation of the intermolecular disulphide bonds (Shull et al., 1992). In terms of molecular weight, two γ-kafirin bands can be separated by SDS-PAGE (Evans et al., 1987). These two bands have been classified as major band of 28 kDa and minor band of 49 kDa. Like the β-kafirins, γ-kafirins are more digestible than α-kafirin in intact kafirin protein bodies due to their peripheral location (Oria et al., 1995).

2.3.3.2.4 *Delta-kafirin*

There is limited information about δ-kafirin as it has not yet been identified at the protein level (Belton et al., 2006). Delta-kafirin displays two sequences that were more equivalent to the δ-zein of molecular weight 14 kDa. These two sub-classes of δ-kafirin are rich in methionine.

2.3.3.3 *Kafirin digestibility*

When cooked, sorghum kafirin protein has inferior digestibility compared to other cereal proteins (Hamaker et al., 1987). There are two causes both of them related to disulphide cross-linking. The first concerns the formation of relatively large polymeric units through intermolecular disulphide bonds. The large size of these polymers make them less susceptible to protease enzymes compared to the other protein fraction of lower molecular weight. The second cause is because of the structure of the kafirin protein bodies. Because of the location of the digestible major kafirin (α-kafirin) in the centre of the protein bodies, sorghum protein digestibility is low (Duodu et al., 2003).

Beta and γ-kafirin disulphide crosslinking can also result in retardation of the α-kafirin digestion (Da Silva et al., 2011a).

Dry heating process such as popping and extrusion have little effect on sorghum protein digestion (Dahlin and Lorenz, 1993; Duodu et al., 2001). However, the presence of water in wet cooking can enhance and facilitate the interaction between protein fractions themselves and between proteins and the other components (Belton et al., 2006). Wet cooking results in increasing of bonding of β- and α-kafirin (Duodu et al., 2001). Increase in temperature also leads to loss of α-helical structure and then protein aggregation occurs to form β-sheets structure. However, the formation of β-sheet structure during wet cooking does not seem to be the only factor that affects sorghum protein digestibility as the phenomenon was observed in genetically modified high protein digestibility sorghums (Da Silva et al., 2011a).

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis of total kafirin showed that the oligomers of kafirin composed mainly of the two main subunits namely α- and γ-kafirin while the β-kafirin seemed to exist in the monomers region only (El Nour et al., 1998). Reduction of γ-kafirin expression through genetic modification results in an increase in protein digestibility (Da Silva et al., 2011a)

2.4 WHEAT STARCH AND ITS ROLE IN FLOUR QUALITY

Starch characteristics play a major role in wheat breads in terms of volume, texture and general consumer acceptability (Gray and BeMiller, 2003; Van Hung et al., 2006). Together with gluten,

starch is involved in the formation of the elastic network in wheat dough and bread. It has been found that high amylose (30–37% amylose) wheat flour produces substantially smaller bread loaf volume than normal (non-waxy) starch (25–28% amylose) wheat flour and waxy (0–3% amylose) wheat (Morita et al., 2002; Van Hung et al., 2006). Waxy wheat flour produced a slightly larger loaf than the ones from normal wheat bread flour. In terms of the characteristics of bread crumb structure, waxy wheat flour results in a more porous crumb than normal wheat bread (Lee et al., 2001; Van Hung et al., 2006). This was ascribed to the greater susceptibility of amylopectin to α-amylase during the fermentation process, which results in a higher gas production while the retention of gas remains low. This low capability to hold the gas is due to the weak dough strength of waxy wheat compared to normal starch wheat flour. It seems from the studies that compared waxy and normal wheat flour, there were different interpretations of the results which can be because there are other factors involved in wheat flour properties and the effect of gluten could be in the foremost (Van Hung et al., 2006). Retrogradation usually referred to as bread staling that is noticed as firming of breadcrumb, and starch is believed to be primarily responsible for this phenomenon (Hug-Iten et al., 1999). However, there is disagreement about which component of starch (amylose or amylopectin) has the major role in retrogradation of bread (Gray & BeMiller, 2003). Bread and particularly the breadcrumb made from waxy wheat flour is more resistant to firmness and maintaining moisture. These properties result in bread from waxy wheat having extended shelf-life compared to bread from normal wheat flour (Van Hung et al., 2006).

2.5 WHEAT PROTEINS AND THEIR ROLE IN FLOUR QUALITY

The viscosity and the extensibility of wheat flour dough are attributed to the gliadin proteins (Shewry et al., 1986). However, the elasticity and cohesiveness of gliadins are low compared to the wheat glutenins. In contrast, the elasticity of the wheat flour dough and its cohesiveness resulted mainly from glutenins (MacRitchie, 1980; Wieser, 2007). The elasticity of glutenins has been ascribed to the β-sheet formation of high molecular weight glutenin subunits which form a loose spiral (Shewry et al., 1992; Wieser, 2007). However, the theory involving the formation of loops and trains by glutenins in wheat gluten being responsible for the elasticity of wheat gluten (Belton, 1999) is more widly accepted, although the β-sheet formation is considered to play a role (Belton, 2005).

Low charge density of wheat gluten and the hydrogen bonds strengthen the association of gluten units and make the dough stable (Shewry et al., 1992; McCann, et al., 2009). The higher amount of helical structure (approx. 60% α-helix) in kafirin (Belton et al., 2006) and the greater hydrophobicity of zein and kafirin (Fevzioglu et al., 2012) are major differences compared to gluten.

2.6 FACTORS RELATED TO FLOUR FUNCTIONALITY

2.6.1 Water absorption and water solubility of flour

Starch, protein and fibre absorb water in flour. Water absorption and swelling power of starch are closely related together and it can be said that they are two sides of the same coin (Jenkins and Donald, 1998). Flour water absorption index (WAI) is the weight (g) of gel per gram of dry flour. The water solubility of flour can be defined as the maximum quantity of flour which will be dissolved in a unit volume of water. The type of crystallinity of starch granules has an influence on the water solubility of starch, the major flour component (Crochet et al., 2005). The A-type crystal has less solubility than the B-type. Both water absorption and water solubility of starch are affected by the strength and the structure of the micellar network within the starch granule (Qian, Rayas-Duarte and Grant, 1989; Udachan et al., 2012). Water solubility and swelling of starch increase with increasing temperature (Yusuf et al., 2007; Udachan et al., 2012). This is attributed to induction of strong vibration of starch granules which results in breaking or release of intermolecular linkages. Therefore more sites inside the starch granule become available to take more water through linking via hydrogen bonds (Udachan et al., 2012). Water absorption of starch rises with increasing extent of starch granule damage (Dexter et al., 1994). Noticeable and rapid increase in water absorption of sorghum starch is in the range of its gelatinization temperature approx. 60-70 °C (Udachan et al., 2012). Determination of WSI and WAI at high temperature enables estimation of the textural character of products based on starch.

Starches of a white (non-pigmented) high amylose sorghum and red (pigmented) low amylose content sorghum showed different swelling power (SP) and WSI at various temperatures in the range 55-95 °C (Boudries et al., 2009). Normally swelling power and WSI of sorghum starch increase with temperature. The sorghum starches with different amylose content exhibited the

same swelling power at temperatures lower than 65 °C (Boudries et al., 2009). Nevertheless, at temperatures above 65 °C sorghum starch of low amylose content displayed higher swelling power than sorghum starch of higher amylose content. This may confirm what has been stated by Tester and Morrison (1990), that swelling power of cereal starches is related to their amylopectin content, while amylose works as a retarder, especially when amylose forms amylose-lipid complexes in the presence of lipids. The WSI of sorghum starches almost stops increasing at about 85-95 °C (Boudries et al., 2009). Furthermore, WSI of high amylose starch from non-pigmented sorghum is higher than that of lower amylose starch from red sorghum. Alkali treatment of sorghum starch resulted in a decrease in swelling temperature, perhaps due to pre-gelatinization of starch (Beta et al., 2000).

It can be said that the WAI and WSI of sorghum increase with temperature (Carcea et al., 1992). However, the swelling powers of some sorghum starches and flours are similar to wheat at a temperature of about 55 °C (Chanapamokkhot and Thongngam, 2007; Phattanakulkaewmorie et al., 2011). However, at temperatures above 75 °C, sorghum starch and flours have a higher swelling power than wheat (Chanapamokkhot and Thongngam, 2007)

2.6.2 Dough rheology

Starch and protein are the major components in the flour that affect dough rheology. During baking of the dough for bread making, protein denatures and releases water for starch to use it for its gelatinization (Therdthai and Zhou., 2003). As with wheat dough, the rheological properties of sorghum dough are affected by the nature of protein (Goodall et al., 2012). These authors found that dough from sorghum of high protein digestibility had higher maximum resistance to extension than normal sorghum.

With regard to the effect of starch, it has been found that starch from red sorghum of lower amylose content exhibited higher peak viscosity (around 4731 cP) than that of high amylose starch from white sorghum (around 4093 cP) (Boudries et al., 2009). This difference in pasting behaviour could be attributed to the amount of amylose rather than the colour of the sorghum. When Beta et al. (2000) tested the pasting properties of starches isolated from 10 sorghum varieties grown in Zimbabwe , they found that sorghum starches had higher peak viscosity (PV) (300-398 RVU)

than commercial maize starch (239 RVU). The total time for peak viscosity to reach its maximum level starting from the beginning of viscosity increase was generally less in sorghum starches, approx. 1.73-3.03 min than maize starch about 3.50 min. While sorghum starch showed a higher rate of shear thinning around 24.4-40.4 RVU/min than maize starch, approx. 21.0 RVU/min. Polyphenol content of the sorghum starch had a positive relationship with starch peak viscosity ($r = 0.75$, $p < 0.05$). Moreover, breakdown and rate of shear thinning of sorghum starch had negative relation with hardness of sorghum starch gel. Sorghum starch from high tannin varieties had a higher rate of shear thinning than sorghum starch from low or non-tannin sorghum. It was concluded that sorghum pasting properties are significantly affected by the genetic diversity and the environmental condition during growing (Beta and Corke, 2001).

The pasting properties of hard wheat, barley and sorghum starches were compared (Ragaee and Abdel-Aal, 2006). The peak viscosity (PV), breakdown viscosity (BV) and setback viscosity (SV) were different between these cereals. With regard to wheat starch, PV, BV and SV were about 1335 cP, 755 cP and 842 cP, respectively. Barley starch had a PV of approx. 1355 cP, BV of 989 cP and SV of 695 cP. Generally, sorghum starch had a lower average peak viscosity (821 cP) than wheat and barley starch. Regarding breakdown viscosity, sorghum starch had the lowest extent (2 cP) compared to wheat and barley which may relate to higher stability. Fruthermore, sorghum starch had the highest degree of setback average 1307 cP compared to wheat and barley.

2.6.3 Kafirin modification and its effect on flour functional properties

Modification of kafirin by suppression of γ-kafirin synthesis has been found to increase protein digestibility (Da Silva et al., 2011b). It appears that to some extent kafirin functionality is affected by kafirin structure. The effect of the high protein digestibility trait in sorghum on the viscoelasticity of sorghum-wheat composite dough and bread quality has been studied (Goodall et al., 2012). For this purpose, high digestibility high lysine (HDHL) sorghum and normal sorghum were studied. Regardless of the percentage of the sorghum flour (30 and 60%) within the sorghum-wheat composite dough, the extensibility and dough breakage time of HDHL were significantly higher than the normal sorghum composite. As the strain hardening can indicate the ability of a dough to trap the gas, it was also tested. In the composite doughs containing 60% sorghum, the

HDHL displayed no significant difference compared to wheat dough in terms of the strain hardening, while the normal sorghum showed significantly lower strain hardening than wheat and HDHL composite doughs. HDHL improved the quality of the bread in terms of loaf volume. Loaf volumes of breads made from HDHL composite doughs with sorghum percentages between 30% to 56% were significantly higher than bread loaf made from normal sorghum. The crumb of breads made with the HDHL sorghums were found to be spongier with lower hardness and compressibility compared to bread made from normal sorghum. Doughs from HDHL and normal sorghum flours with the addition of 18% vital wheat gluten were also evaluated. By naked eye, HDHL flour with 18% gluten gave a dough with better viscoelastic properties than normal sorghum.

It is thus clear that whether in wheat or sorghum, starch and storage protein structure have direct effects on flour, dough and bread quality.

2.7 ANALYTICAL TECHNIQUES FOR FLOUR COMPOSITION AND QUALITY

2.7.1 Microscopy

Microscopy is the major food analysis visualization technique (Kaláb et al., 1995). It can be considered as the most suitable method to study and evaluate food structure as microscopy provides visual images. Microscopy can be used to identify the effects of many types of processing on food structure. The main principle of microscopy is to magnify and enlarge particles of food materials to facilitate and enable their study.

2.7.1.1 *Scanning Electron Microscopy (SEM)*

Compared to light microscopy, the resolution of electron microscopy is significantly improved (Bozzola and Russell, 1999).The principle of SEM can be summarized the use an electron beam instead of visible light as is used in light microscopy (LM). The electron beam is generated by a suitable source which is usually a tungsten filament, and accelerated by a high voltage and passed through an electromagnetic field to scan the surface of the object. The emitted electrons from the specimen are collected by an appropriate detector and recorded, normally digitally. The key feature of SEM is that it generates three dimensional images (Aguilera and Stanley, 1999). It provides a

massive depth of field around 500 times greater than that of LM at the same magnification (Table 2.2). Depth of field also called effective focus range, is the distance between the nearest and farthest objects at which an image can be focused without loss of clarity. SEM covers the magnification gap (20× to 100,000×) between LM and transmission electron microscopy (TEM). Also sample preparation for SEM is easier than for LM or TEM as no sectioning is required, which can result in numerous artifacts that can affect the quality and reliability of the images. The main drawback of conventional SEM is the need for a high vacuum during scanning of the samples which means the samples have to be totally dehydrated. However, the Cryo SEM can now be applied for the hydrated samples. Cryo SEM is a type of SEM in which sample is studied at cryogenic temperatures, normally liquid nitrogen temperatures. Ultra SEM is a developed SEM with higher resolution.

Table 2.2 Features comparison of light microscopy, transmission electron microscopy and scanning electron microscopy

(Flegler et al., 1993; Aguilera and Stanley, 1999)

Factor	**LM**	**TEM**	**SEM**
General use	Surface structure and sections	Thin sections	Surface structure
Resolution (nm)	200-500	0.2-1	3-6
Magnification (×)	10-1500	200-500,000	20-100,000
Depth of field at 500× (µm)	2	800	1000
Illumination	Visible light	Hi-speed electrons	Hi-speed electrons
Lens	Glass or quartz	Electromagnetic	Electromagnetic
Specimen preparation	Easy	Difficult	Easy
Thickness	Thick	Very thin	Reflectance
Environment	Versatile	Vacuum	Vacuum
Available space	Small	Small	Large

There have been many studies involving sorghum where SEM was used. For example, the effect of crop rotation and soil amendment on sorghum grain quality was examined by using SEM (Mady Kaye et al., 2007). It was found that the nodulating soybean rotation influenced sorghum grain quality. The nodulating soybean rotation resulted in harder sorghum endosperm compared to sorghum produced without soil amendment by soybean. SEM of the soft sorghum showed less compact starch granules within the endosperm, while the hard sorghum had closely packed starch granules within the endosperm.

2.7.1.2 *Transmission electron microscopy (TEM)*

As with SEM, TEM can be considered as analogous to LM. Since TEM is an electron optical instrument, the visualization of the objects does not occur by light illumination, but by using an electron beam (Klang et al., 2012). For this reason, i.e. use of electrons, TEM needs to be operated under vacuum to avoid electron deviation by air molecules. TEM has an electron gun on top of a column used to generate the electron beam. As with SEM, electromagnetic lenses concentrate the electron beam on the specimen. Electron scattering i.e. the interaction of electrons with specimen is responsible for the image contrast in TEM. The resolution of TEM is positively correlated with the acceleration voltage of the electrons. However, continuous acceleration voltage leads to poor image contrast due to a decrease in electron scattering at high velocity (Kuntsche et al., 2011).

TEM can be used to visualize the internal structure of food specimens (Mady Kaye et al., 2007). Samples should be sectioned to a thickness of 15-90 nm after being embedded in epoxy resin or platinum-carbon. The magnified image of the specimens can be viewed on a fluorescent screen or photographed digitally. TEM has, for example, been used to investigate sorghum protein bodies (Da Silva et al., 2011a). TEM facilitated understanding of the effect of suppression of γ-kafirin synthesis on protein body structure. It was found that the genetically modified sorghums with high protein digestibility due to reduction in γ-kafirin had invaginated protein bodies.

2.7.1.3 *Confocal laser scanning microscopy (CLSM)*

CLSM can be considered as an advanced technique of light microscopy (Mady Kaye et al., 2007). Unlike conventional LM, the light in CLSM is generated by a laser. One of the merits of CLSM is that the specimen points are illuminated by parallel light simultaneously, i.e. uniform illumination.

This feature of CLSM enables the obtaining of a clear image and optical sectioning of the object due to focusing the light at desired levels beneath the surface. CLSM is generally used in the fluorescence mode. Various fluorescent dyes can be applied to the specimen, then the image is collected from the interaction between the light from the laser and the emitted fluorescence from the specimen. By focusing the light at specific levels, slices (as images) of the specimen can be obtained and a 3-D image or recombined video may be made from these slices. By using the florescence mode, a thick sample can be viewed easily, unlike with conventional LM where the sample must be thin. Another advantage of CLSM compared to LM is that it requires little sample preparation, which means that artifacts in the images are minimised. Because of these advantages CLSM is used commonly in the field of food science in general and cereal science in particular. For example, CLSM was used in a study of the effect of sourdough fermentation on the quality of gluten-free sorghum bread (Schober et al., 2008). The sourdough eliminated defects (flat top bread and holes in the crumb) which appeared in the bread without sourdough fermentation. CLSM was used to view the bread crumb microstructure to identify the effect of the sourdough fermentation. Proteins in CLSM images appeared brighter compared to other bread components such as starch, fibre and fat with use of fluorescein 5(6)-isothiocyanate (FITC) as fluorochrome. Another application of CLSM which investigated the effect of protease on rice bread crumb structure showed the protein as red and the starch as green (Renzetti and Arendt, 2009). In this study to stain protein as red/yellow, Rhodamine B (0.2%) was used, while Fluorescein isothiocyanate (1% in acetone) was used to stain starch (green). By using various types of dyes CLSM can display the same food components in different colours to study food microstructure. CLSM was also used to study the effect of the maize sourdough fermentation on dough and bread structure (Falade et al., 2014). CLSM showed that maize sourdough had a more cohesive structure compared to the unfermented maize dough. Also, CLSM revealed that bread made with maize sourdough had larger gas cells compared to the unfermented treatment.

2.7.1.4 *Fourier transform infrared (FTIR) spectroscopy*

The range of the mid-infrared (400–5,000 cm^{-1}) that is applied using FTIR has become a commonly used and effective tool to provide information concerning the chemical bond types in food substances (Carbonaro and Nucara, 2010). Strictly speaking, it can be said that FTIR indicates the

structure and conformation of the chemical substances. The molecular framework of proteins, including cereal proteins, is much studied using FTIR spectroscopy. One of the advantages of FTIR compared to other spectroscopic techniques is that FTIR does not need a large sample size and at the same time affords high quality spectra. The secondary structure of proteins can be studied by FTIR through the investigation of the fraction of peptide bonds commonly in the Amide I band region (1600–1700 cm^{-1}). The infrared spectrum of proteins consists of nine well-known amide absorption bands that are related to the peptide bond due to the presence of CONH grouping, however, the Amide I band is the region that is most commonly used for protein secondary structure study (Arrondo et al., 1993). In the Amide I region, α-helices, β-sheets and β-turns can be determined. The principle of FTIR can be summarised as relying on vibrational energy of bonds, i.e. determination of vibrational transitions usually between the zero-point energy (ground state) and the first excited state (Arrondo et al., 1993). It has been found that the summation of electronic, vibrational, rotational, and translational energies of a molecule gives the total energy of that molecule. The Bohr equation describes how the change in the molecular vibrational energy can be calculated when the infrared energy measured by FTIR is absorbed or emitted.

Energy Difference (ΔE) = $E_2 - E_1 = hf = hc\nu$

Where: E_1 and E_2 are the initial and final energy, respectively. H is the Planck's constant, c is the velocity of light, f is vibrational frequency in sec^{-1} and ν is the wavenumber in cm^{-1}.

With regard to the application of FTIR in the study of sorghum proteins, FTIR has, for example, been used to determine the effect of wet cooking on the secondary structure of zein and kafirin (Duodu et al., 2001). FTIR showed that α-helical conformation predominated when compared to β-sheet when the proteins were in their raw (uncooked) state. When they were wet cooked the proportion of β-sheet was increased. This increase in β-sheet was at the expense of α-helix, i.e. the latter unfolded to form β-sheet structure.

2.7.2 Rheometry

Rheometry is the science that refers to the experimental techniques used for the measurement of rheological properties of a material (Menjivar, 1990). Rheology is defined as the study of flow and deformation of a material and its response to the applied forces in a form of stress or strain

(Dobraszczyk and Morgenstern, 2003). Rheometers are instruments that measure the rheological behaviour of moderately viscous non-Newtonian materials such as flour dough, particularly the elongational viscosity (McKinley and Sridhar, 2002). One of the most effective rheometric measurements is by oscillatory techniques. If the rheological properties of a dough are obtainable within the linear viscoelastic region (LVE), then oscillatory techniques can assess the viscosity and the elasticity as a non-destructive, linear, and dynamic characterization (Steffe, 1992). In oscillatory tests, an oscillatory stress or strain is applied to the dough. The dough sample is held between two parallel plates, where the lower plate is fixed and the top one is moving sinusoidally. Parameters such as the storage modulus, G' (so-called elastic modulus), can be calculated so as to relate to the recoverable mechanical energy held in the material. Another major parameter that is obtained is the shear loss modulus, G" (viscous modulus), which indicates the loss of energy due to the viscous dissipation. Through G' and G", the viscoelastic behaviour of a material can be determined as the loss tangent δ. Dough rheological characteristics are strongly related to the bread making process and product quality (Dobraszczyk and Morgenstern, 2003). As an example of the application of rheology to understand dough characteristics, the effect of sourdough fermentation of maize has been investigated (Falade et al., 2014). The oscillatory technique was used with a rheometer with parallel plate geometry. Under amplitude sweep analysis, sourdough had a lower storage modulus than unfermented maize dough, indicating that the sourdough had lower elasticity and a softer structure. Temperature sweep analysis showed that maize sourdough initially had lower elasticity but had a higher final loss tangent. These data indicated that it had better tolerance to gas expansion pressure at baking temperature so that the bread structure was more resistant to crumbling.

2.7.3 Electrophoresis

Electrophoresis separates proteins through application of an electric field that affords the migration of the protein fractions and separates them (Yada et al., 1996). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates the components in a protein mixture based on the apparent molecular size of these components (Garfin, 1990). The charged molecules of the proteins migrate within an electric field from the negative electrode to the positive one. By denaturing the protein before loading this eliminates the effect of the protein shape in the

separation as the protein loses its secondary, tertiary or quaternary structure. Proteins are treated with SDS (an anionic detergent) to give them a negative charge and this eliminates the effect of the charge factor as almost all the molecule is covered by SDS and all molecules will have equal charge. That means, the resistance of the gel to the migration of the protein molecules is almost entirely the single factor responsible for separation. According to the pore size of the gel, smaller molecules have less resistance to migrate and therefore they move faster and vice versa for the larger molecules.

SDS-PAGE has been applied to separate kafirin fractions based on their molecular size (El Nour et al., 1998). SDS-PAGE gave effective separation of intact kafirins. Two dimensional (2-D) electrophoresis is a much more powerful analytical technique as it comprises two electrophoresis systems, isoelectric focusing (IEF) followed by SDS-PAGE (Friedman et al., 2009). IEF separates the proteins depending on their differences in the isoelectric point (p*I*). 2-D electrophoresis has been applied to compare the prolamin fractions of teff with maize and sorghum (Adebowale et al., 2011). 2-D PAGE showed that teff had more different protein fractions than maize and sorghum prolamins. The authors attributed this to differences in chromosome number, which is higher in teff compared to maize and sorghum. Also, 2-D PAGE showed that the number of the polypeptide spots within the acidic range (p*I* 3.0-6.5) were higher than the spots in the basic range (p*I* 7.5-10.0) for the three cereal grains. However, teff had a higher proportion of basic polypeptides (47%) than maize (34%) or sorghum (43%).

2.8 CONCLUSIONS

There is very limited research into the changes in flour functionality of sorghum mutants with altered kafirin synthesis. Although there is some research into the functional properties of waxy type sorghums, there is little on flour functionality. A major issue is that no research has been conducted which has resulted in successful formation of a dough from only kafirin protein. Thus, there is need to study the effect of the combined waxy-and high protein digestibility traits and their effect on sorghum flour functionality and try to form dough from kafirin and subsequently from flour. Also, there is limited research on effect of suppression of kafirin synthesis on sorghum flour funcutionlity.

3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

1. The combination of high protein digestibility trait due to kafirin alteration and waxy trait in novel sorghum lines will lead to an improvement in flour functionality especially an increase in pasting viscosity.

It has been found that sorghum with the high protein digestibility trait had a floury endosperm (Da Silva et al., 2011a). It was assumed that the novel sorghum lines had their high protein digestibility due to reduction of the cysteine-rich γ-kafirin sub-class. Also, it was found that sorghum with high protein digestibility had invaginated protein bodies (Oria et al. (2000). The invagination in the protein bodies increases the accessibility of water to the most hydrophilic kafirin sub-class, α-kafirin, in the centre of the protein body. The floury endosperm of sorghum would result in smaller flour particles and that will increase the overall available flour particle surface susceptibility to water interaction. Due to the branches in the amylopectin of waxy starch, it has the ability to trap more water through hydrogen bonding. The availability of the water and the ability to trap it by the starch will result in greater starch granule expansion when the hydrated flour is subjected to heating during pasting. This higher expansion of the starch granules will appear as high peak viscosity.

2. Suppression of γ-kafirin synthesis in genetically modified sorghums with high protein digestibility will increase water solubility and protein-starch interaction in the dough and improve dough elasticity.

With the kafirin sub-classes, γ-kafirin is always decribed as the most hydrophobic class compared to the α- and β-kafirins (Duodu et al., 2003). The reduction of γ-kafirin will result in higher water solubility of the flour. Gamma-kafirin is involved in kafirin polymerization due to disulphide cross linking (Da Silva et al., 2011b). The polymerization of kafirin results in a compact protein matrix that surrounds the starch granules closely. The reduction of the γ-kafirin will result in less packed starch granules in a hydrophilic or less hydrophobic protein matrix. The looser protein matrix will lead to better distribution of the protein in the dough system and thereby enable better interaction

with starch, which will result in more cohesive dough. This cohesion will result in higher dough elasticity.

3. The use of glacial acetic acid as kafirin solvent will enable the formation of a kafirin dough.

Solubilisation of kafirin in glacial acetic acid enabled kafirin microparticle formation (Taylor, 2008). It was assumed that the acetic acid unfolded the kafirin structure and resulted in β-sheet structure at the expense of the α-helical secondary structure conformation. The same is expected to happen in the process of kafirin dough formation.

3.2 OBJECTIVES

The overall objective of this study is to look for effective approaches that enable the utilization of sorghum flour at the commercial level for various products making especially leavened wheat-free bread.

Specific objectives:

1. To determine the effect of the combination of waxy and high protein digestibility traits in novel sorghum lines on flour pasting, solubility and related flour functional properties

2. To determine whether reduced γ-kafirin expression affects sorghum flour dough quality

3. To determine whether kafirin dough can be prepared using glacial acetic acid as a solvent

4 RESEARCH

4.1 NOVEL BIOFORTIFIED SORGHUM LINES WITH COMBINED WAXY (HIGH AMYLOPECTIN) STARCH AND HIGH PROTEIN DIGESTIBILITY TRAITS: EFFECTS ON ENDOSPERM AND FLOUR PROPERTIES

4.1.1 Abstract

Novel biofortified sorghum lines have been developed with both waxy starch (high amylopectin) and high protein digestibility traits. Eight sorghum lines with different combinations of waxy, non-waxy, high- and normal-protein digestibility traits were studied in relation to flour properties. Lines with the high protein digestibility trait had loosely packed starch granules and floury endosperm, irrespective of whether they were waxy or non-waxy. In terms of thermal properties, combined waxy-high digestibility lines had the highest onset endothermic temperature as well as endothermic energy compared to non-waxy, normal protein digestibility lines. The waxy-high protein digestibility lines had higher paste viscosity and formed much softer and less sticky pastes than the non-waxy, normal protein digestibility lines. Flours of the combined waxy-high protein digestibility sorghum lines had much higher solubility than the non-waxy, normal protein digestibility lines. At 30^{o}C, flour water solubility, of waxy-high protein digestibility sorghum lines flour was similar to commercial wheat bread flours. The high flour water solubility, high pasting viscosity and soft paste of sorghum lines with combined waxy and high protein digestibility traits indicates that their flours may have better properties for making dough-based food products than normal non-waxy, normal protein digestibility sorghums.

4.1.2 Introduction

Sorghum is the fifth most important cereal crop in terms of production, about 58 million tonnes in 2011, with Africa being the major producing region accounting for > 40% of world production (FAOSTAT, 2013). Sorghum is highly suited for cultivation in the semi-arid and sub-tropical regions of Africa as it is one of the most drought-tolerant cereal crops (Srinivas et al., 2009). Further, sorghum does not elicit an adverse reaction in coeliacs (Ciacci et al., 2007). However, despite its high production and its applicability in gluten-free foods, the commercial utilization of sorghum is limited, particularly as flour for dough-based food products, and especially in bread. This is largely due to the fact that its flour does not form a gas-holding dough (Taylor et al., 2006).

This drawback of sorghum presents a major challenge in Africa, where increasing dependence on wheat to produce bread to meet the needs of the rapid expanding urban population is negatively affecting the continent's economic situation. According to the International Food Policy Research Institute, in 2013 African countries were spending US $12 billion to import 40 million tons of wheat (IFPRI, 2013).

The major reason for the inferior bread-making properties of sorghum is that kafirin, the sorghum prolamin protein, does not exhibit the viscoelastic properties of wheat gluten in normal dough systems (Taylor et al., 2014). However, Goodall et al. (2012) showed that flour of sorghum with a high protein digestibility trait (Weaver et al., 1998), resulting from modified kafirin prolamin protein body development (Oria et al., 2000), produced better quality sorghum-wheat composite doughs and breads than normal sorghum flour. This was attributed to formation of an improved dough protein network. Furthermore, the starch granules in the corneous endosperm of sorghum are surrounded by hydrophobic matrix proteins (Munck, 1995), comprising the kafirin protein bodies and glutelins (Taylor et al., 1984). This matrix of hydrophobic proteins may reduce the extent of water absorption and solubilisation of sorghum starch (Chandrashekar and Kirleis, 1988; Ezeogu et al., 2005). In turn, this may lead to inadequate functionality of sorghum flour because in wheat flour starch water holding is related to dough functionality (Dexter et al., 1994).

Novel biofortified sorghum lines which combine the high protein digestibility trait with the waxy (high amylopectin) starch trait, hence having high starch digestibility (Rooney and Pfugfelder,

1986), have recently been developed by Texas A&M University through conventional breeding (Jampala et al., 2012). These lines were developed from a cross between a waxy endosperm parental line and the high protein digestibility line described above, which was developed by Purdue University (Weaver et al., 1998). However, research into the end-use functionality of these waxy-high protein digestibility sorghum lines has been very limited.

The objective of this work was to examine the effects of sorghum lines with the waxy and high protein digestibility traits individually and in combination on characteristics related to flour functionality with the aim of determining the potential of these novel biofortified sorghum lines for making good quality dough-based food products.

4.1.3 Materials and Methods

4.1.3.1 *Sorghum samples*

Eight sorghum lines, developed and bred through conventional plant breeding by Texas A&M University, were studied. All the lines were of the white tan-plant type. They comprised: two non-waxy-normal protein digestibility lines coded 199 and 200, two waxy-normal protein digestibility lines (coded 175 and 179) and one non-waxy-high protein digestibility line (coded 106), and three waxy-high protein digestibility lines coded 109, 142 and 146). Sorghum lines coded 109, 142 and 146 were obtained via crossing lines Tx2907 and P850029 (Jampala et al., 2012). Tx2907 was released from the Texas A & M AgriLife Research sorghum breeding program as a waxy and normal protein digestibility sorghum (Miller et al., 1996). P850029, a high protein digestibility line, was developed at Purdue University from a population derived from the high lysine line P721Q (Weaver et al., 1998). The lines were increased at Halfway, Texas in 2012. The lines were blocked using rows of photosensitive lines as pollen breaks to avoid cross pollination.

MR Buster, a red, non-tannin, non-waxy, normal protein digestibility commercial hybrid sorghum was used as a standard. It was cultivated in Botswana and kindly supplied by the National Food Technology Research Centre in Botswana.

4.1.3.2 *Grain endosperm and protein body structure*

Twenty sound grains from each sorghum line were dissected longitudinally. Endosperm texture was recorded by stereo light microscopy. By reference to sorghum endosperm type illustrations (ICC, 2011), the relative proportion of corneous endosperm to floury endosperm was estimated for each line. Endosperm structure was evaluated using scanning electron microscopy (SEM). Whole sorghum grains were dissected longitudinally with a scalpel after freezing in liquid nitrogen. Then samples were mounted on aluminium stubs using poster gum and sputter coated with gold and then viewed using a Zeiss Evo LS15 SEM (Carl Zeiss, Oberkochem, Germany) operated at an acceleration voltage of 8 kV. Protein body structure was investigated using Transmission Electron Microscopy (TEM) as described (Da Silva et al., 2011a,b).

4.1.3.3 *Flour preparation*

Sound grains of each line (20 g) were decorticated using a Tangential Abrasive Dehulling Device (TADD, Venables Machine Works, Saskatoon, Canada), removing approx. 20% by weight of the grain outer layers. A laboratory hammer mill (Mikro-Feinmuhle-Culatti MFC Grinder, Janke and Kunkel, Staufen, Germany) with a 500 µm opening screen was used to mill the decorticated grain.

4.1.3.4 *Flour moisture content*

Moisture content was determined by NIR (DA 7200 NIR analyser, Perten Instruments Springfield, IL) using the supplier's calibration for sorghum.

4.1.3.5 *Protein content*

Protein content (N x 6.25), was determined by Dumas combustion according to AACC method 46-30 (AACC International, 2000).

4.1.3.6 *Starch amylose content*

Amylose content was determined by the Megazyme amylose/amylopectin assay kit procedure (Megazyme Ireland International, Bray, Ireland). Amylopectin in the sample is specifically precipitated by the addition of the lectin concanavalin A (Con A) and removed by centrifugation.

The concentration of amylose in the sample is determined colorimetrically using the glucose oxidase-peroxidase (GOPOD) reagent (Wong et al. 2009).

4.1.3.7 *In vitro protein digestibility*

In vitro protein digestibility of the flours was determined according to the pepsin digestibility method of Hamaker et al. (1986) as modified by Da Silva et al. (2011b). After pepsin digestion of the sample under specific conditions, the quantity of protein remaining is measured as a percentage of total protein in the sample and percentage digestibility calculated.

4.1.3.8 *Flour thermal properties*

Differential scanning calorimetry (HP DSC 827e (Mettler Toledo, Schwerzenbach, Switzerland) was used to determine the thermal properties of the sorghum flours, as described by Beta et al. (2000). Sorghum flour (9 mg dwb) was weighed into a 100 µl aluminium DSC pan and deionized distilled water was added to a total weight of 36 mg. After sealing, the sample was equilibrated at room temperature for 2 h. Each sample was scanned from 30 to 120ºC at a heating rate of 10ºC/min. Nitrogen was used at normal air pressure with flow rate of 30 ml/min. Onset (T_0), peak (T_p) and conclusion endotherm (T_c) temperatures were measured and endothermic enthalpy was calculated.

4.1.3.9 *Flour pasting properties*

The pasting properties of the sorghum flours were determined using a Physica MCR 301 Rheometer (Anton Paar, Ostfildern, Germany) using a cup and a stirrer. Flour and distilled water were mixed to a ratio 1: 9. Samples were stirred for 30 s at 50°C before measurement. The pasting programme was: hold for 1 min at 50°C, heat to 91°C over 7 min at a rate of 5°C/min, hold for 10 min, cool down to 50 °C over 7 min and then hold for 5 min. Pasting temperature, Peak viscosity (mPa.s), Holding strength, Breakdown, Setback and Final viscosity were measured.

4.1.3.10 *Gel texture properties of flour*

The gel texture properties were performed according to D'Silva et al. (2011). In brief, the pasted samples from the rheometic analysis were allowed to stand overnight at 25°C to allow gelation to take place. Gel texture was determined using a TA-XT2 texture analyser (SMS Stable Microsystems, Godalming, UK) using a cylindrical plunger of 20 mm diameter. at a test speed of

05 mm/s. Samples were compressed to a distance of 5 mm. Hardness was recorded as maximum force on the compression phase. Adhesiveness was recorded as negative force area of the curve resulted from withdrawing the probe.

4.1.3.11 *Flour WAI and WSF*

Water absorption index (WAI) and water soluble fraction (WSF) were measured at 30°C and 60°C essentially as described by Anderson et al. (1970). The WAI and WSF of two commercial wheat white bread flours (Golden Cloud, Tiger Brands, Bryanston, South Africa and Snowflake, Premier Foods, Isando, South Africa) were measured for comparison.

4.1.3.12 *Statistical analysis*

All experiments were performed at least two times. Data were analysed by one-way analysis of variance (ANOVA) in the case of amylose content and protein digestibility and two-way ANOVA for the other analyses, at a confidence level of $P < 0.05$. Means were compared by Fisher's least significant difference (LSD) test. Principal Component Analysis (PCA) for all numerical results was performed using STATISTICA version 12 (StatSoft, Tulsa, OK, USA).

4.1.4 Results and discussion

4.1.4.1 *Waxy and high protein digestibility traits*

As expected, the five waxy lines (109, 142, 144, 175 and 179) had a much lower percentage of amylose then the normal lines (Table 4.1.1). However, since all the waxy lines contained some amylose it would appear from comparison with the findings of Sang et al. (2008) that all were heterowaxy types, containing at least one recessive waxy gene.

Also, as expected, all the four lines that had the high protein digestibility trait (106, 109, 142 and 146) had significantly higher ($p < 0.05$) in vitro protein digestibility in raw flour and higher or generally higher (line 142) in cooked flour when compared to the four lines with the normal protein digestibility trait. However, the cooked flour protein digestibilities of the high protein digestibility lines (36.5-48.1%) were considerably lower than the values given by Weaver et al. (1998) (72.5-80.8%) in the first report of these high protein digestibility sorghum lines. The pepsin digestibility

assay procedure used in this present work and in that of Da Silva et al. (2011a,b), appears to give much lower absolute values. A single sample of a high protein digestibility sorghum line assayed using the procedure gave a cooked protein digestibility of only 55% (Da Silva et al., 2011a), closer to the values found for the high protein digestibility lines in this present work. Notwithstanding this, the cooked protein digestibilities of the high protein digestibility lines in this present work were rather lower than observed with transgenic sorghum with suppressed kafirin synthesis, but similar to their null controls (Da Silva et al., 2011b). Also, as observed with transgenic sorghum and with this non-transgenic high protein digestibility mutant (Da Silva et al., 2011a), the in vitro protein digestibilities of the cooked flours were considerably lower than those of the raw flours. Nevertheless, two of the three combined waxy-high protein digestibility lines (146 and 109) had substantially higher cooked and raw protein digestibility than all the normal protein digestibility lines including cultivar MR Buster, the commercial hybrid sorghum standard.

Of significance is that the waxy trait did not appear to influence protein digestibility as line 142 (waxy-high protein digestibility) had lower digestibility than line 106 (non-waxy, high protein digestibility). Wong et al. (2009) investigated two closely related sorghum lines, one of which was waxy and had high starch and protein digestibilities and the other was non-waxy and had low digestibilities. These authors observed that the protein bodies were more numerous and more tightly associated with the starch granules in the corneous endosperm of the low digestibility line than in the high digestibility line and suggested that interaction between the starch granules and protein bodies could have contributed to its low digestibility. However, with the lines investigated in this present work, the high protein digestibility trait was clearly associated with a floury (soft) endosperm trait (see below), as has been found previously (Tesso et al., 2006).

Table 4.1.1 Starch amylose content and in vitro pepsin protein digestibility of waxy and high protein digestibility sorghum lines and their controls (199 and 200) and normal red sorghum cultivar MR Buster

Line	Starch type	Protein digestibility trait	Amylose (%)	Protein digestibility of raw sorghum flour (%)	Protein digestibility of cooked sorghum flour (%)
109	Waxy	High	3.9^{a}	72.8^{f} ± 1.1	46.7^{f} ± 0.2
142	Waxy	High	7.3^{c}	58.1^{c} ± 0.3	36.5^{de} ± 0.0
146	Waxy	High	5.8^{b}	68.6^{e} ± 0.2	48.1^{f} ± 1.7
175	Waxy	Normal	12.1^{d}	55.4^{ab} ± 0.3	32.3^{ab} ± 1.1
179	Waxy	Normal	7.9^{c}	54.3^{a} ± 0.3	31.9^{a} ± 1.1
106	Non-waxy	High	23.2^{e}	64.1^{d} ± 0.0	38.7^{e} ± 0.9
199	Non-waxy	Normal	25.7^{f}	55.4^{ab} ± 0.1	32.7^{abc} ±0.1
200	Non-waxy	Normal	27.8^{g}	56.3^{b} ± 0.1	35.4^{cd} ± 1.2
MR Buster	Non-waxy	Normal	30.8^{h}	57.9^{c} ± 0.2	35.0^{bcd} ± 1.2

Means with different superscript letters within a column are significantly different ($p < 0.05$).

n = 2

4.1.4.2 *Grain endosperm texture and structure*

All the four high protein digestibility lines, lines 109, 142 and 146 with waxy trait and line 106 (non-waxy) had a floury endosperm texture (Figure 4.1.1). The floury endosperm texture of transgenic high protein digestibility sorghum (Da Silva et al., 2011a) and the soft endosperm character of this non-transgenic high protein digestibility mutant (Tesso et al., 2006) have been reported previously. In contrast, and as expected, the corneous endosperm of the two waxy-normal protein digestibility lines (175 and 179) had the typical "waxed floor-like" appearance of waxy sorghum described by Rooney and Miller (1982). Also, the two non-waxy- normal protein digestibility lines (199 and 200) had an intermediate (part corneous) endosperm texture. Thus, both the high protein digestibility and waxy traits modified the endosperm texture. Notably, however, the high protein digestibility trait seemed to override the waxy trait when they were in combination, resulting in a floury endosperm.

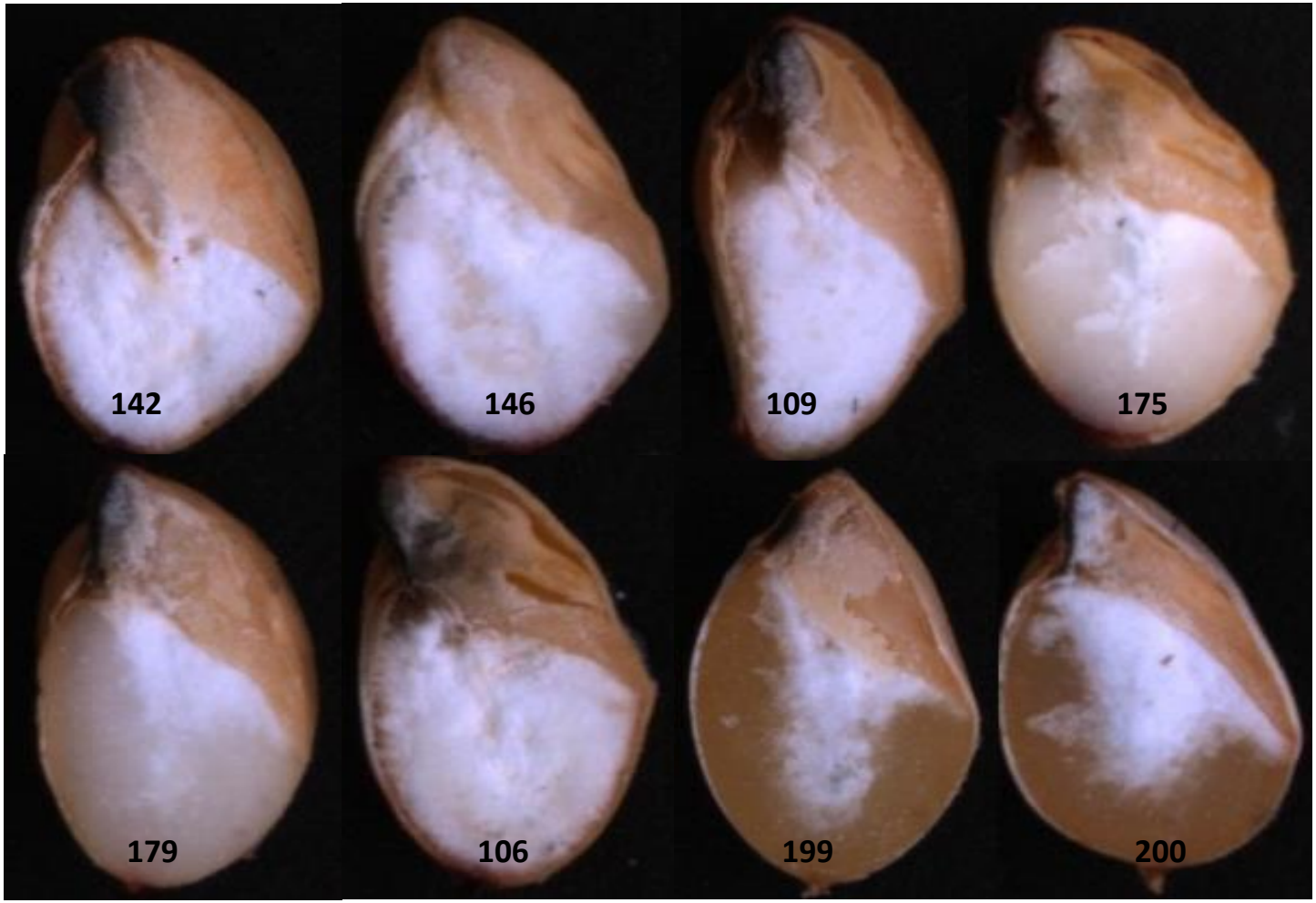

Figure 4.1.1 Endosperm texture and structure of waxy and high protein digestibility sorghum lines A: Longitudinal sections through the grains

142,146 and 109 - waxy, high protein digestibility; 175 and 179 - waxy, normal protein digestibility; 106 - non-waxy, high protein digestibility; 199 and 200 - non-waxy, normal protein digestibility.

SEM showed that the peripheral starchy endosperm cells of all four high protein digestibility lines, both waxy (109, 142 and 146) and non-waxy (106), contained loosely packed starch granules (Figure 4.1.3), hence their floury endosperm texture (Figure 4.1.1). In contrast, the starch granules were tightly packed in all the four normal protein digestibility lines (175, 179, 199 and 200), irrespective of whether they had the waxy trait. The floury endosperm texture of the high protein digestibility mutants is a consequence of the altered kafirin synthesis, which causes the kafirin containing protein bodies to have a folded (invaginated) structure (Figure 4.1.2), as first described by Oria et al. (2000). This in turn results in an incomplete protein matrix surrounding the starch granules in the outer floury endosperm.

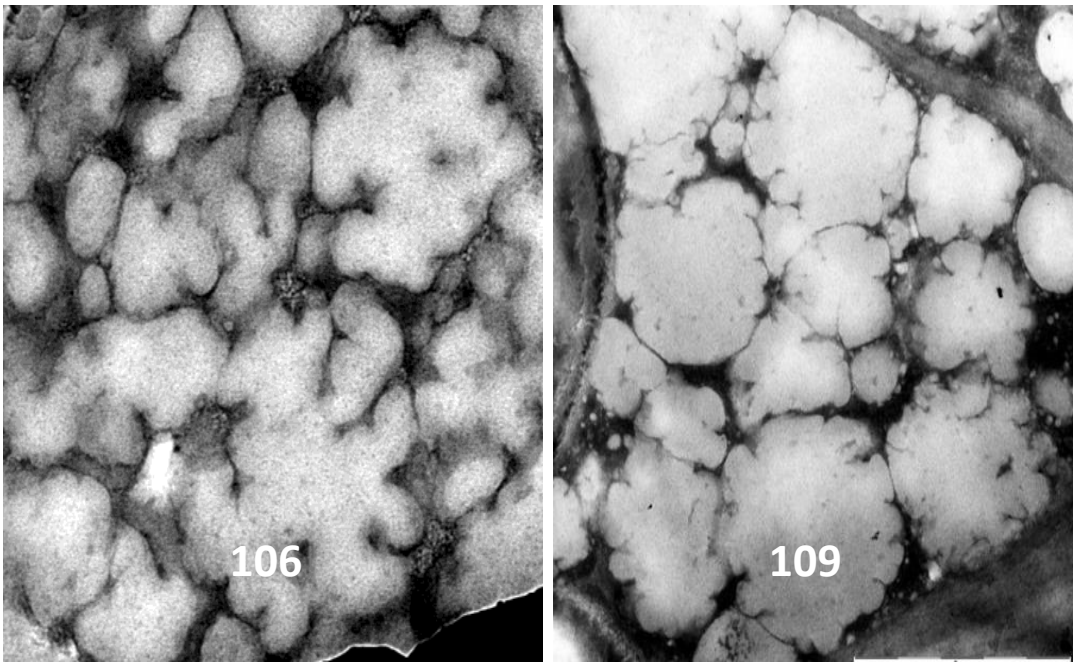

Figure 4.1.2 Endosperm texture and structure of waxy and high protein digestibility sorghum lines: TEM of protein bodies

106 – non-waxy , 109- waxy

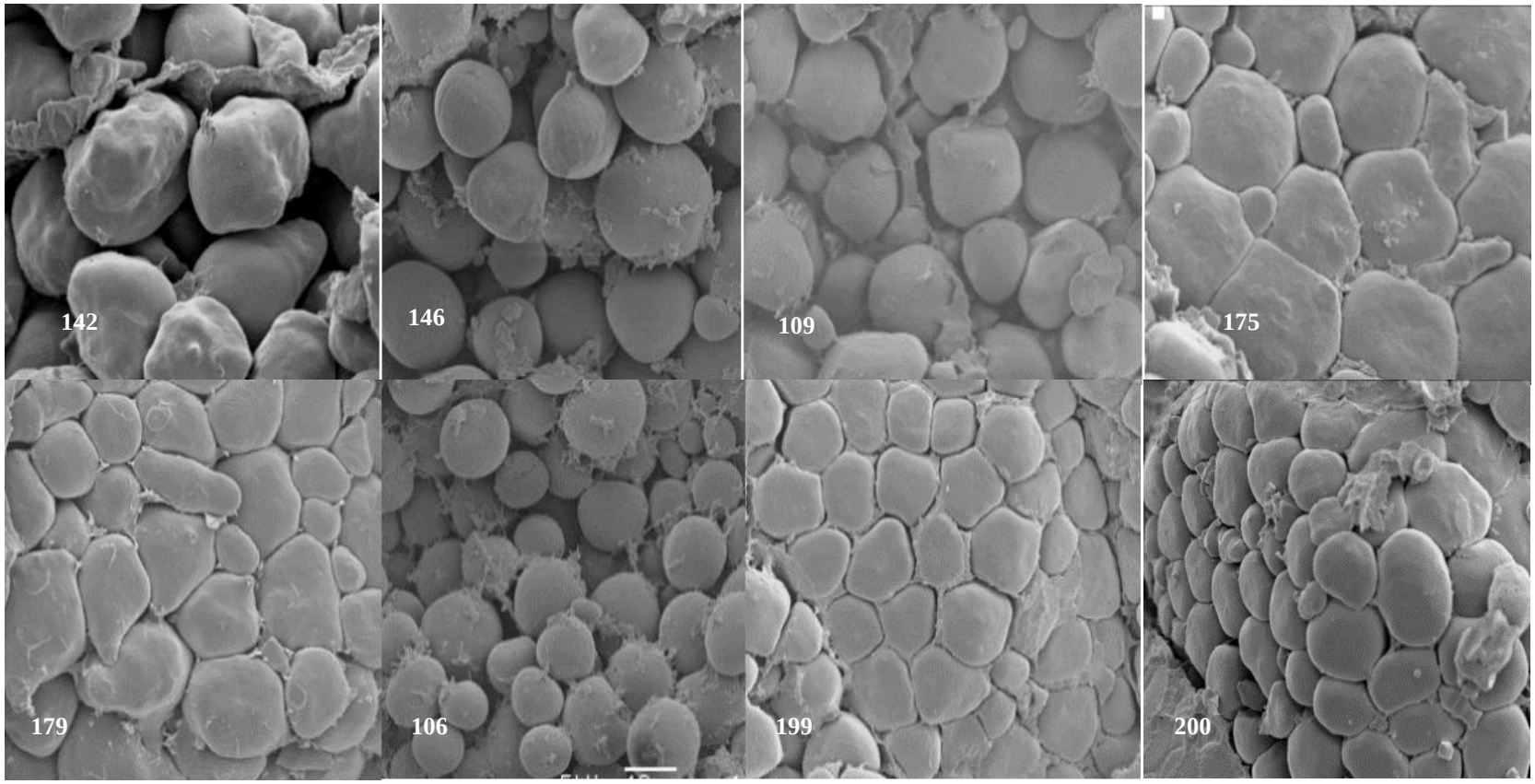

Figure 4.1.3 Endosperm texture and structure of waxy and high protein digestibility sorghum lines: SEM of starch granules

142, 146 and 109 - waxy-high protein digestibility; 175 and 179 - waxy, normal protein digestibility; 106 - non-waxy, high protein digestibility; 199 and 200 - non-waxy, normal protein digestibility

4.1.4.3 *Flour thermal properties*

Both the waxy trait and the high protein digestibility trait affected the sorghum flour endotherms (Table 4.1.2.A). The waxy trait significantly ($p<0.05$) increased the onset, peak and endset endotherm temperatures by approx. 2^{o}C, and the enthalpy by approx.1 J/g (Table 4.1.2.B). The high protein digestibility trait significantly ($p<0.05$) increased the onset temperature (by approx. 1^{o}C) but did not affect the peak and endset temperatures or the enthalpy. Overall, the waxy-high digestibility sorghums had the highest onset endotherm temperature and the non-waxy, normal protein digestibility sorghums had the lowest, or among the lowest, onset, peak and endset temperatures and least enthalpy. This was clearly illustrated by Principal Component Analysis (PCA) (Figure 4.1.4). The first PCA component, which accounted for 60.2% of the variation separated the waxy-high protein digestibility lines on the left side of the plot and the non-waxy-normal protein digestibility lines (including the standard hybrid MR Buster) on the right hand side of the plot.

The higher endotherm temperatures and larger enthalpy of the flours from the sorghum lines with the waxy trait is in agreement with work where starches isolated from waxy and normal sorghum were examined (Sang et al., 2008). It was suggested by these authors that peak gelatinization temperature is an indicator of crystallite quality which is related to amylopectin double helix length. In support of this, they found that the low degree of polymerisation fraction (DP 6-15) of amylopectin was present in slightly higher proportion in waxy sorghum starch compared to normal sorghum starch. The higher onset temperature of the high protein digestibility lines suggests that the floury texture resulting from this trait impacted on the gelatinization of the starch. Possibly, it resulted in increased competition for available water, thus delaying the onset of change in molecular order in the starch granules.

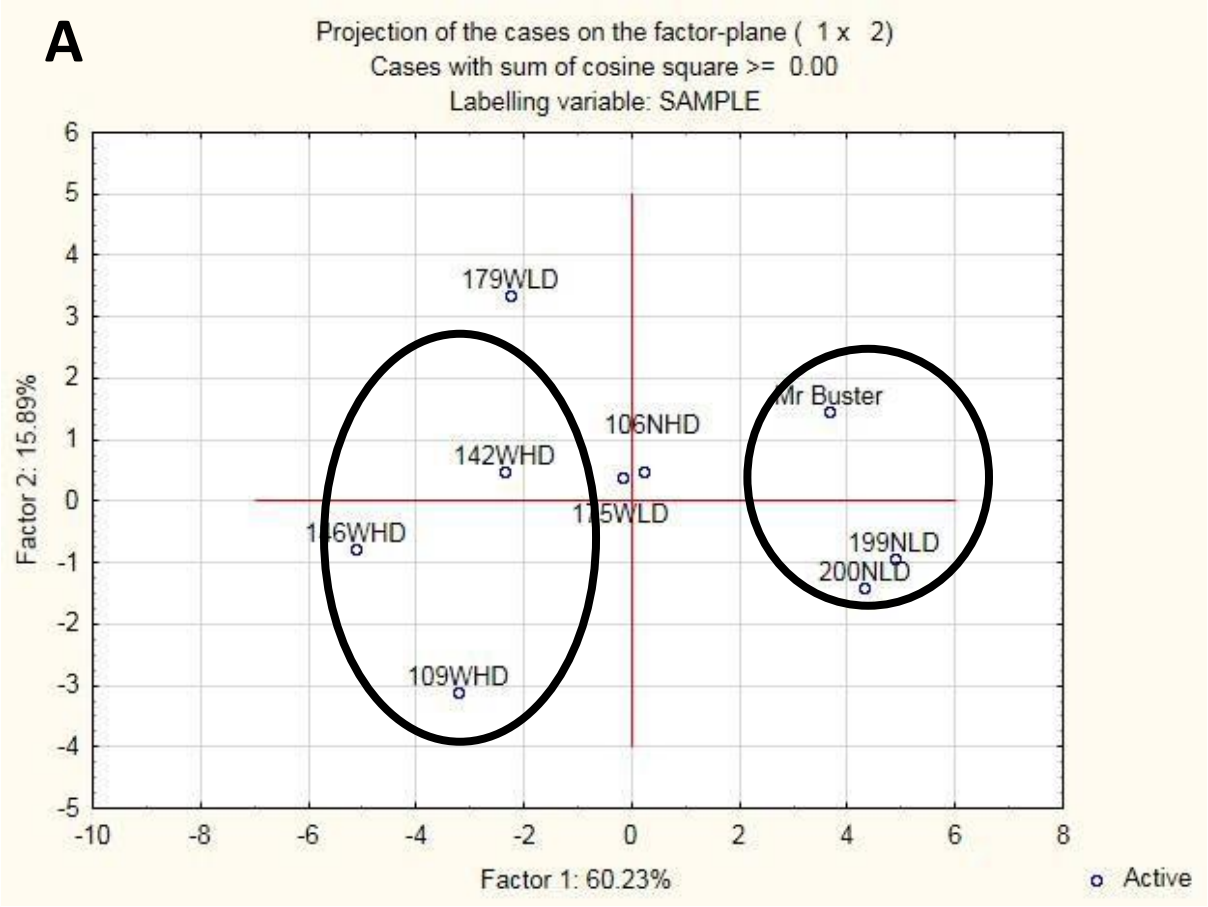

Figure 4.1.4 PCA showing the correlations between the sorghum lines with the different traits and starch type, protein digestibility, flour thermal properties, pasting and gel properties and WAI and WSF

Figure 4.1.4.A Sample scores

WHD- waxy, high protein digestibility, WLD-

waxy, low (normal) protein ddigestibility,
NHD- non-waxy, high protein digestibility, NLD- non-waxy, low (normal) protein digestibility

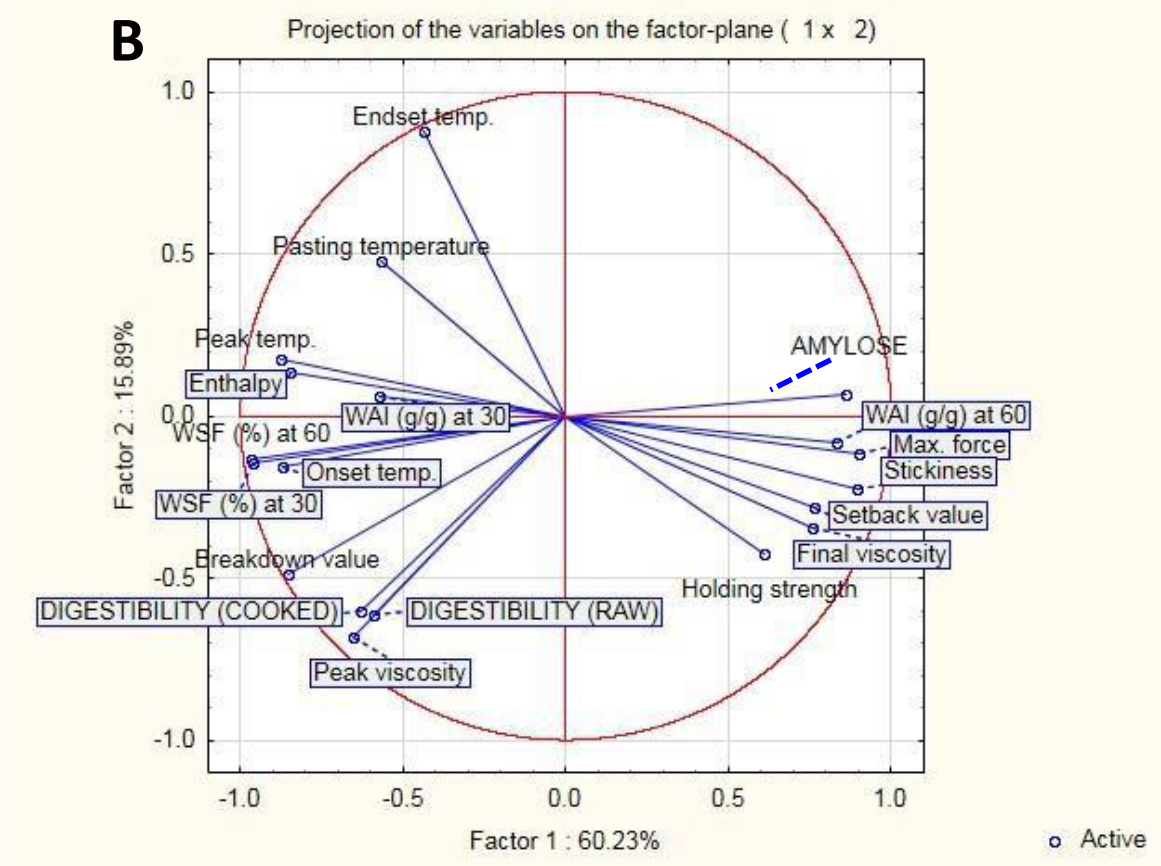

Figure 4.1.4.B PCA, loadings

Table 4.1.2.A Thermal properties of waxy and high protein digestibility sorghum lines and their controls and normal red sorghum cultivar MR Buster. A: Onset, peak, endset temperatures and enthalpy

Line	Starch type	Protein digestibility trait	Onset temp. (℃)	Peak temp. (℃)	Endset temp. (℃)	Enthalpy (J/g)
109	Waxy	High	71.67^{d}	76.44^{c}	84.10^{ab}	4.48^{a}
142	Waxy	High	71.81^{d}	77.50^{e}	87.95^{d}	4.35^{a}
146	Waxy	High	72.87^{e}	77.82^{e}	86.54^{c}	4.35^{a}
175	Waxy	Normal	70.64^{c}	77.10^{d}	86.44^{c}	3.73^{b}
179	Waxy	Normal	71.63^{d}	77.66^{e}	89.79^{e}	4.82^{a}
106	Non-waxy	High	70.15^{c}	76.45^{c}	86.31^{c}	3.34^{c}
199	Non-waxy	Normal	69.58^{b}	75.00^{b}	84.71^{b}	3.36^{bc}
200	Non-waxy	Normal	69.65^{b}	75.33^{b}	83.57^{a}	3.35^{c}
MR Buster	Non-waxy	Normal	67.23^{a}	74.15^{a}	86.61^{c}	3.28^{c}

Means with different superscript letters within a column are significantly different ($p < 0.05$).

n = 3

Table 4.1.2.B Thermal properties of waxy and high protein digestibility sorghum lines and their controls and normal red sorghum cultivar MR Buster. B: Effects of starch type and protein digestibility over the eight lines

Parameter	Protein digestibility trait	Starch type		Means for high or normal protein digestibility sorghums	Means for waxy and non-waxy sorghums	
		Waxy	Non-waxy		Waxy	Non-waxy
Onset (℃)	High	72.12^{c}	70.15^{a}	71.63^{b}	71.72^{b}	69.79^{a}
	Normal	71.14^{b}	69.61^{a}	70.37^{a}		
Peak (℃)	High	77.25^{c}	76.45^{b}	77.05^{a}	77.03^{b}	75.59^{a}
	Normal	77.38^{c}	75.16^{a}	76.27^{a}		
Endset (℃)	High	86.20^{b}	86.31^{abc}	86.23^{a}	86.96^{b}	84.67^{a}
	Normal	88.11^{c}	84.14^{a}	86.13^{a}		
Enthalpy (J/g)	High	4.43^{a}	3.34^{b}	4.15^{a}	4.37^{a}	3.33^{b}
	Normal	4.28^{a}	3.30^{b}	3.81^{a}		

Means with different superscript letters within a cell are significantly different ($p < 0.05$).

n = 3

4.1.4.4 *Flour pasting and gel properties*

Table 4.1.3 shows that the pasting temperatures of the flours from the sorghums with the various traits (69.4-71.2^{o}C) were not significantly different ($p \geq 0.05$). This pasting temperature range is very similar to the 67.9-70.3^{o}C found for starches isolated from Zimbabwean sorghums (Beta et al., 2000). However, the flours with the combined waxy-high protein digestibility traits (lines 109, 142 and 146) gave the highest paste peak viscosity ($p<0.05$), whereas only one of the waxy, normal protein digestibility lines (175) gave a higher peak viscosity than the non-waxy lines. All the waxy lines (normal and high protein digestibility) and the non-waxy-high protein digestibility waxy line (106) had lower paste holding strength than the two non-waxy-normal protein digestibility lines, but not compared to MR Buster, the red non-waxy, normal protein digestibility standard. These effects on peak viscosity and paste holding strength are also clearly illustrated by PCA (Figure 4.1.4). Paste holding strength is clearly associated with the two non-waxy- normal protein digestibility lines (199 and 200). The impacts of the high protein digestibility trait on sorghum flour pasting properties have not previously been described, although it has been found that both isolated waxy and heterowaxy sorghum starches (Sang et al., 2008) and their flours (Wu et al., 2010) had much higher peak viscosities than non-waxy sorghum.

In terms of flour gel texture, all the waxy lines (high and normal protein digestibility) and the non-waxy-high protein digestibility line (106) were far softer and less sticky ($p<0.05$) than the non-waxy, normal protein digestibility lines (Table 4.1.3). The findings with regard to the effects of the waxy trait on gelling are similar to those of Sang et al. (2008), who observed that whereas isolated non-waxy sorghum starch formed a strong gel, heterowaxy sorghum starch only formed a very weak gel and waxy sorghum starch remained a paste. The same effect of the high protein digestibility trait on sorghum flour gel properties has not been previously reported. The very soft, non-sticky gel texture of all the lines with the high protein digestibility trait is probably related to their floury endosperm texture. The floury endosperm of the high protein digestibility sorghums may have resulted from the reduction of β-kafirin, which is hydrophobic (Belton et al., 2006). It is rich in cysteine (10 cysteine residues/mole) (Belton et al., 2006). Therefore, reduction of the β-kafirin would increase the hydrophilicity of sorghum protein, which could have resulted in greater water holding for longer time during the gelling period. This may have delayed the hardening of the paste to make a gel.

Table 4.1.3 Pasting properties and gel texture characteristics of waxy and high protein digestibility sorghum lines and their controls and normal red sorghum cultivar MR Buster

Line	Starch type	Protein digestibility trait	Pasting temperature (°C)	Peak viscosity (mPa.s)	Holding strength (mPa.s)	Breakdown value (mPa.s)	Setback value (mPa.s)	Final viscosity (mPa.s)	Max. force calc. at entire areas (N)	Stickiness (N)
109	Waxy	High	69.4^{a}	1759^{i}	909^{d}	850^{f}	478^{b}	1387^{b}	0.122^{a}	0.039^{c}
142	Waxy	High	70.2^{a}	1424^{g}	959^{e}	466^{d}	505^{b}	1463^{c}	0.125^{a}	0.045^{c}
146	Waxy	High	71.2^{a}	1557^{h}	801^{a}	756^{e}	389^{a}	1190^{a}	0.125^{a}	0.038^{c}
175	Waxy	Normal	70.9^{a}	1394^{f}	950^{e}	444^{d}	868^{e}	1818^{e}	0.215^{ab}	0.076^{c}
179	Waxy	Normal	71.1^{a}	1099^{c}	819^{b}	279^{c}	392^{a}	1212^{a}	0.123^{a}	0.045^{c}
106	Non-waxy	High	70.1^{a}	1066^{b}	807a^{b}	259^{c}	714^{d}	1521^{d}	0.238^{b}	0.081^{c}
199	Non-waxy	Normal	69.0^{a}	1147^{d}	1003^{f}	143^{b}	903^{f}	1907^{f}	2.609^{e}	0.300^{a}
200	Non-waxy	Normal	70.2^{a}	1267^{e}	1131^{g}	136^{b}	980^{g}	2110^{g}	1.754^{c}	0.232^{ab}
MR Buster	Non-waxy	Normal	69.7^{a}	879^{a}	874^{c}	5^{a}	537^{c}	1411^{b}	1.996^{d}	0.136^{bc}

Means with different superscript letters within a column are significantly different ($p < 0.05$).

4.1.4.5 *Flour water absorption and solubility*

Both the waxy and high protein digestibility traits significantly affected flour WAI and WSF ($p<0.05$) (Table 4.1.4). Overall, at 30°C, flours of the high protein digestibility lines had slightly higher WAI (Table 4.1.4.B) The higher WAI of the high protein digestibility lines is presumably related to their floury endosperm texture (Figure 4.1.1). As explained, thereduction in β-kafirin in the floury endosperm may have reduced the hydrophobicity of sorghum protein. In contrast, at 60°C, just below the sorghum starch gelatinization temperature (Delcour and Hoseney, 2010), the non-waxy lines had slightly higher WAI (Table 4.1.4.B). This may be related to the lower endotherm temperature of the non-waxy lines (Table 4.1.2.B). Heat would break the hydrogen bonds between starch molecules, enabling binding of more water. In case of the waxy lines, there was need for greater thermal energy as their amylopectin clusters would be tightly bound with each other, which would result in reduction of WAI compared to non-waxy sorghum lines.

The effects of the traits on flour WSF were much greater. Both the waxy and the high protein digestibility traits increased WSF by approx. 60% (Table 4.1.4.B). Hence, at both 30 and 60°C, on average, the WSF of the combined waxy-high protein digestibility lines was more than twice that of the non-waxy-normal protein digestibility lines (Table 4.1.4.A), and the combined waxy-high protein lines were strongly associated with a high WSF (Figure 4.1.4). In fact, at 30°C the WSF of the flours of these sorghum lines was similar to that of the wheat flours. At 60°C the WSF of the waxy-high protein digestibility lines was substantially higher than the wheat flours at 30°C. However, it was lower than that of the wheat flours at 60°C, but this was as a consequence of the wheat starch gelatinizing at this temperature (Delcour and Hoseney, 2010).

The high water solubility of the flours of the combined waxy-high protein digestibility lines seems to be related their floury (less dense) endosperm texture (Figures 4.1.1 and 4.1.3), high amylopectin content and also their unique endosperm protein composition. It has been shown that sorghum lines with this conventionally bred high protein digestibility trait, although having a similar protein content to normal digestibility types, have a much lower proportion of kafirin proteins, which are water insoluble, relative to total protein (Da Silva et al., 2011a), approximately 33% compared to 40-50% in normal sorghums.

Table 4.1.4.A Water Absorption Index (WAI) and Water Soluble Fraction (WSF) of waxy and high protein digestibility sorghum lines and their controls and normal red sorghum cultivar MR Buster.

A: WAI and WSF at 30°C and 60°C

Line/name	Starch type	Protein digestibility	WAI (g/g) at 30°C	WAI (g/g) at 60°C	WSF (%) at 30°C	WSF (%) at 60°C
109	Waxy	High	2.55^{de}	2.65^{ab}	8.24^{e}	9.92^{d}
142	Waxy	High	2.59^{f}	2.72^{bc}	8.45^{ef}	9.87^{d}
146	Waxy	High	2.63^{f}	2.68^{ab}	9.88^{g}	12.02^{e}
175	Waxy	Normal	2.55^{de}	2.69^{ab}	5.96^{c}	6.89^{b}
179	Waxy	Normal	2.52^{cde}	2.58^{a}	6.71^{d}	8.13^{c}
106	Non-waxy	High	2.61^{f}	2.82^{cd}	6.21^{c}	7.15^{b}
199	Non-waxy	Normal	2.49^{c}	2.91^{de}	4.21^{b}	4.83^{a}
200	Non-waxy	Normal	2.51^{cd}	2.82^{cd}	3.95^{a}	4.31^{a}
MR Buster	Non-waxy	Normal	2.58^{ef}	2.96^{e}	4.02^{ab}	4.57^{a}
***Golden Cloud**	NA	NA	2.06^{a}	3.24^{f}	6.92^{d}	18.46^{g}
***Snow Flake**	NA	NA	2.13^{b}	3.59^{g}	8.52^{f}	15.50^{f}

Means with different superscript letters within a column are significantly different ($p < 0.05$). n = 2,

*Wheat bread flour, NA = not applicable

Table 4.1.4.B Water Absorption Index (WAI) and Water Soluble Fraction (WSF) of waxy and high protein digestibility sorghum lines and their controls and normal red sorghum cultivar MR Buster.

B: Effects of starch type and protein digestibility individually and in combination on WAI and WSF

Parameter	Protein digestibility trait	Starch type		Means for high and normal protein digestibility sorghums	Means for waxy and non-waxy sorghums	
		Waxy	Non-waxy		Waxy	Non-waxy
WAI at 30°C	High	2.59^{b}	2.61^{b}	2.60^{b}	2.57^{a}	2.54^{a}
	Normal	2.54^{a}	2.50^{a}	2.52^{a}		
WAI at 60°C	High	2.68^{a}	2.82^{b}	2.72^{a}	2.66^{a}	2.85^{b}
	Normal	2.63^{a}	2.86^{b}	2.75^{a}		
WSF at 30°C	High	8.86^{c}	6.21^{b}	8.20^{b}	7.85^{b}	4.79^{a}
	Normal	6.34^{b}	4.08^{a}	5.21^{a}		
WSF at 60°C	High	10.61^{c}	7.15^{b}	9.74^{b}	9.37^{b}	5.43^{a}
	Normal	7.51^{b}	4.57^{a}	6.04^{a}		

Means with different superscript letters within a cell are significantly different ($p < 0.05$). n = 2

4.1.5 Conclusions

The novel biofortified sorghum lines with combined waxy and high protein digestibility traits have much higher flour water solubility, high pasting viscosity and form much softer and less sticky pastes than regular non-waxy, normal protein digestibility sorghums. Hence, their flours may have better properties for making dough-based food products.

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4.2 EFFECTS OF GENETICALLY MODIFIED SORGHUMS WITH SUPPRESSED GAMMA-KAFIRIN SYNTHESIS ON THEIR FLOUR AND DOUGH RHEOLOGICAL CHARACTERISTICS

4.2.1 Abstract

Gamma-kafirin synthesis suppression in two genetically modified sorghum (GM-HD) lines of high protein digestibility (PD) trait and their null (N) controls was investigated to determine the effect of altered kafirin on sorghum flour properties for bread making. Generally, GM-HD and N sorghums had a starch amylose content in the range 19-20% except GM-HD-2 which had a low starch amylose content of only 15%. As expected, the raw flour PD of GM-HD was 23.5% higher than N sorghums, while, cooked flour PD of GM-HD was 27% higher than N lines. GM-HD had a slightly and significantly ($p < 0.05$) higher flour water soluble fraction (WSF) compared to N sorghums. At 30°C the WSF of GM-HD and N sorghums were 6 % and 5.1 %, while at 60°C WSF were 6.4 % and 5.9 %, respectively. Peak viscosity (PV) and holding strength of GM-HD during pasting were also significantly higher than N sorghums, while pasting temperature and setback of GM-HD were significantly lower than N sorghums. The GM-HD had PV 41 % higher and pasting temperature 13 % lower than N sorghums. There was no significant difference between GM-HD and N sorghums in terms of thermal properties of their flours as well as the texture of their gels after pasting. CLSM revealed that GM-HD had a less compact endosperm protein matrix surrounding the starch compared to N sorghums in their slurries and pastes. Dough rheology showed that the storage modulus (G′) and loss modulus (G″) of GM-HD were higher than N sorghum doughs during amplitude sweep and temperature sweep analysis which means higher viscoelasticity. GM-HD sorghums had stronger dough and shorter relaxation time than N during stress relaxation. The improvements that occurred in flour and dough properties of GM-HD indicate that kafirin suppression can be considered as a factor in sorghum flour quality.

4.2.2 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a staple food of over 300 million people in Africa (Bennetzen et al., 2001). Inferior sorghum protein nutritional quality particularly in terms of its deficiency in the essential amino acid lysine and low protein digestibility can be considered as one of the reasons that limit its utilization when compared to wheat (Taylor and Taylor, 2011). The other main problem in sorghum kafirin protein is that it does not have viscoelastic properties like wheat gluten (Dahir et al., 2015). Absence of the viscoelastic property prevents dough formation that is suitable for leavened bread making. This is why sorghum is generally used for local and traditional food making only (Taylor and Taylor, 2011). In 2009, the Africa Biofortified Sorghum (ABS) consortium developed genetically modified transgenic sorghums with improved nutritional quality (Biosorghum (2010). The aim of the ABS project was to produce biofortified sorghum lines in terms of improved digestibility and nutritional quality of protein and micronutrient content and availability using genetic engineering techniques (Henley et al., 2010). These genetically modified sorghums have improved lysine content and protein digestibility due to suppression of synthesis of specific kafirin storage proteins. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) of the genetically modified sorghums was doubled compared to the null control (Henley et al., 2010). Recombinant DNA technology (genetic engineering) using Agrobacterium gene transfer was used to produce the ABS where the gene responsible for γ-kafirin synthesis was supressed (Da Silva et al., 2011b).

This work was undertaken to corroborate the findings in Chapter 4.1 that waxy-high protein digestibility (WHD) sorghum produced by conventional breeding had improved flour quality. This current study is important because the ABS sorghums with improved protein quality are compared directly with their null controls which makes the comparison more accurate than the study with conventionally bred high protein digestibility sorghums. Furthermore, any improvement in the functional properties of the flour will improve the commercial utility of these ABS sorghums. The objective of this study was to determine the effect of the suppression of γ-kafirin synthesis on sorghum flour functionalities and dough characteristics such as pasting profile, flour water absorption and solubility and dough quality.

4.2.3 Materials and Methods

4.2.3.1 *Sorghum samples*

Crushed grain of two genetically modified (GM-HD) sorghums (GM-HD-1: 26206 and GM-HD-2: 26234) which were produced by the ABS project consortium and their null controls (N1: 26241and N2: 26236) were investigated. The GM-HD sorghums were produced through recombinant DNA technology and they expressed suppressed kafirin synthesis particularly of the γ-kafirin sub-class (Henley et al., 2010; Da Silva et al., 2011b).

4.2.3.2 *Sorghum milling*

A laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) fitted with a 250 μm opening screen was used to mill each line separately to give whole grain sorghum flour. The milled flours were then kept at 10°C until used for the analysis.

4.2.3.3 *Protein content*

Dumas combustion was used to determine the protein content (N x 6.25) following AACC method 46-30 (AACC International, 2000).

4.2.3.4 *Starch amylose content*

The Megazyme amylose/amylopectin assay kit (Megazyme Ireland International, Bray, Ireland) was used to measure amylose/amylopectin ratio as described in Chapter 4.1.

4.2.3.5 *In vitro pepsin protein digestibility*

In vitro protein digestibility of the flours was determined according to the pepsin digestibility method of Hamaker et al. (1986) as modified by Da Silva et al. (2011b) as detailed in Chapter 4.1.

4.2.3.6 *Differential scanning calorimetry (DSC) of flour thermal behaviour*

The method described by Beta et al. (2000) was used as described in Chapter 4.1.

4.2.3.7 *Flour pasting profile*

Using the rheometer and heating programme as in Chapter 4.1, slurries of the samples were analysed to determine their pasting properties.

4.2.3.8 *Gel strength (texture)*

A TA-XT2 texture analyser was similarly used as in Chapter 4.1 to determine flour gel texture.

4.2.3.9 *Flour WAI and WSF*

As in Chapter 4.1 the method described by Anderson et al. (1970) was used to determine Water absorption index (WAI) and water soluble fraction (WSF) of the GM-HD sorghum flours and their null controls at 30°C and 60°C.

4.2.3.10 *Stress relaxation behaviour of doughs*

The relaxation properties of the sorghum dough were determined according to the method of Singh et al. (2006) as modified by Falade et al. (2014). A texture analyser (EZ-L, Shimadzu, Kyoto, Japan) was used. Sorghum dough was prepared with 1 g flour and 0.9 g water. Homogeneous discs of doughs of diameter 19 mm and height 7 mm were made using a syringe. To compress the dough disc, a plastic rod (43 mm diameter and 10 mm height) was used at a 25% strain to compress the sorghum dough for 5 s, then the dough was left to relax over a period of 180 s and the maximum force was recorded. Relaxation time (RT) was calculated as the time required for the maximum force to drop to 36.8% of its value (Singh et al., 2006).

4.2.3.11 *Dynamic rheological analysis*

A Physica MCR 101 Rheometer (Anton Paar, Ostfildern, Germany) was used for dynamic rheological analysis as described by Falade et al. (2014). A parallel plate configuration (PP25) of 25 mm diameter and 2 mm gap between the top and bottom plates was used. Sorghum dough was prepared with 1:1 (w/w) flour: water. Dough samples were placed between the parallel plates and the edges of the dough pieces were trimmed using a thin spatula. Oscillatory measurements were performed in two steps. Amplitude sweep was measured at constant temperature and at constant frequency (6.3 rad/s) within the linear viscoelastic range (LVE). Storage shear modulus (G′) and the loss shear modulus (G″), and damping factor/loss tangent (tan δ=G″/G′) were recorded at increasing strain from 0.01 to 100%. Temperature sweep was determined as a dough baking process simulation at the same constant frequency as the amplitude sweep analysis. A constant strain of 0.1% was applied to measure the temperature sweep in the LVE as above. A temperature range from 25 to 150°C for 20 min at a heating rate of 6.25°C/min was applied. Mineral oil (paraffin) was used to cover the edges of the dough samples to prevent dehydration.

4.2.3.12 *Confocal laser scanning microscopy (CLSM)*

Confocal laser scanning microscopy (CLSM) Zeiss 510 META system (Jena, Germany) with a Plan-Neofluar 10 × 0.3 objective under natural fluorescence at an excitation wavelength of 405

nm was used. Water (4 g) was added to 1 g sorghum flour to prepare a slurry. The slurries were incubated in test tubes at 75°C for 25 minutes in a water bath. They were stirred every 5 minutes using a glass rod. A layer of about 2 mm thickness of each slurry was placed on a glass slide and left for 3 minutes at ambient temperature. Approx. three drops 0.02% Acid Fuchsin dye in 1% acetic acid was added to the slurries to stain the kafirin protein (Autio et al., 2005). The samples were then incubated for 1 minute in an oven at 60°C to fix the dye before CLSM.

4.2.3.13 *Statistical analysis*

Data were analysed using IBM SPSS Statistics 22 (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was applied at a confidence level of $p = 0.05$ to determine the effect of the kafirin alteration on the applicable parameters. Means were compared by Fisher's least significant difference (LSD) test. Each experiment was repeated at least two times.

4.2.4 Results and discussion

4.2.4.1 *Starch amylose content*

GM-HD-1, N1 and N2 had a normal starch amylose content in the range of 19-20.1% (Table 4.2.1). GM-HD-2 displayed a heterowaxy trait with a starch amylose content of 14.9%. This heterowaxy trait could be due to an accidental mutation during the genetic modification process. Hence, these GM-HD sorghums were not waxy (one of them had normal starch while the other one had heterowaxy starch) unlike the WHD sorghum lines in Chapter 4.1 which were waxy sorghums.

4.2.4.2 *Protein digestibility*

Table 4.2.1 shows as expected, the GM-HD sorghums had significantly higher ($p < 0.05$) in vitro protein digestibility than their null controls in both raw and cooked flour. These findings are similar to those of Da Silva et al. (2011b) where the authors reported that the suppression of γ-kafirin synthesis in ABS sorghums resulted in high protein digestibility. The cooked sorghum flours in general had lower protein digestibility than the raw flours. Protein digestibility of uncooked GM-HD sorghum flours was about 25% higher than their uncooked null control, while cooked flour of GM-HD sorghums had approx. 16% higher protein digestibility than cooked flours of their null controls. This increase in the protein digestibility of the GM-HD sorghum compared to their N controls, has been ascribed to alteration in kafirin expression (Henley et al., 2010). The suppression of γ-kafirin synthesis resulted in absence of or reduced expression of this sub-class of kafirin (Da

Silva et al., 2011a). Hence, the low digestibility of the normal sorghums is related to the presence of γ-kafirin (Da Silva et al., 2011b) due to its disulphide cross-linking property. This parallels where the higher protein digestibility of conventionally bred HD sorghum lines in Chapter 4.1 was also attributed to kafirin modification.

4.2.4.3 *Thermal characteristics*

The general trend of the thermal flour parameters (Onset (T_0), peak (T_p) and conclusion (T_c) temperatures and enthalpy (ΔH)) measured by DSC was that the GM-HD sorghums had no clear difference in terms of thermal properties compared to their N controls (Table 4.2.1). Thermal properties have been found to be affected by the starch type (Sang et al., 2008). In Chapter 4.1 the waxy trait significantly ($p < 0.05$) increased the thermal properties of the WHD sorghum flour. However, this current finding indicated that the thermal properties may also be affected by kafirin suppression. This means it might be there was also an effect of kafirin modification in WHD sorghum lines alongside waxy trait on the increase of thermal properties in Chapter 4.1.

Table 4.2.1 Starch amylose content, in vitro pepsin protein digestibility and thermal properties of the genetically modified sorghums and their null controls

Sorghum type	Amylose (%)	Protein digestibility trait	Protein digestibility of raw flour (%)	Protein digestibility of cooked flour (%)	Onset temp. (℃)	Peak temp. (℃)	Endset temp. (℃)	Enthalpy (J/g)
GM-HD-1	19.3^{b} ±0.0	High	89.5^{b} ± 0.5	73.7^{b} ± 0.7	70.44^{b} ±0.17	75.59^{b} ± 0.12	81.79^{b} ±0.40	3.1^{b} ± 0.2
GM-HD-2	14.9^{a} ±0.1	High	90.2^{b} ± 0.6	70.5^{b} ± 1.8	69.74^{a} ±0.06	75.00^{ab} ±0.47	81.39^{ab} ±0.48	3.1^{b} ± 0.0
N1	19.0^{b} ±1.3	Normal	68.4^{a} ± 1.0	52.0^{a} ± 2.0	69.39^{a} ±0.01	74.92^{ab} ±0.12	81.43^{ab} ±0.13	2.7^{a} ± 0.1
N2	20.1^{b} ±0.0	Normal	69.4^{a} ± 2.0	54.3^{a} ± 3.3	69.37^{a} ±0.20	74.59^{a} ±0.12	80.74^{a} ±0.20	3.0^{b} ± 0.1

Means with different superscript letters within a column are significantly different ($p < 0.05$).

n = 2, for PD n= 3

4.2.4.4 *Pasting profile and gel properties*

GM-HD sorghums had significantly higher ($p < 0.05$) peak viscosity, holding strength, breakdown and final viscosity than their null controls (Table 4.2.2 and Figure 4.2.1). The GM-HD sorghums also had significantly shorter peak time and lower pasting temperature and setback. Peak viscosity of GM-HD sorghums was about 41% higher than their null controls. The higher peak viscosity of the GM-HD sorghums can be attributed to the loose or non-compact protein matrix in these mutants (Figures 4.2.7 and 4.2.9). The loose protein matrix was found also in the HD sorghum lines (Chapter 4.1). The loose protein matrix presumably resulted in better interaction between the protein network and starch (Figures 4.2.7 and 4.2.9) to form better consistency of dough of higher viscosity. The sorghum protein matrix in the normal sorghum can act as a barrier and retard starch granule expansion (Ezeogu et al., 2008). This protein barrier was presumably the reason for the longer peak time and higher pasting temperature of the N controls compared to GM-HD sorghums. When the protein matrix is loose this would provide more space for the starch granules to swell and lead to higher peak viscosity. GM-HD-2 had significantly higher peak viscosity than its counterpart GM-HD-1 (Table 4.2.2). This was probably due to the heterowaxy trait of GM-DH-2. A similar result was obtained in Chapter 4.1 where sorghum lines with waxy (low amylose content) displayed higher peak viscosity. The pasting temperature of the GM-HD sorghums was some 10^{o}C lower than that of their null controls (Table 4.2.2). This is in contrast to what was found in Chapter 1 where the WHD had lower final viscosity compared to normal sorghum of low PD, the GM-HD sorghums had higher final viscosity than their null controls. This indicates that in the absence of the waxy trait paste final viscosity which paralleled the retrogradation behaviour, the high PD trait due to kafirin alteration increased the final viscosity of the sorghum paste.

Gel texture (Table 4.2.2) showed no significant difference between GM-HD lines and their null controls in terms of their flour gel strength. However, GM-HD-2 with heterowaxy trait showed the lowest gel strength which agree with finding in Chapter 4.1 where the sorghum lines with lower amylose content had lower gel strength. This can be attributed to the slow retrogradation of

Table 4.2.2 Pasting characteristics, gel texture and dough relaxation of the genetically modified sorghums and their null controls

Sorghum type	Protein digestibility trait	Peak time min	Pasting temp. (°C)	Peak viscosity (mPa.s)	Holding strength (mPa.s)	Breakdown (mPa.s)	Setback (mPa.s)	Final viscosity (mPa.s)	Max. force gel texture (N)	Max. force dough relaxation (N)
GM-HD-1	High	5.70^{a} ±0.09	74^{a} ±1	1985^{b} ±9	1776^{b} ±5	209^{b} ±4	120^{b} ±4	1656^{c} ±9	0.17^{a} ±0.01	2.00^{b}±0.02
GM-HD-2	High	5.50^{a} ±0.07	73^{a} ±0	2149^{c} ±12	1786^{b} ±16	363^{c} ±5	127^{b} ±3	1659^{c} ±14	0.14^{a} ±0.01	2.37^{b} ±0.07
N1	Normal	7.36^{b} ±0.11	84^{b} ±1	1472^{a} ±4	1405^{a} ±12	67^{a} ±8	148^{b}±24	1257^{a} ±36	0.17^{a} ±0.02	1.10^{a} ±0.10
N2	Normal	7.49^{b} ±0.07	85^{b} ±0	1458^{a} ±12	1389^{a} ±8	69^{a} ±4	58^{a} ±8	1331^{b} ±0	0.15^{a} ±0.01	1.21^{a} ±0.03

Means with different superscript letters within a column are significantly different ($p < 0.05$).

n = 2

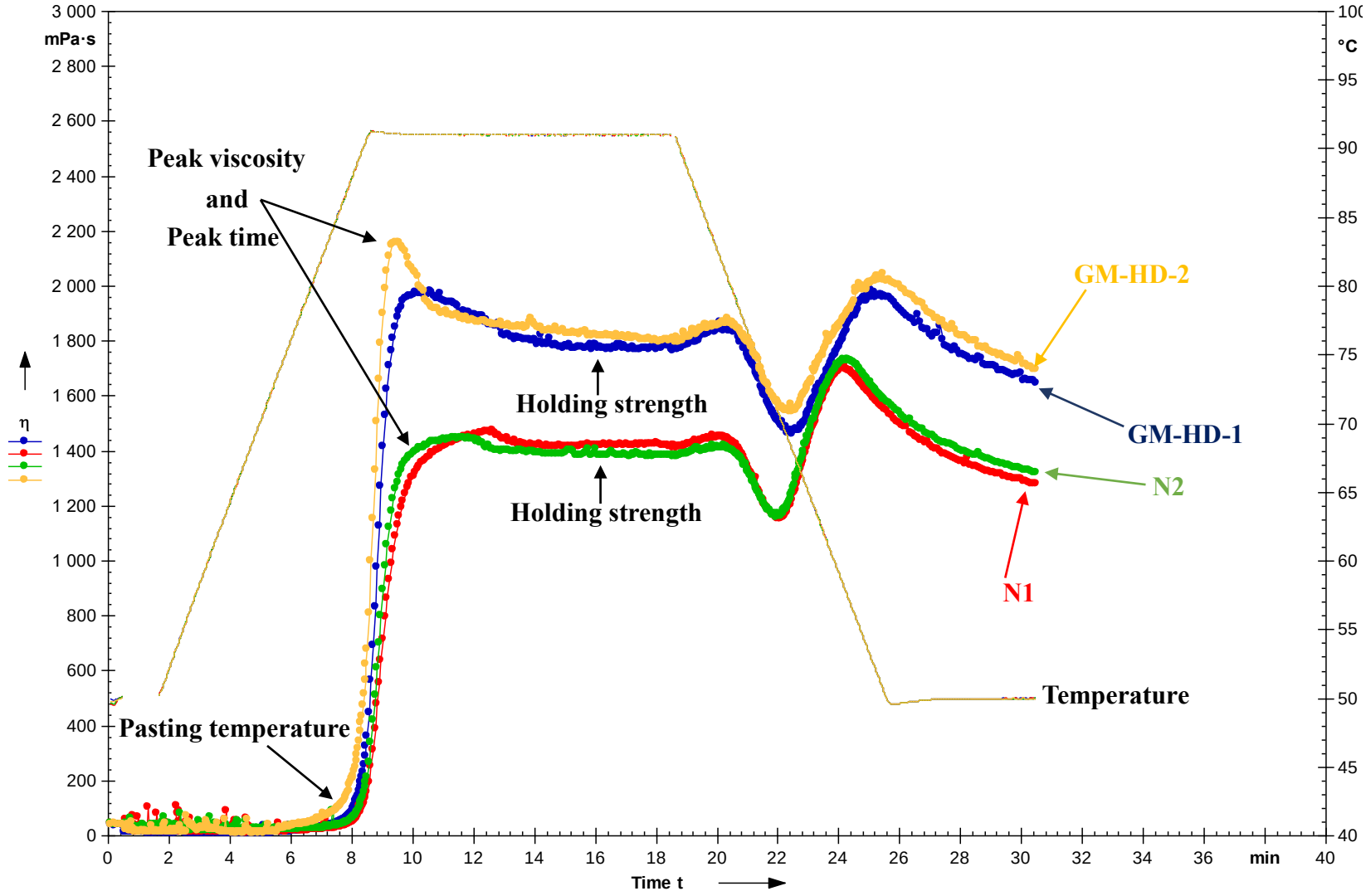

Figure 4.2.1 Pasting curves of the GM-HD sorghums and their null controls showing the general trend of higher viscosity of the GM-HD sorghum

amylopectin compared to amylose. Sang et al. (2008) found that normal sorghum starch retrogrades more rapidly than heterowaxy and waxy sorghum starches. This current study showed that the gel texture of sorghum flour was affected by starch type more than protein digestibility.

4.2.4.5 *Water absorption and solubility*

Table 4.2.3 shows that GM-HD sorghums had a significantly higher ($p < 0.05$) water soluble fraction (WSF) than their null controls at both 30°C and 60°C. At 30°C, the GM-HD sorghums had WSF of approx. 18% higher than N sorghums. GM-HD-2 had a higher WSF than GM-HD-1 which can be ascribed to the higher amylopectin content of the GM-HD-2 (Table 4.2.1). In waxy type starch the amylopectin branches can bind with more water molecules through hydrogen bonding (Wootton and Bamunuarachchi, 1978). At 60°C, GM-HD-1 had a significantly higher WSF than N1 but a lower WSF than N2. The reason why GM-HD-1 had a lower WSF than N2 could be that the HD trait (due to kafirin modification) made the GM sorghum to some extent less sensitive to heating. Since GM-HD-1 needed higher temperature to disassociate that means there were less available sites for hydrogen bonding to occur with water. The GM-HD-2 at 60°C in contrast, had significantly higher WSF (about 14%) than both N controls. The higher WSF of GM-HD-2 can be attributed to its heterowaxy trait as seen in Chapter 4.1 where there was increase in WSF in the waxy lines. The heterowaxy trait in GM-HD-2 overshadowed the effect of the higher thermal properties due to modified kafirin expression. The increase in the WSF of GM-HD sorghum flour was presumably due to the higher hydrophilicity, or strictly speaking reduction of the hydrophobicity of the GM-HD kafirin. Cystine is a hydrophobic amino acid (Nagano et al., 1999). When γ-kafirin expression which is rich in cystine is suppressed, the hydrophobicity would be reduced. Increasing the WSF may affect the dough consistency, where the higher WSF may increase the softness of the dough (Falade et al., 2014).

There was no clear difference between GM-HD lines and their null controls in terms of flour WAI.

Table 4.2.3 Water Absorption Index (WAI) and Water Soluble Fraction (WSF) at 30°C and 60°C of the genetically modified sorghums and their null controls

Sorghum type	Protein digestibility	WAI (g/g) at 30°C	WAI (g/g) at 60°C	WSF (%) at 30°C	WSF (%) at 60°C
GM-HD-1	**High**	**2.42^{a} ±0.30**	**2.34^{a} ±0.05**	**5.66^{c} ±0.06**	**5.97^{b} ±0.16**
GM-HD-2	**High**	**2.32^{a} ±0.04**	**2.53^{b} ±0.03**	**6.21^{d} ±0.14**	**6.68^{d} ±0.07**
N1	**Normal**	**2.31^{a} ±0.01**	**2.47^{b} ±0.00**	**4.92^{a} ±0.04**	**5.53^{a} ±0.03**
N2	**Normal**	**2.33^{a} ±0.00**	**2.46^{b} ±0.02**	**5.25^{b} ±0.01**	**6.34^{c} ±0.02**

Means with different superscript letters within a column are significantly different ($p < 0.05$).

n = 2

4.2.4.6 *Amplitude and temperature sweeps*

The storage modulus (G′) (Figure 4.2.2) of GM-HD and N control flours was higher than their loss modulus (G″) (Figure 4.2.3) in the amplitude sweep mode analysis. Higher G′ than G″ relates to a strong, more elastic dough as was found with maize dough (Falade et al., 2014). . Shear stress plotted against strain amplitude showed that the N sorghums had lower shear stress which can be due to their softer slurry compared to the GM-HD flour. A similar explanation was given by Falade et al. (2014) where maize dough containing sourdoughs had a lower shear stress than untreated maize dough. In this work, the softer dough related to a less elastic behaviour as indicated by the lower G′ of the N sorghums when compared to the GM-HD sorghums. Storage modulus (G′) andcomplex viscosity (η*) of the GM-HD sorghums were higher than their null controls in temperature sweep measurement, while there was no clear difference in terms of loss tangent (tan δ) (Figures 4.2.5, 4.2.6 and 4.2.7). Generally, G′ and η* behaved similarly during temperature sweep analysis. The storage modulus of the GM-HD lines were higher than their N controls within the range of 25 to about 90^{o}C and then started to decline to be similar or slightly lower than the G′ of the N sorghums. The lower G′ of GM-HD lines dough during the latter stages of the temperature sweep can be attributed to their more rapid drying compared to their null controls which resulted in dough hardening due to the decrease of the moisture in their doughs. Drying of the samples was observed after rheological analysis, where the GM-HD lines in particular showed signs of thermal damage and had become stiff. As stated, the modification of endosperm protein results in floury endosperm, the milling process may have resulted in a flour with finer particles compared to normal sorghum of intermediate or corneous endosperm. The smaller flour particles increased the surface area of flour that subjected to heat and due to this the evaporation of water (drying) was higher in GM-HD lines compared to normal sorghums. Complex viscosity of the GM-HD sorghums was higher than their N controls until the temperature reached about 90^{o}C and then the η* of GM-HD sorghums started declining to be lower than the N sorghums (Figure 4.2.6). The higher complex viscosity of the GM-HD sorghum dough compared to the N sorghums indicates the greater viscoelastic characteristics of the GM-HD lines, as explained by Edwards et al. (2003) with reference to durum semolina viscoelastic properties.

Alteration of kafirin composition by suppression of γ-kafirin synthesis in the GM-HD sorghums affected the dynamic rheological properties of their doughs. The change in kafirin composition improved the GM-HD sorghums dough rheological behaviour as it increased the

viscoelastic properties. That means kafirin modification had an impact on the sorghum properties as it would improve flour quality for bread making.

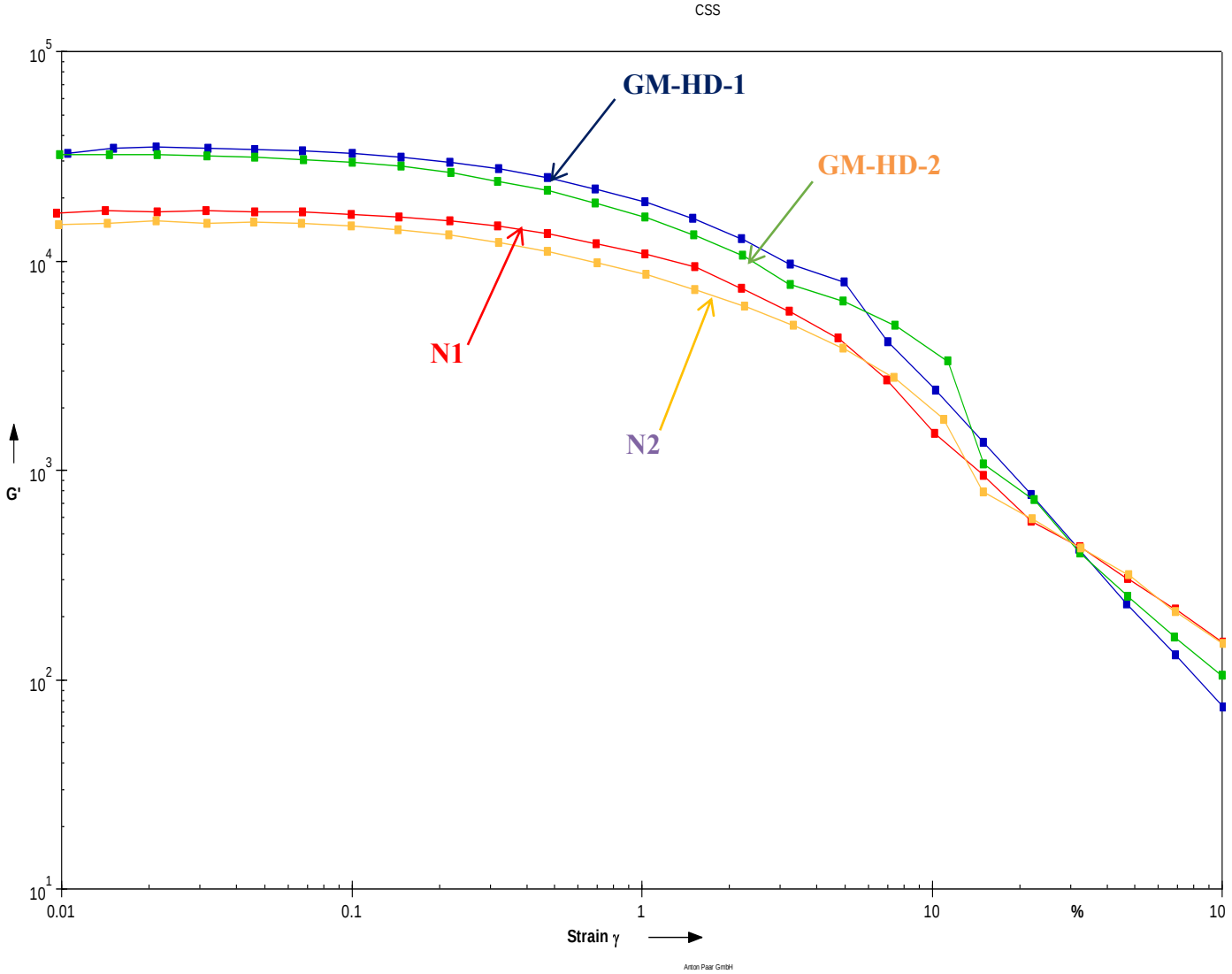

Figure 4.2.2 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as storage modulus through amplitude sweep mode within a strain of 0.01-100%

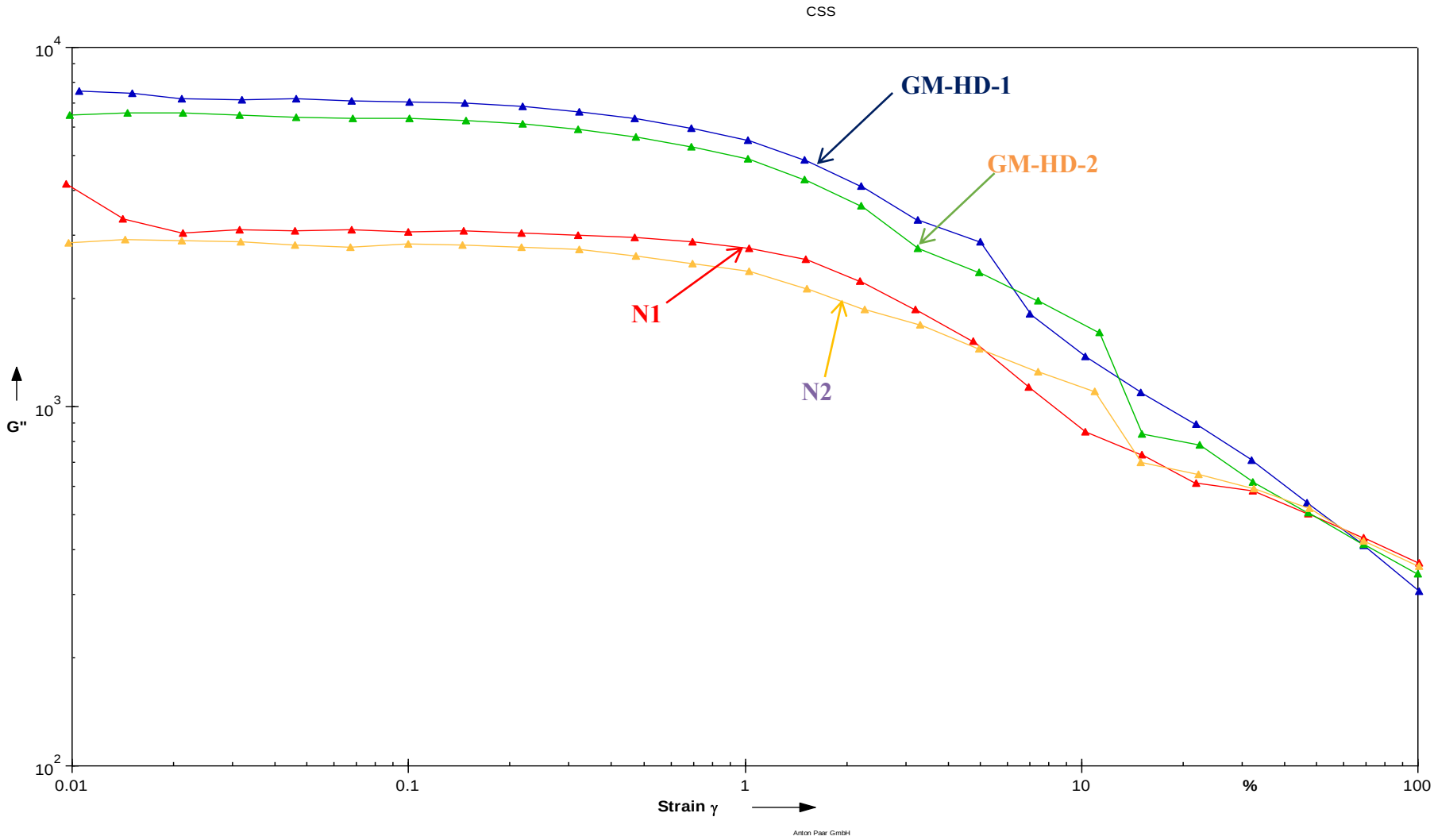

Figure 4.2.3 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as loss modulus through amplitude sweep mode within a strain of 0.01-100%

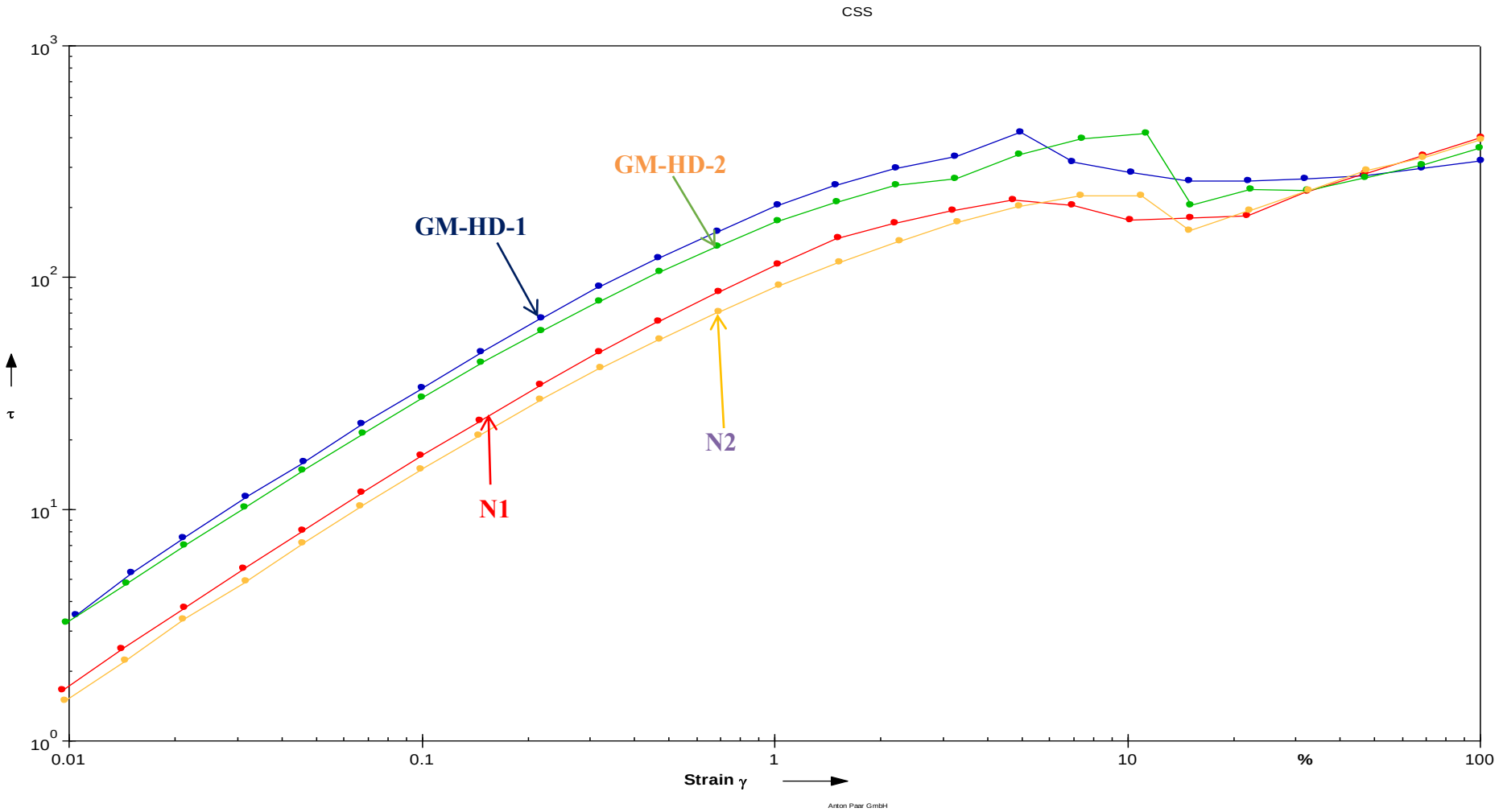

Figure 4.2.4 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as shear stress through amplitude sweep mode within a strain of 0.01-100

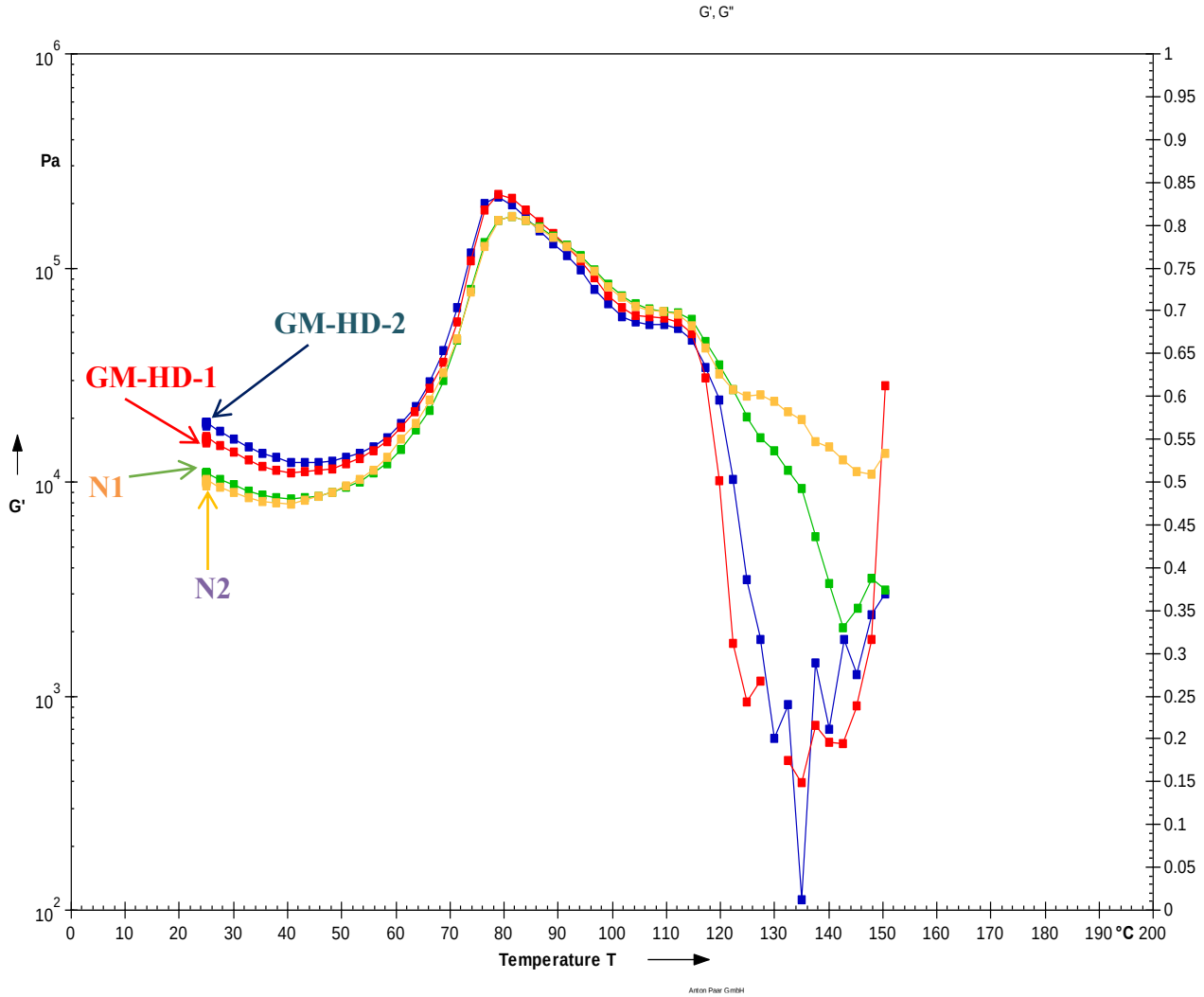

Figure 4.2.5 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as storage modulus through temperature sweep mode within a temperature range of 25-150°C

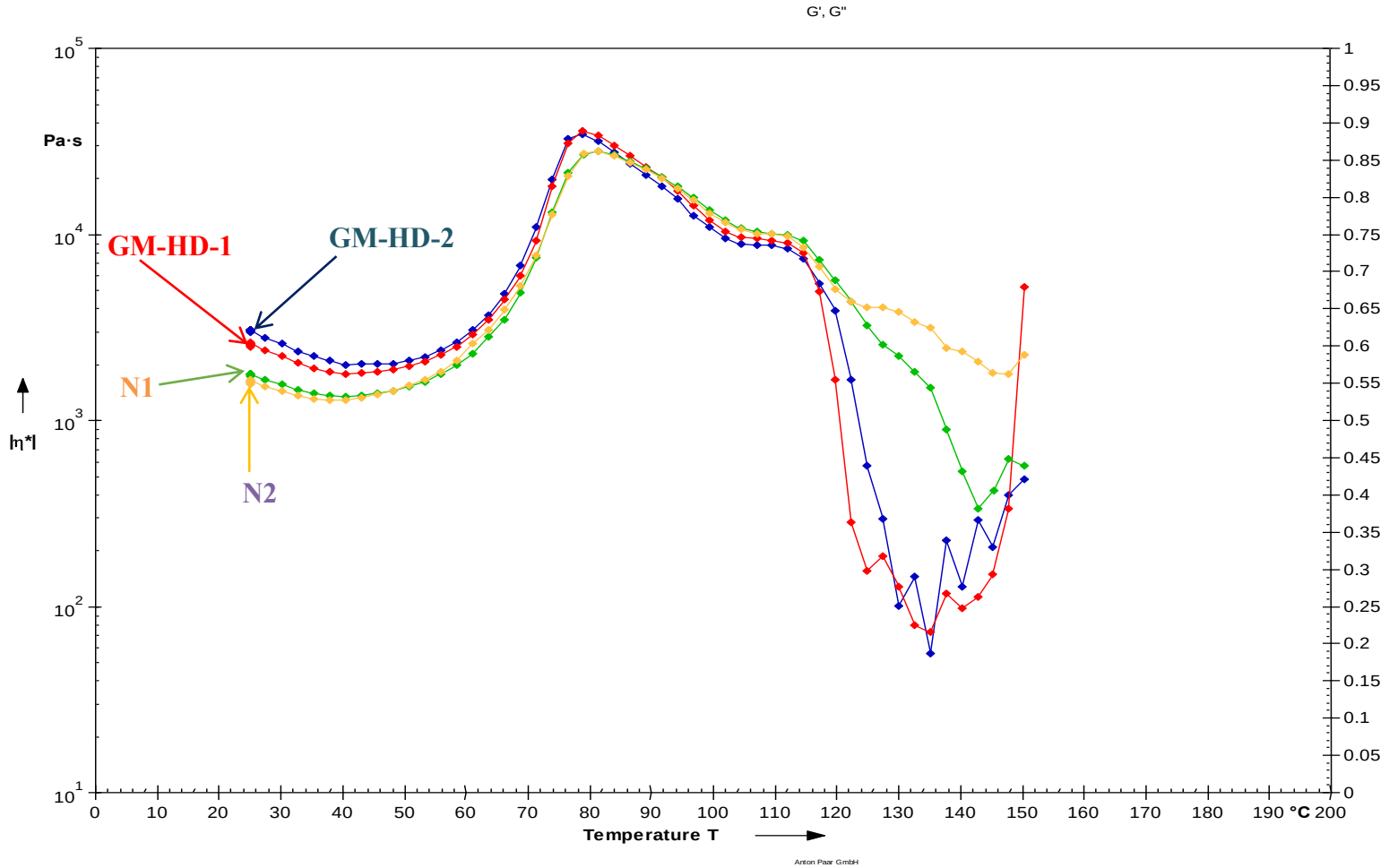

Figure 4.2.6 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as complex viscosity through temperature sweep mode within a temperature range of 25-150°*c*

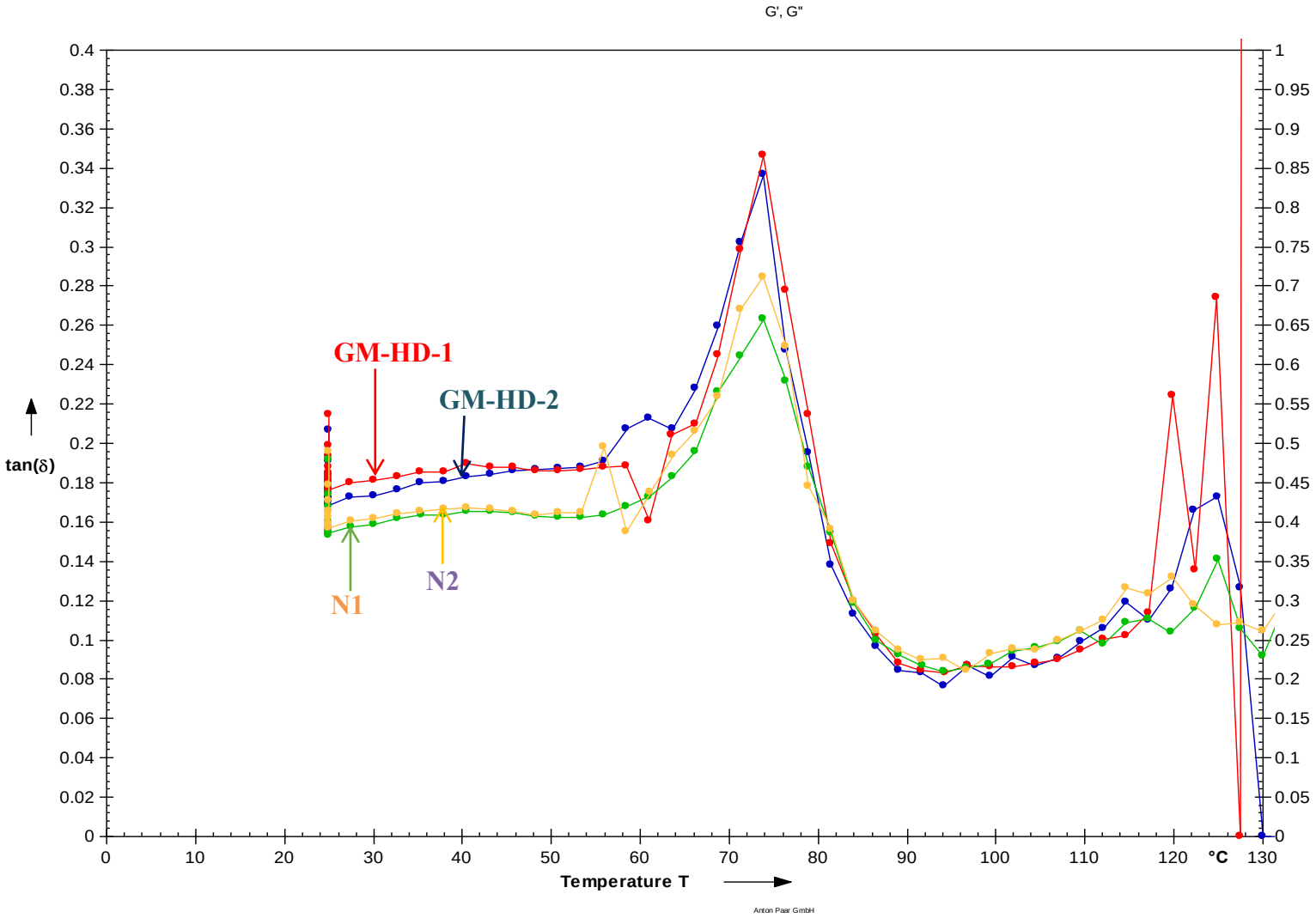

Figure 4.2.7 Dynamic rheological properties of the doughs from the genetically modified sorghums and their null controls measured as complex viscosity through temperature sweep mode within a temperature range of 25-150°C

4.2.4.7 *Stress relaxation*

GM-HD sorghums had higher maximum force during dough relaxation than N sorghums (Table 4.2.2). The higher force of GM-HD sorghums dough compared to their N controls indicates stronger GM-HD sorghum doughs. This can be ascribed to the better protein-starch network formation (Figure 4.2.10). Both GM sorghums exhibited lower (average 5.4 seconds) relaxation time than the N 1 (6.05 seconds) and N 2 line (6.6 seconds) (Figure 4.2.8). The lower relaxation time indicates a higher viscosity (Mejia et al., 2012). So, it can be said that the GM sorghums because of kafirin modification exhibited to some extent, higher viscosity.

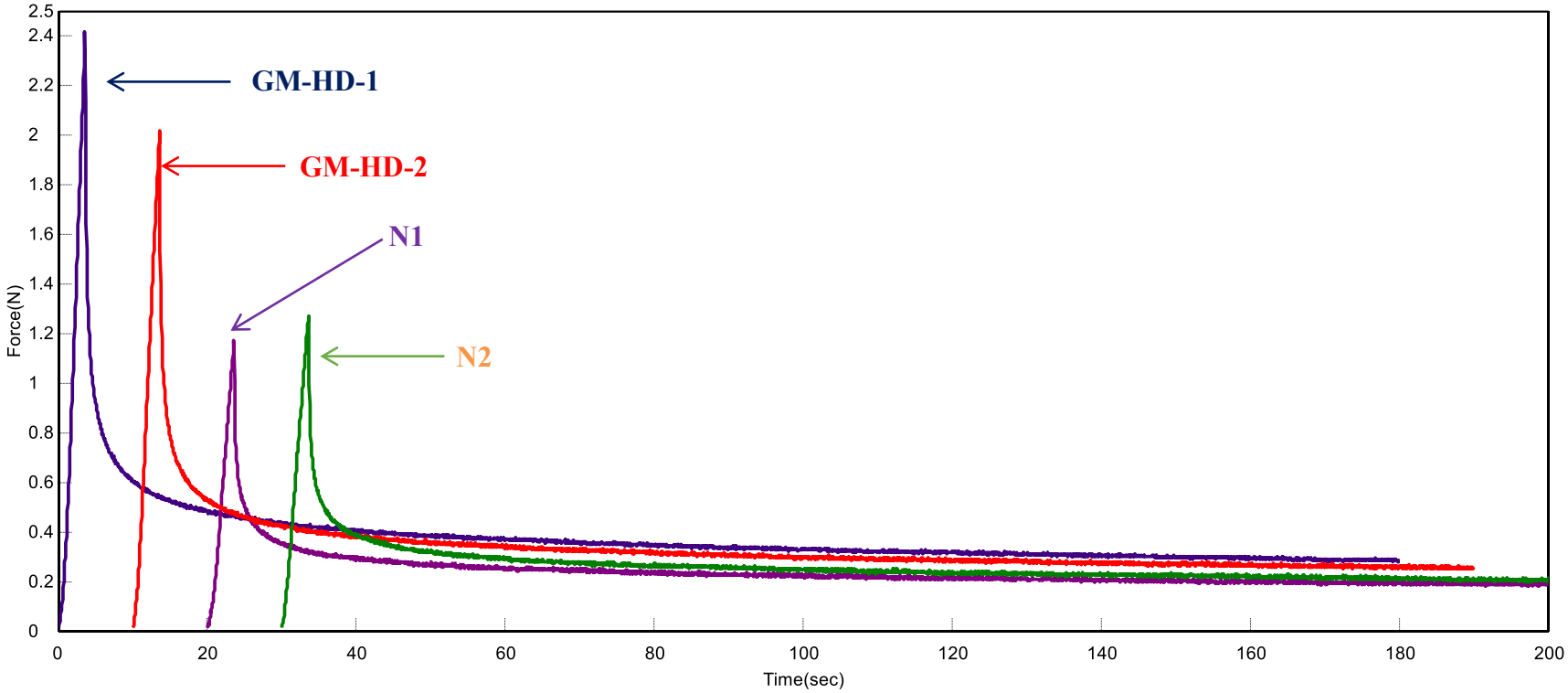

Figure 4.2.8 Dough relaxation of the GM-HD sorghums and their null controls

4.2.4.8 *Sorghum dough (thick slurry) microstructure*

CLSM of the sorghum doughs showed that both GM-HD and N sorghum doughs prepared at ambient temperature had less protein-starch interaction than their heated counterparts. The degree of protein-starch interaction is indicated by less red kafirins protein distribution within the dough, i.e. the protein in the doughs prepared at ambient temperature appeared compact as protein matrix. In the doughs prepared at ambient temperature, the GM-HD sorghums displayed a more loose protein network compared to the N sorghums. Also, GM-HD sorghums displayed greater protein-starch interaction compared to the N sorghums in the dough prepared at 70^{o}C. The higher protein-starch interaction was expressed as greater protein distribution within the GM-HD doughs. The better protein-starch interaction in GM-HD dough compared to their null controls can be the reason for the higher paste peak viscosity of GM-HD doughs. The improvement of the protein-starch interaction of the GM-HD sorghum can be attributed to the absence of the γ-kafirin. Reduction of the γ-kafirin resulted in floury endosperm (Da Silva et al., 2011b) which led to higher paste peak viscosity as found in chapter 4.1.

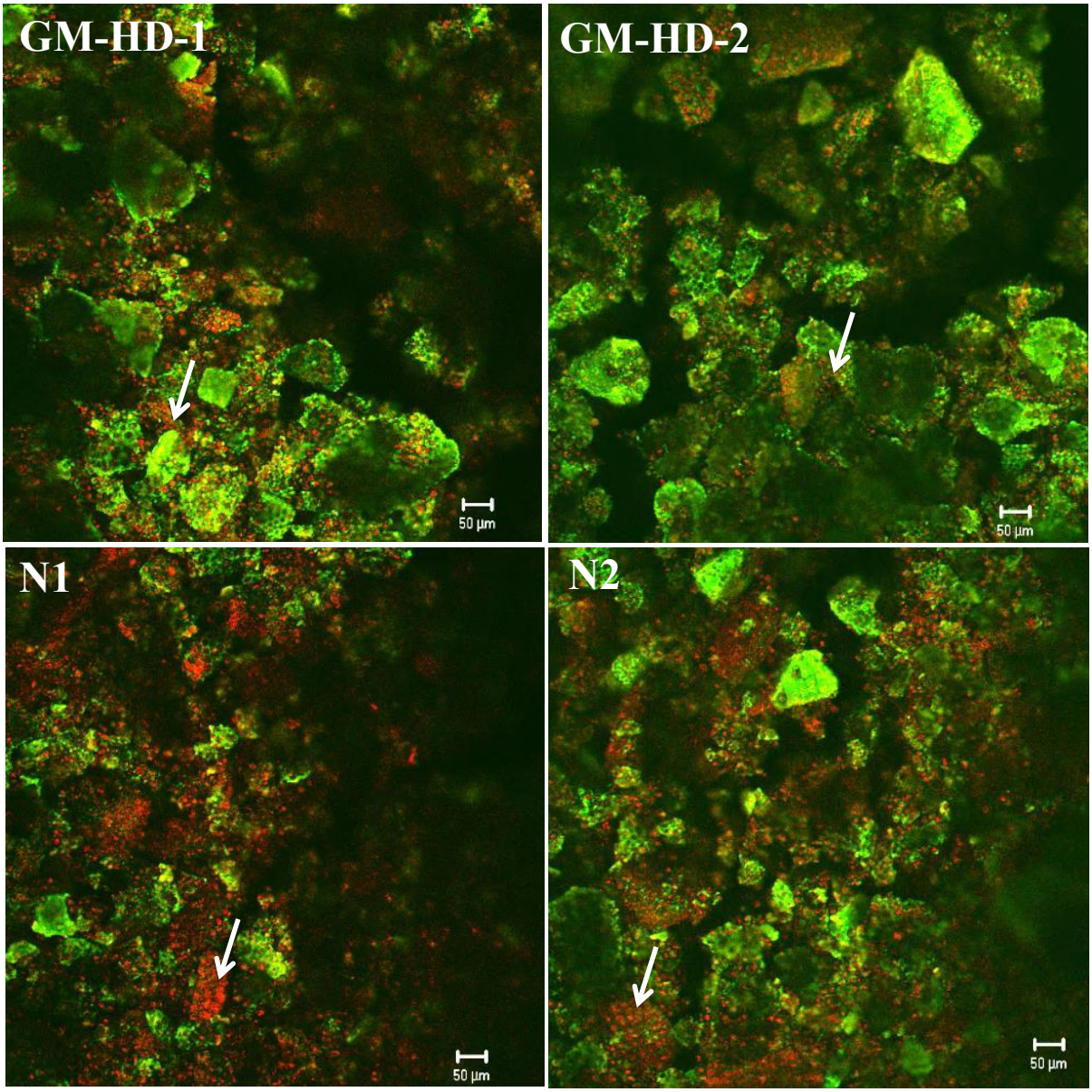

Figure 4.2.9 CLSM of the microstructure of the doughs from the genetically modified sorghums and their null controls prepared at ambient temperature

GM-HD-1 and GM-HD-2, arrows show less compact (loose) protein network (red colour). N1 and N2, arrows show more compact protein network (red colour)

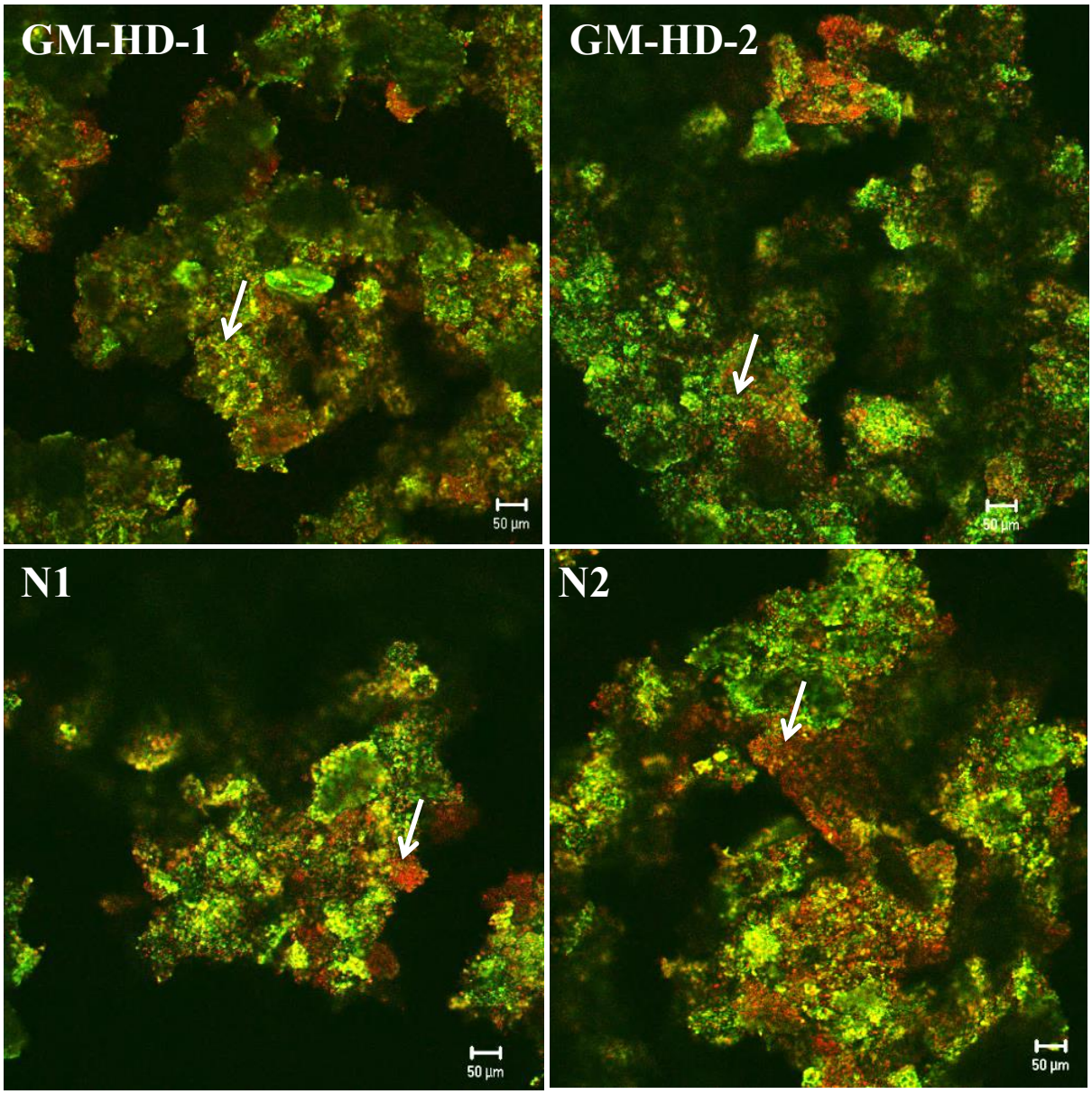

Figure 4.2.10 CLSM of the microstructure of the pastes from the genetically modified sorghums and their null controls prepared at 70°C

GM-HD-1 and GM-HD-2, arrows show more protein (red)-starch interaction. N1 and N2, arrows show less protein (red)-starch interaction (protein network still compacted together

4.2.5 Conclusions

In addition to improved protein digestibility due to alteration of kafirin composition, suppression of γ-kafirin synthesis can be considered as one of the factors that can improve sorghum flour properties. It impacts positively (increase) on Water Soluble Fraction, peak viscosity during pasting and dough elasticity. As the protein-starch interaction occurs through hydrogen bonding in the presence of the water, it is assumed that the reduction in the level of hydrophobic γ-kafirin provided more hydrophilicity in the GM-HD kafirin which resulted in better protein-starch interaction. However, it seems that the combination of the waxy trait and kafirin modification is more effective in improving sorghum flour and dough quality compared to the sorghum with altered kafirin only, i.e. with non-waxy starch.

4.2.6 References

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4.3 CHEMICAL AND DOUGH FORMING PROPERTIES OF KAFIRIN EXTRACTED FROM CONVENTIONALLY BRED AND GENETICALLY MODIFIED SORGHUMS WITH ALTERED KAFIRIN SYNTHESIS

4.3.1 Abstract

As prolamin protein is a key factor in the dough formation system and to date no study has showed successful dough formation from kafirin only, this study was conducted to determine the effect of glacial acetic acid in kafirin dough formation. Two different (conventionally bred and genetically modified) groups of kafirins with modified composition were studied and compared. Doughs from all these different kafirins were formed using glacial acetic acid as solvent. SDS-PAGE and 2-D PAGE showed that the kafirin fractions of the genetically modified-high digestibility (GM-HD) and waxy-high digestibility (WHD) samples were different from each other and from their controls. SDS-PAGE of GM-HD sorghums showed a missing band of molecular weight about 23 kDa that was indicated to be γ-kafirin. SDS-PAGE also revealed that kafirin from high digestibility (HD) (109, 142, and 146) sorghums had a missing band of MW 18.5 kDa approx., that was indicated to be β-kafirin. The 2-D PAGE showed that all samples had more protein fractions of basic p*I* than of acidic p*I*. There were missing spots of MW about 27 kDa in the GM-HD 2-D PAGE pattern unlike their null controls. However, there was no difference between HD-CB kafirins compared to their null controls. However, missing spots in their null control of about 21.5 kDa could be observed compared to HD-CB. FTIR showed that α/β ratio increased in kafirin doughs prepared with glacial acetic acid compared to kafirin doughs prepared with dilute acetic acid. CLSM, Ultra SEM and stereomicroscopy showed fibrils in all kafirin doughs with differences between the various kafirin samples. This work indicated that there is potential to form viscoelastic dough from sorghum flour.

4.3.2 Introduction

Climate change is one of the biggest challenges that the world is encountering in the recent era. The most vulnerable countries at risk of the impacts of climate change are in sub-Saharan Africa (Haile, 2005). As these people depend on rain in their agriculture, they will be greatly affected by drought caused by climate change. An alternative crop is sorghum, which is highly suited for cultivation in the semi-arid regions as it is one of the most drought-tolerant cereal crops (Hattori et al., 2005). However, a problem with the sorghum lies in inability of kafirin, its prolamin proteinto exhibit viscoelastic properties that are required in many important food products such as bread.

Flours from two different groups of sorghum (conventionally bred and genetically modified) have been investigated in Chapter 4.1 and 4.2 of this study. In this chapter, kafirin will be extracted from these two sorghum groups for their possibility to form doughs through application of a coacervation procedure using glacial acetic acid.

Disulphide cross-linking in kafirin has an influence on sorghum flour properties in terms of its dough viscoelastic characteristics (Goodall et al., 2012). The low protein digestibility of sorghum is attributed to high levels of disulphide cross-linking (Duodu et al., 2002). Further, the secondary structure of kafirin is affected by the different processes that kafirin may be subjected to especially wet cooking (Duodu et al., 2001). Compared to uncooked kafirin, antiparallel intermolecular β-sheet tend to increase at the expense of the α-helix conformation when the kafirin is cooked in wet condition. Acetic acid has been used in different concentrations to prepare dough from α-zein (Sly et al., 2014). The extensibility of the zein doughs increased with increasing acetic acid concentration. FTIR analysis showed that there was trend of increasing of α-helical conformation with increasing acetic acid concentration. CLSM showed that the acidified zein dough had ordered linear fibril network unlike zein dough prepared with water. Another study also applied acidification to investigate its effect on maize dough properties (Falade et al., 2014). In this study, lactic acid was used. Stress relaxation analysis revealed that the chemically acidified maize dough with lactic acid had a longer relaxation time. The longer relaxation time related to the higher elasticity of the acidified dough than the dough prepared with water, i.e. without lactic acid.

Hence, Goodall et al. (2012) managed to prepare improved kafirin doughs but as a composite dough such as gluten-kafirin dough. Also, acetic acid has been used to prepare dough that exhibited

fibrils but in zein (Sly et al., 2014). However, no study has shown the possibility of preparing dough from kafirin alone whether by using acetic acid or not.

4.3.3 Materials and Methods

4.3.3.1 *Sorghum lines*

The two groups of conventionally bred and genetically modified sorghum lines and their controls as described in Chapters 4.1 and 4.2 were used.

4.3.3.2 *Kafirin extraction*

Total kafirin was extracted from both sorghum groups using the method described by Taylor et al. (2005) with modifications. In brief, clean whole grain from each sorghum line (220 g) was milled using a laboratory hammer mill (Mikro-Feinmuhle-Culatti MFC Grinder, Janke and Kunkel, Staufen, Germany) fitted with a 500 µm opening screen. The extractant was 70% (w/w) ethanol, containing 0.35% (w/w) glacial acetic acid and 0.5% (w/w) sodium metabisulphite. Extraction was carried out at 70˚C in a water bath with vigorous stirring for 1 hour. The supernatant was collected after centrifugation at 1000 g at ambient temperature for 5 min. The process was repeated for the precipitate and the supernatant added to the first extract. Sequential extraction of kafirin-1 and kafirin-2 was conducted with non-reducing and reducing solvents (Da Silva et al., 2011b). For non-reducing extraction, 40 ml aqueous tert-butanol 60% (v/v) was added to 8 g sorghum flour and mixed for 5 h at ambient temperature. Samples were then centrifuged into the same tube at 2000 g for 10 min, the supernatant was collected. The pellet was re-suspended in 40 ml of the same solvent and extracted overnight with continuous stirring. After centrifugation at 2000 g for 10 min and supernatant was collected and added to the previous one and designated kafirin-1 (K1). For reducing extraction, the pellet (residue) from non-reducing extraction was re-suspended in 40 ml 60% (v/v) aqueous tert-butanol plus 5% (v/v) 2-mercaptoethanol. Samples were then centrifuged into the same tube at 2000 g for 10 min and the supernatant collected. The pellet was re-suspended in 40 ml of the same solvent with continuous stirring for an additional 3 h. After centrifugation at 2000 g for 10 min, the supernatant was added to the previous one and designated kafirin-2 (K2). The residues and supernatants were freeze dried in their tubes and then stored at ±8°C.

4.3.3.3 *Kafirin dough formation*

The method was based on the kafirin microparticle preparation technique of Taylor (2008); Taylor et al. (2009); Taylor and Taylor, 2010). Glacial acetic acid (5 ml) was pipetted into a 50 ml beaker containing a magnetic stirrer bar. One gram dry total kafirin preparation was placed in the beaker with constant stirring. The mixture was heated slowly with continuous stirring to 50°C (within 5 min). The beaker was covered during heating. Then, the magnetic stirrer bar was removed and immediately a volume of 20 ml distilled water at 15°C was rapidly added to the kafirin solution. A soft net of kafirin dough formed, which was collected using a spatula. This was kneaded into a dough by hand.

4.3.3.4 *Electrophoresis*

4.3.3.4.1 *Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE under reducing and non-reducing conditions was conducted to determine the profile of the kafirin fractions and their degree of polymerization. A XCell SureLock™ Mini-Cell electrophoresis unit (Invitrogen Life Technologies, Carlsbad, CA) was used, as described by Anyango et al. (2013). Pre-prepared NuPAGE 4-12% Bis-Tris gradient gels of 1 mm thickness were used to run the samples. The molecular weight marker used was an Invitrogen Mark 12TM unstained standard (2.5-200 kDa). Before loading, samples were suspended into sample buffer containing SDS and 2-mercaptoethanol and then placed in a boiling water bath for at least 15 min with vigorous vortexing every 5 min to ensure that all the protein was completely dissolved. Sample loading was 10 µl at a concentration of 1 µg protein/1µl. Coomassie Brilliant Blue R-250 was used to stain the gels. A flat-bed scanner was used to photograph the gels.

4.3.3.4.2 *Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)*

Two-D PAGE was conducted to further characterise the kafirin fractions by separation of the kafirins firstly based on their isoelectric points and subsequently based on their molecular size. Unless otherwise specified, all the equipment and reagents were obtained from Invitrogen™. First, isoelectric focusing was conducted using the ZOOM® IPG System in accordance with the manufacturer's instruction manual (Invitrogen, 2012). The dry kafirin preparations were dialyzed using the method described by Anyango et al. (2013) to remove salts. ZOOM® Carrier Ampholyte (pH 3-10) (60 µl) was added to 3 ml pre-prepared solubilization buffer (DeStreak Rehydration

Solution) before the latter was used to solubilize the kafirin sample to obtain a concentration of 2 µg protein/µl. A 7 cm ZOOM® IPG Strip (pH 3-10) was rehydrated using 140 µl (2µg/µl) of the dissolved kafirin for 1 h using the ZOOM® IPGRunner™ Cassette. A ZOOM® IPG Runner Mini-cell chamber was used to perform the IEF. IEF conditions were 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and then 2000 V for 45 min. After IEF separation, the strips were equilibrated for 15 min in 9 ml NuPAGE® LDS Sample Buffer (1x) plus 1 ml Sample Reducing Agent using the ZOOM® Equilibration Tray. Alkylation was then performed in the Equilibration Tray after discarding the equilibration solution. The alkylating solution (125 mM) was prepared fresh by dissolving 232 mg iodoacetamide in 10 mL 1X NuPAGE® LDS Sample Buffer for 15 min. Then, the strip was placed and fixed into the well of a NuPage® Novex 4–12% Bis-Tris ZOOM® Gel by adding approx. 400 µL 0.5% agarose solution. SDS-PAGE was performed in the second dimension as described under 4.3.3.4.1.

4.3.3.5 *Fourier Transform Infrared Spectroscopy (FTIR)*

FTIR was performed on the dry kafirin preparations and their doughs (kafirin doughs formed with glacial acetic acid only and kafirin doughs formed by addition of water to kafirin solutions in glacial acetic acid) to determine any changes in secondary structure. The spectra were obtained with a Vertex 70v FTIR spectrophotometer (Bruker Optik, Ettlingen, Germany), with a zinc selenide crystal using 32 scans, 8 cm^{-1} bandwidth, and an interval of 1 cm^{-1} (Anyango et al., 2013). The Attenuated Total Reflectance (ATR) mode in the wavenumber range of 400−4000 cm^{-1} was used. The angle of incidence for the ATR crystal was 45°. Fourier self-deconvolution (FSD) was carried out using the resolution enhancement factor of 2 and a 6 cm^{-1} bandwidth. Samples (approx. 2 mg) were spread using spatula on the ATR crystal and squeezed by a screw to be in contact with the crystal.

4.3.3.6 *Microscopy*

4.3.3.6.1 *Stereomicroscopy*

To examine the features of the doughs, which had a complex surface topography, a Nikon stereo light microscope (Nikon SMZ 800, Tokyo, Japan) fitted with a Nikon DXM 1200 digital camera

was used at 400x magnification. Each kafirin dough piece of approx. size 5 mm × 3 mm and thickness 1.5 mm was placed and stretched on a glass slide and then released before it was imaged.

4.3.3.6.2 *Confocal Laser Scanning Microscopy (CLSM)*

CLSM was used to investigate the internal structure of the kafirin doughs. Each dough piece of approx. size 5 mm × 3 mm and thickness 1.5 mm was stretched onto a glass slide. The doughs were imaged using a Zeiss 510 META confocal laser scanning microscope ((Jena, Germany) fitted with a Plan-Neofluar 10 × 0.3 objective at an excitation wavelength of 405 nm with natural fluorescence (Sly et al., 2014).

4.3.3.6.3 *Scanning Electron Microscopy (SEM)*

For further investigation of kafirin dough microstructure, an Ultra high resolution Field emission SEM (JEOL 6000F FEGSEM Tokyo, Japan) was used. Samples were mounted on aluminium stubs using carbon glue and partially dried by incubation for three days at 4°C before they were sputter coated with gold.

4.3.3.7 *Statistical analysis*

Each experiment was repeated at least two times. The t-test at a confidence level of $P < 0.05$ was applied to analyse the data using IBM SPSS Statistics 22 (SPSS, Chicago, IL).

4.3.4 Results and discussion

4.3.4.1 *Kafirin dough formation*

Visual observation and stereomicroscopy (Figures 4.3.1.A, 4.3.1.B and 4.3.1.C) revealed that doughs were successfully made from kafirin by coacervation of a solution of kafirin in glacial acetic acid by rapid addition of cold water. This is a novel finding as it is the first time that kafirin alone, i.e. not as composite protein, has been shown to form a dough. It is speculated that a kafirin dough was obtained rather than microparticles, as formed by Taylor et al. (2009) by coacervation of a solution of kafirin in glacial acetic acid with water addition, because during the addition of the water to the solution of kafirin in glacial acetic acid in this present work there was no stirring. Also, the rapid addition of the cold water (~15°C) helped in interacting of the maximum amount of the soluble kafirin with water instead of aggregating into small microparticles. With rapid cold

water addition, a network of insoluble kafirin fibrils was formed. By kneading this substance, it coalesced into a dough. Schober et al. (2011) found that kafirin aggregated in warm water in the presence of a reducing agent. However, these authors observed that the gluten-like substance of kafirin quickly became firm and lost its extensibility. In this current study, the glacial acetic acid resulted in much improved kafirin dough properties in terms of elasticity as observed visually compared to those found by Schober et al. (2011).

Also, of importance was the finding that the kafirin doughs remained elastic even after they were stored in a zip lock bag at 10°C (below the T_g of hydrated kafirin) for a week. The elasticity of the kafirin dough was evaluated by stretching by hand and not instrumentally, nevertheless the elastic properties of the dough were clearly evident. These findings concerning the effect of acetic acid fit in with the finding of Sly et al. (2014) that dilute acetic acid improved zein dough properties when it was used to prepare dough from commercial zein (α-zein).

Furthermore, it was observed that doughs prepared from kafirin extracted from WHD and GM-HD sorghum lines showed greater fibril formation than their controls (Figures 4.3.1.B and 4.3.1.C).

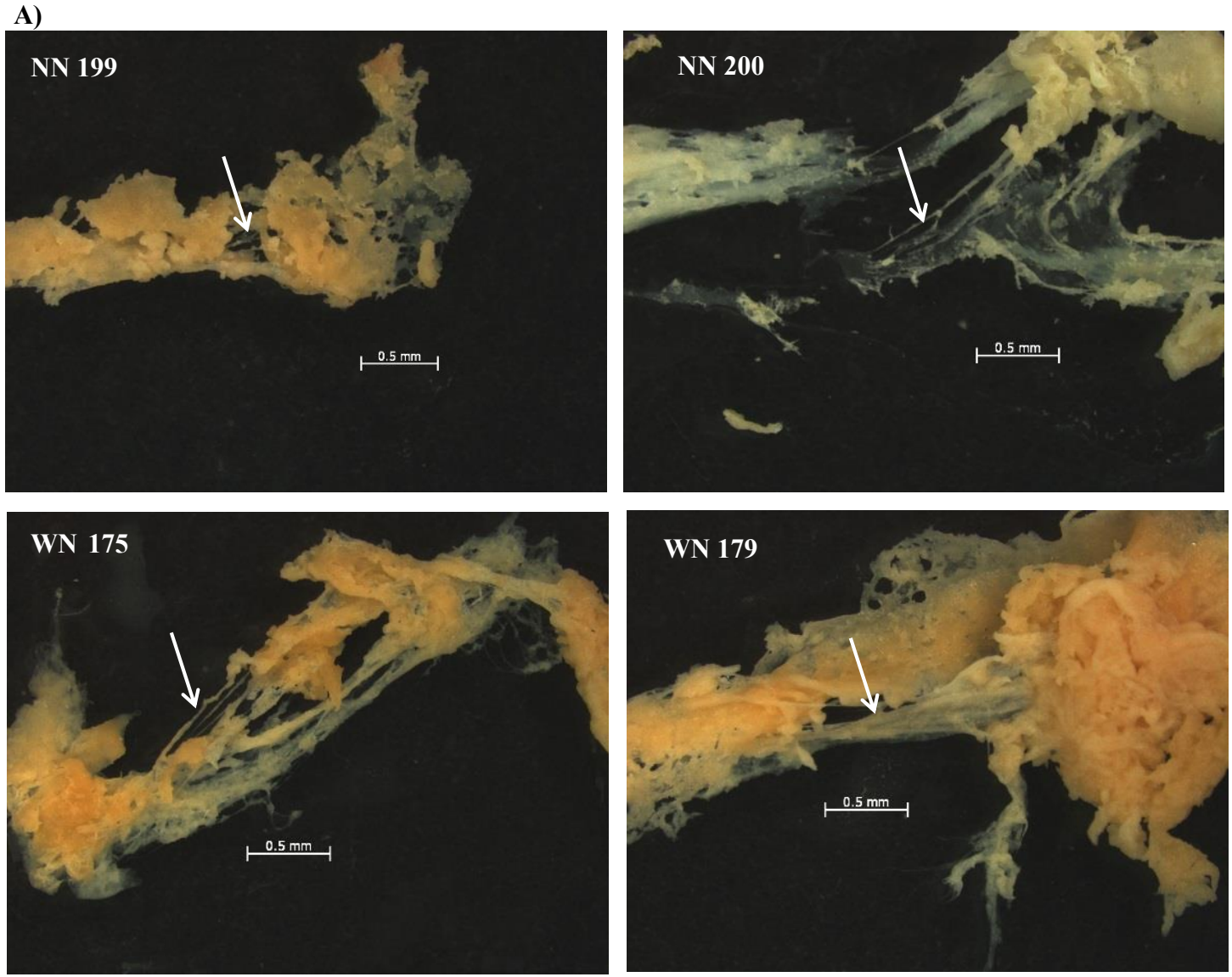

Figure 4.3.1.A Stereomicroscopy of doughs made from total kafirin. A: sorghum lines of normal protein digestibility

Arrows show the fibrils within the doughs formed by stretching

B)

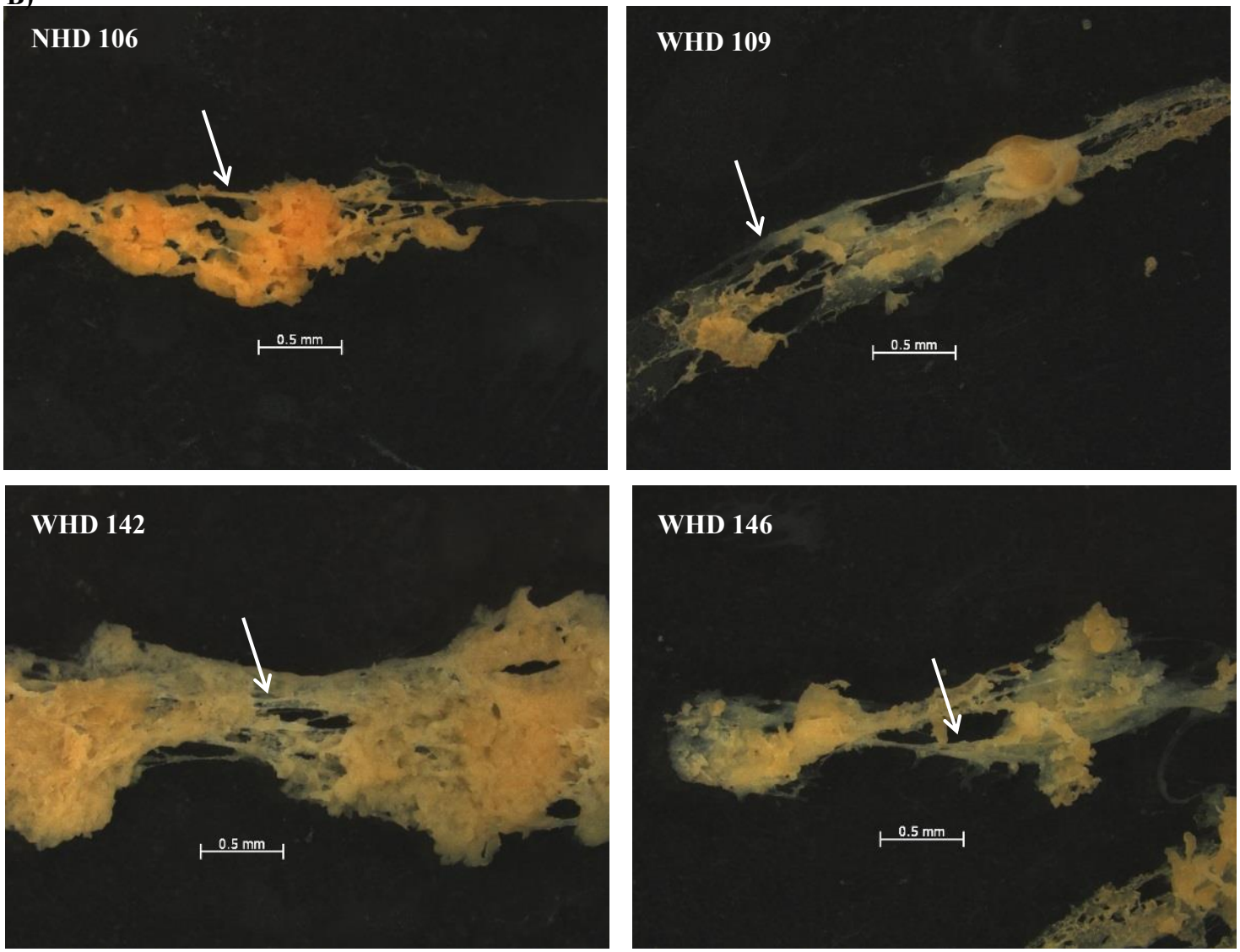

Figure 4.3.1.B Stereomicroscopy of doughs made from total kafirin. B: sorghum lines of high protein digestibility

Arrows show the fibrils within the doughs formed by stretching

C)

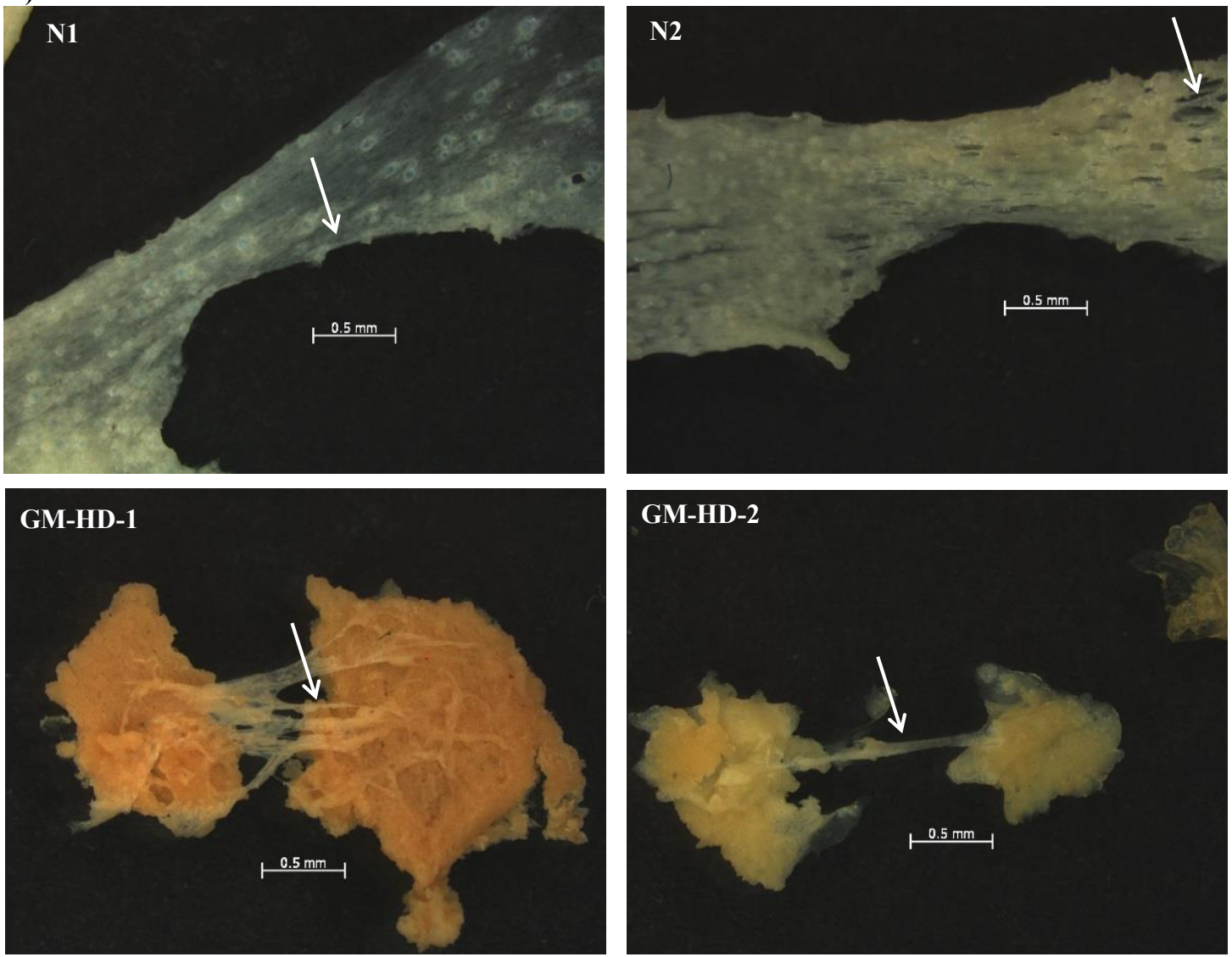

Figure 4.3.1.C Stereomicroscopy of doughs made from total kafirin. C: GM-HD sorghum lines and their null controls

Arrows show the fibrils within the doughs formed by stretching

Microstructure of the kafirin doughs observed by CLSM (Figures 4.3.2.A, 4.3.2.B and 4.3.2.C) confirmed the presence of the fibrils within the doughs from all the various kafirins. This means the fibrils were not just present on the exterior of the doughs as shown by stereomicroscopy (Figure 4.3.1). This finding is similar to that of Sly et al. (2014) who also observed the presence of fibrils within zein doughs as a result of dough preparation with acetic acid. The fibrils in the non-GM and GM high protein digestibility lines 106 (HD), 109 (HD), 142 (HD), 146 (HD), GM-HD-1 and GM-HD-2 (Figures 4.3.2.B and 4.3.2.C) were more evident, being thinner, and compact and had a consistent order compared with the fibrils in 199 (normal digestibility (N)), 200 (N), 175 (N), 179 (N), N1 (N) and N2 (N) (Figures 4.3.2.A, 4.3.2.C). These were less evident, broader, less compact and less well ordered. This difference in the fibril characteristics between kafirins from high protein digestibility and normal sorghum was similarly noted in terms of better dough formation and viscoelasticity of wheat-sorghum and gluten-kafirin composite doughs prepared from high protein digestibility sorghum lines (Goodall et al., 2012).

Ultra SEM of the kafirin doughs confirmed the presence of the fibrils and showed additional differences between the doughs for the kafirins extracted from the different sorghum lines (Figure 4.3.3). Lines 106 (HD), 109 (HD), 142 (HD), 146 (HD), GM-HD-1 and GM-HD-2 (Figures 4.3.3.B and 4.3.3.C) displayed greater fibril formation (organised in ribbons) compared to the kafirins from the normal digestibility sorghums (Figure 4.3.3.A).

A)

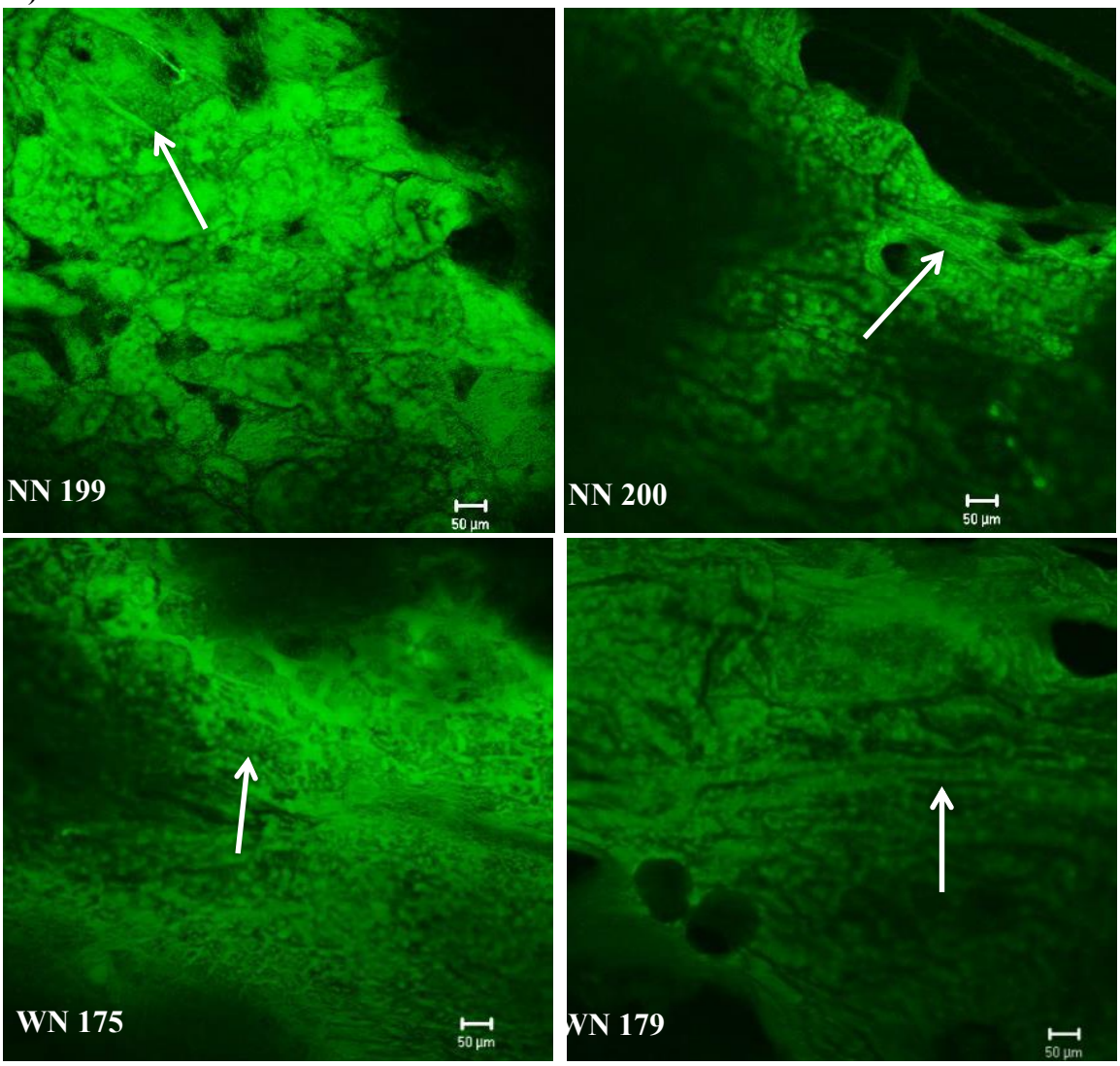

Figure 4.3.2.A CLSM of dough made from total kafirin. A: Conventionally bred waxy and non-waxy sorghum lines of normal protein digestibility

Arrows show fewer, wide, non-compact and non-consistently ordered fibrils within the kafirin dough

B)

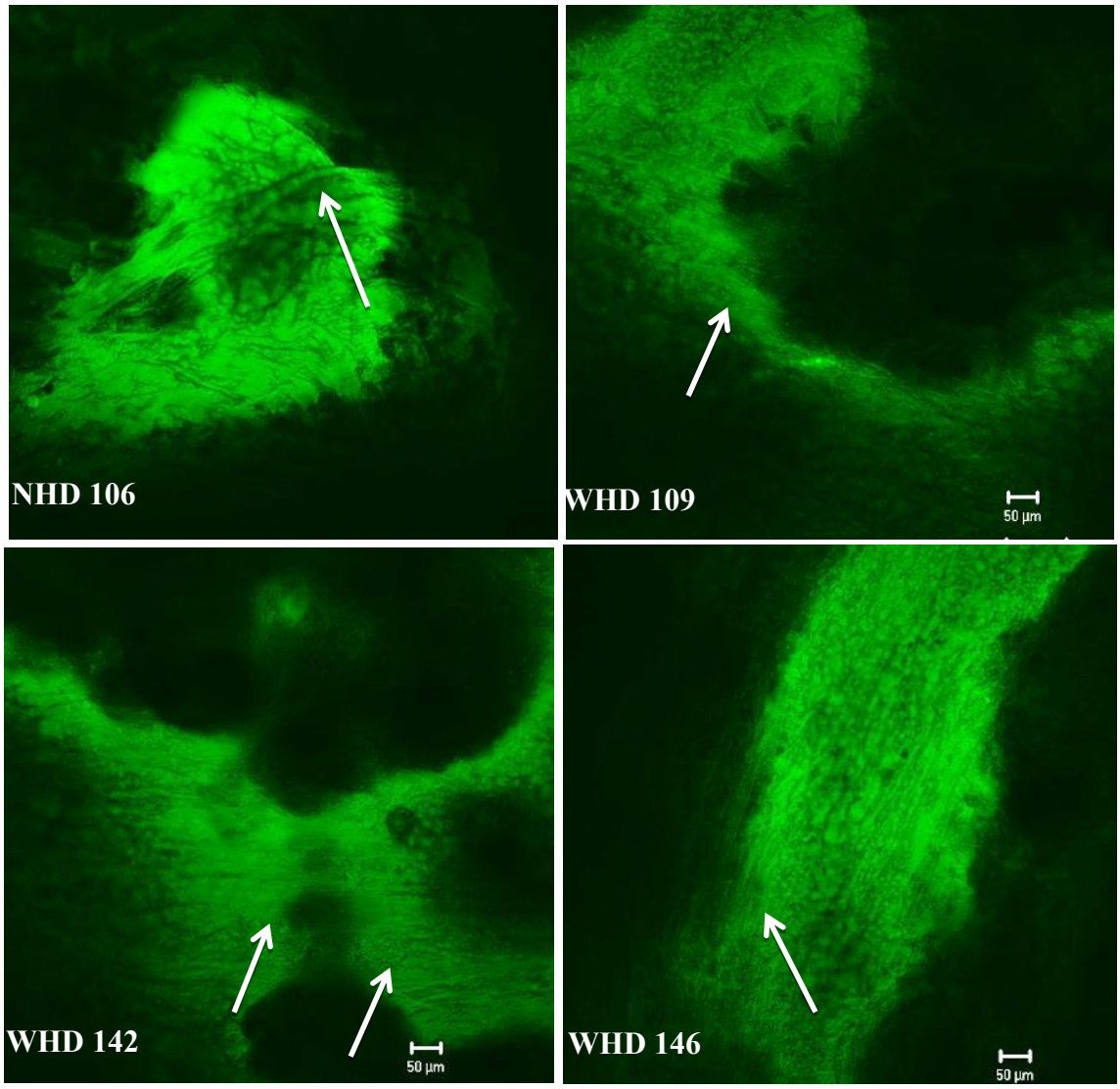

Figure 4.3.2.B CLSM of dough made from total kafirin. B: Conventionally bred waxy and non-waxy sorghum lines of high protein digestibility

Arrows show more, thin, compact and consistently ordered fibrils within the kafirin dough

C)

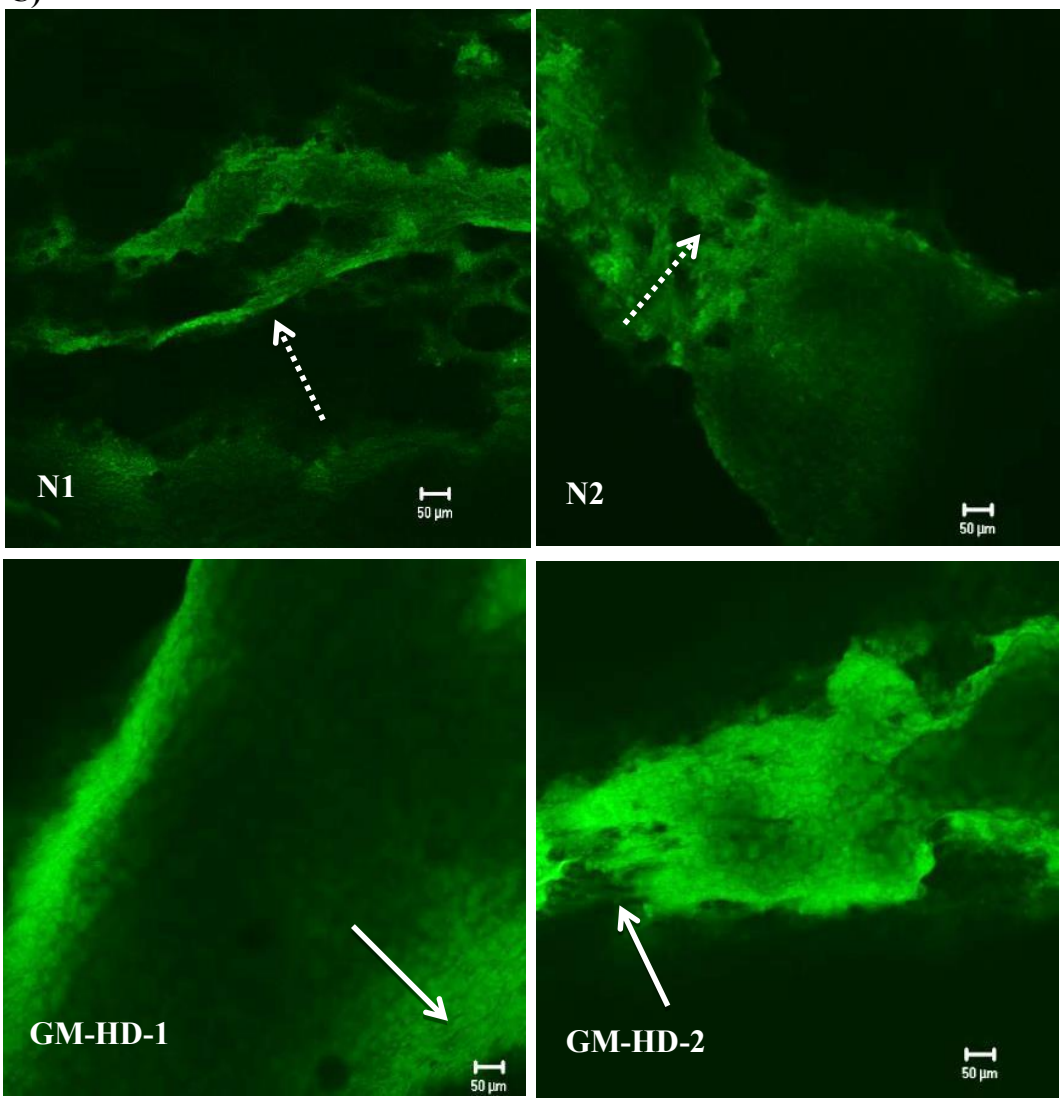

Figure 4.3.2.C CLSM of dough made from total kafirin. C: GM-HD sorghum lines and their null controls

Dotted arrows show fewer, wide, non-compact and non-consistently ordered fibrils within the kafirin dough
Solid arrows show more, thin, compact and consistently ordered fibrils within the kafirin

A)

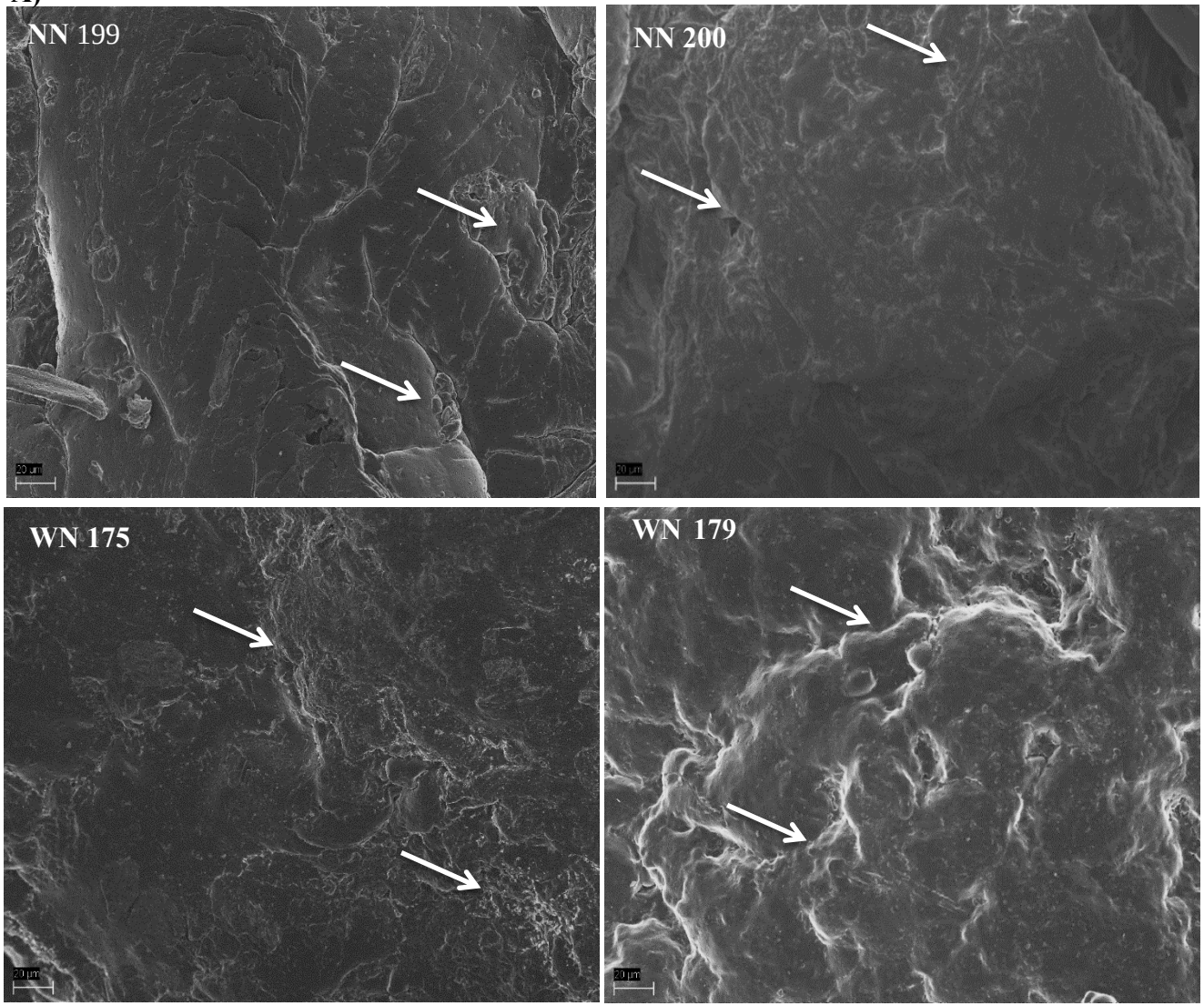

Figure 4.3.3.A Ultra SEM of doughs made from total kafirin. A: Conventionally bred waxy and non-waxy sorghum lines of normal protein digestibility

Arrows show fewer fibril-like shapes within the kafirin dough.

B)

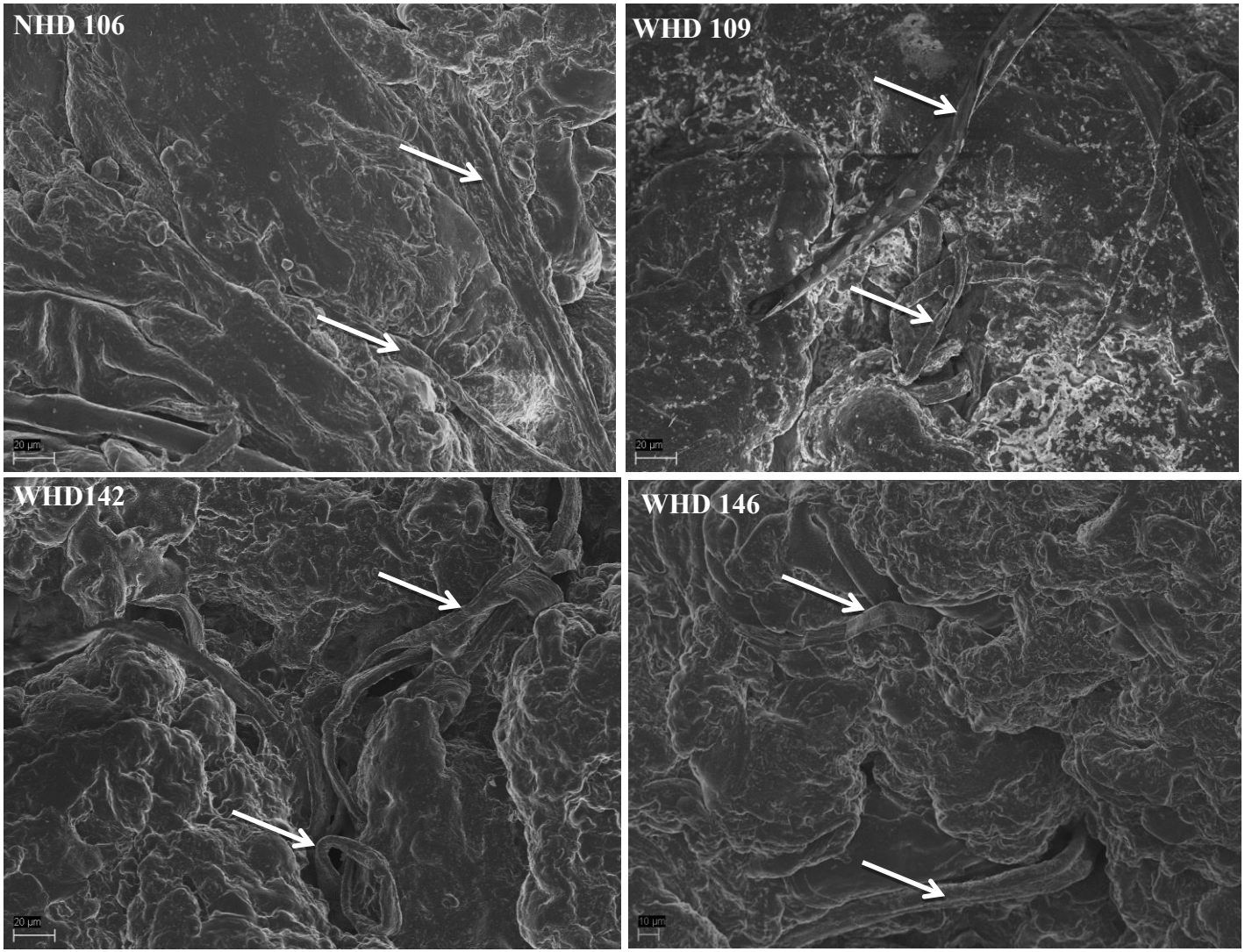

Figure 4.3.3.B Ultra SEM of doughs made from total kafirin. B: Conventionally bred waxy and non-waxy sorghum lines of high protein digestibility

Arrows show more fibril-like shapes within the kafirin dough.

C)

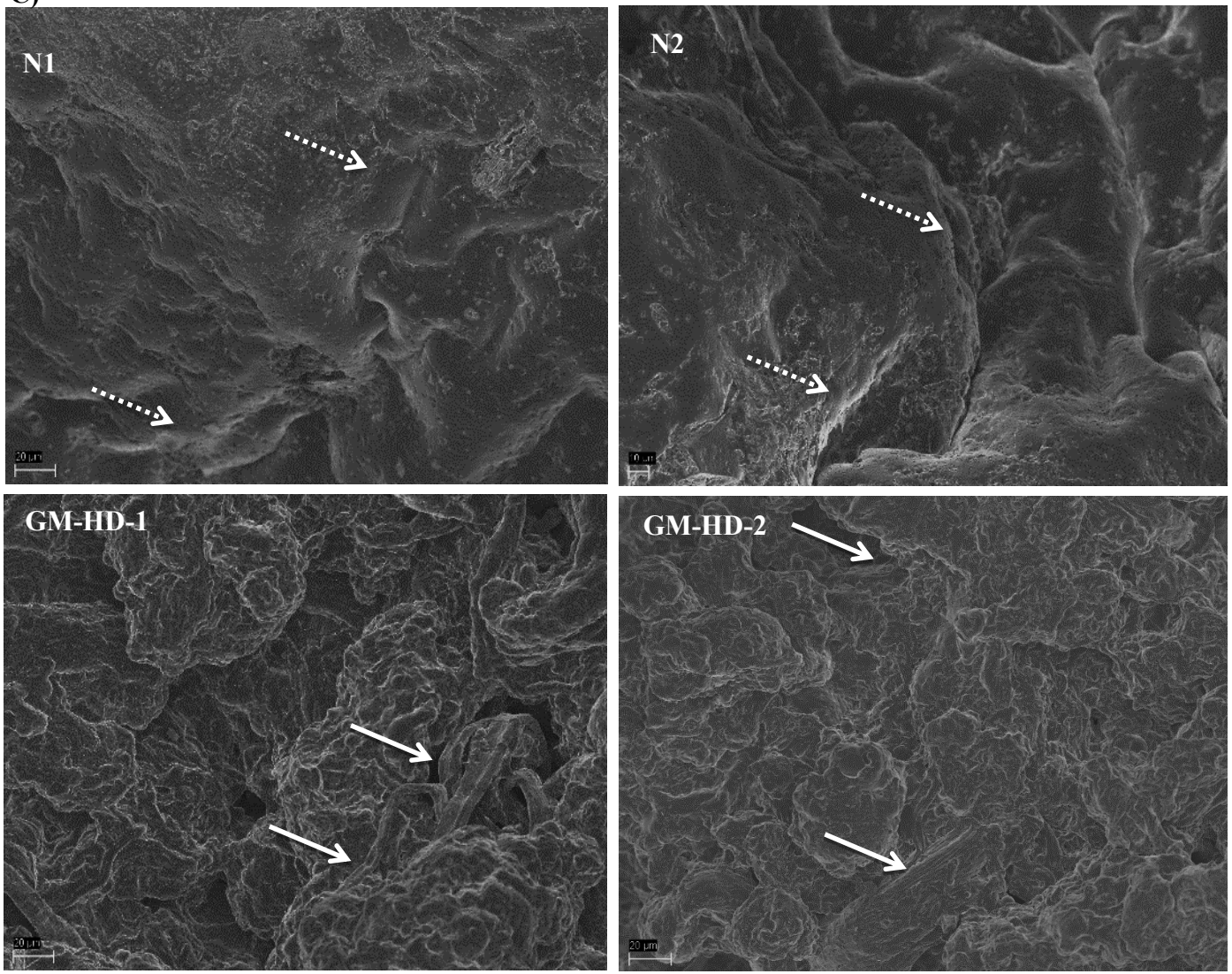

Figure 4.3.3.C Ultra SEM of doughs made from total kafirin.C: GM-HD sorghum lines and their null controls
Dotted arrows show fewer fibril-like shapes within the kafirin dough.
Sorowslid ar show more fibril-like shapes within the kafirin dough.

4.3.4.2 *Secondary structure by FTIR*

FTIR (Figures 4.3.4.A and 4.3.4.B and Table 4.3.1) in the Amide I region indicated that there were no substantial differences in the secondary structure between the dry total kafirin preparations that were extracted from the different sorghum lines. The α/β ratios and relative α-helical conformations of the dry total kafirin preparations were essentially the same for all the dry kafirins. When a small quantity of glacial acetic acid was added to these dry total kafirin preparations dough-like substances were formed in all cases and the proportion of α-helical conformation greatly increased compared to their dry total kafirin preparations. However, there were no great differences in conformation between the different dough-like substances that were prepared from the different dry kafirins. When water was added to the kafirin dissolved in glacial acetic acid that formed the dough, the α-helical conformation decreased again and became substantially lower compared to the dough-like substances that were formed by just glacial acetic acid. However, there were no clear and consistent differences between the true kafirin doughs and the dry total kafirins from which they came in terms of α/β ratio. This indicates that there was no correlation between the dough formation and α/β ratio. These findings are similar to that of Sly et al. (2014), where the proportion of α-helical conformation in zein doughs increased under acidic conditions and this was attributed to deamination. This conformational change was assumed to be responsible for the considerably improved zein dough properties. In contrast, Mejia et al. (2007) found that β-sheet increased with the viscoelasticity of α-zein dough. It can be speculated that the absence of a consistent difference in α/β ratio between the kafirin doughs and their dry total kafirins may have been due to interference in the FTIR spectra caused by the water molecules present in the kafirin doughs.

A)

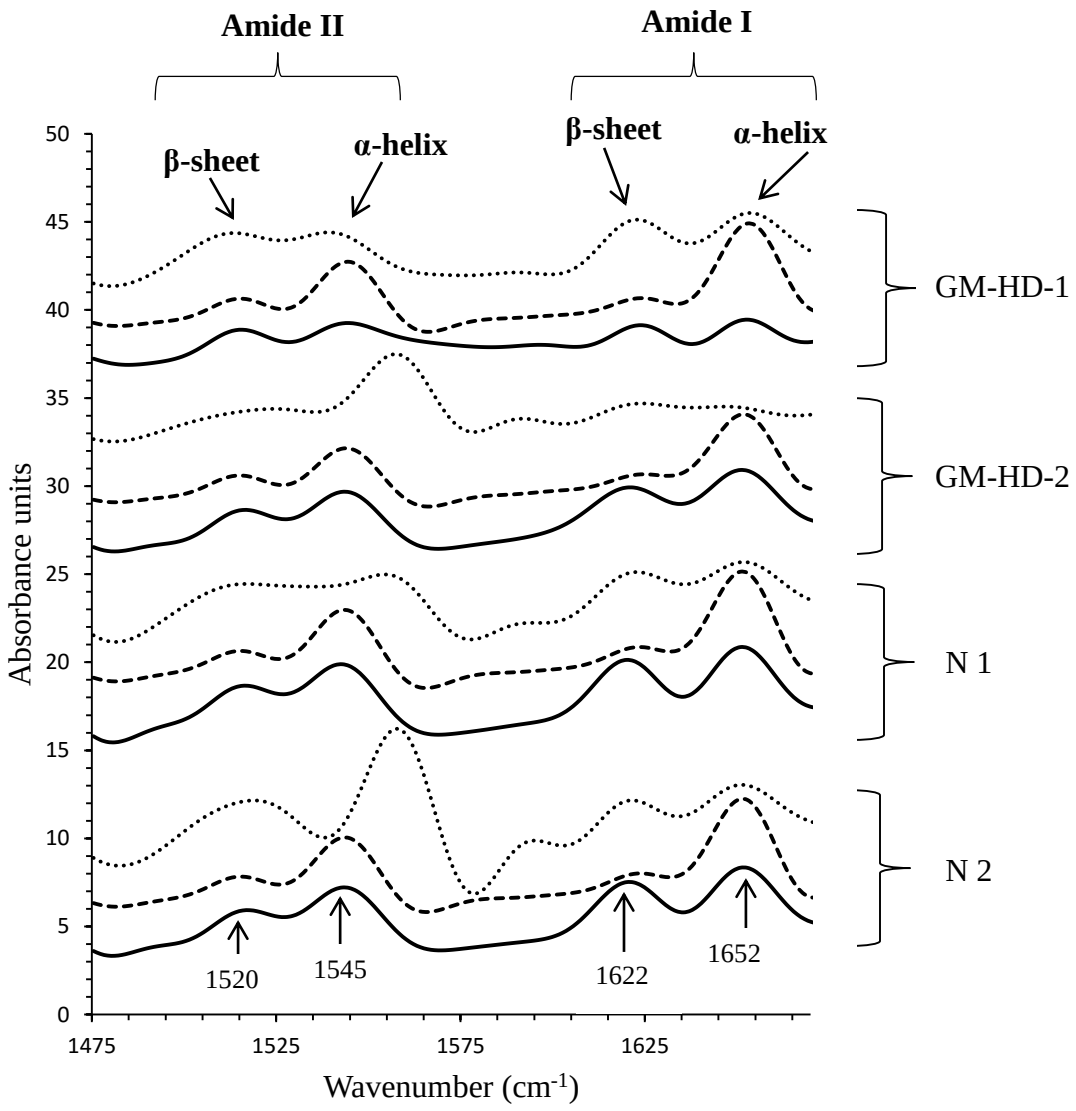

Figure 4.3.4.A FTIR spectra of dry total kafirin (dotted lines), kafirin dough-like substance formed with glacial acetic acid only (dashed lines) and true kafirin doughs formed by addition of water to kafirin solutions in glacial acetic acid (solid lines). A: modified kafirin from GM-HD lines and normal kafirin from their null controls.

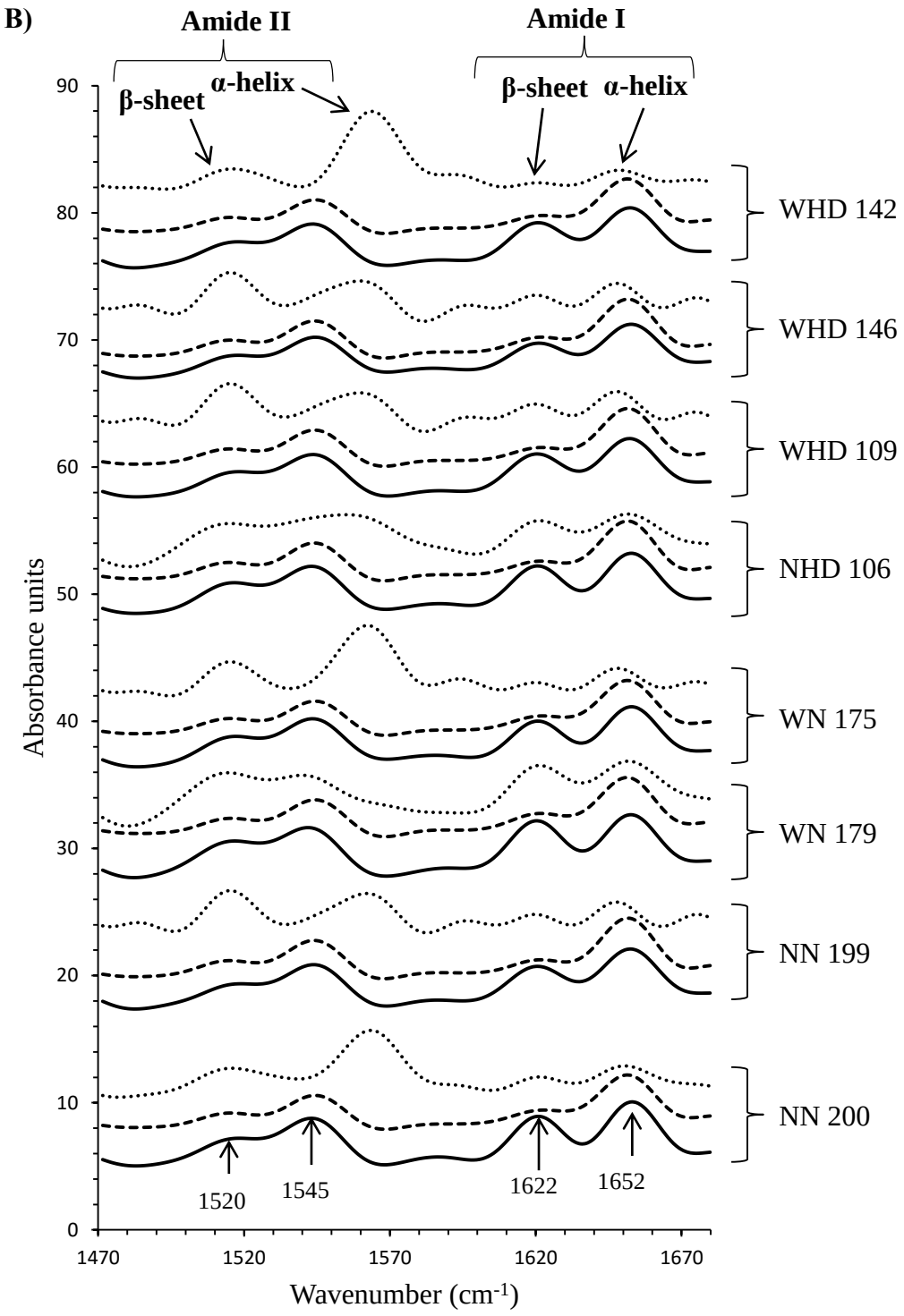

Figure 4 .3.4.B FTIR spectra of dry total kafirin (dotted lines), kafirin dough-like substance formed with glacial acetic acid only (dashed lines) and true kafirin doughs formed by addition of water to kafirin solutions in glacial acetic acid (solid line). B: modified kafirin from WHD lines and normal kafirin from waxy and non-waxy N sorghums.

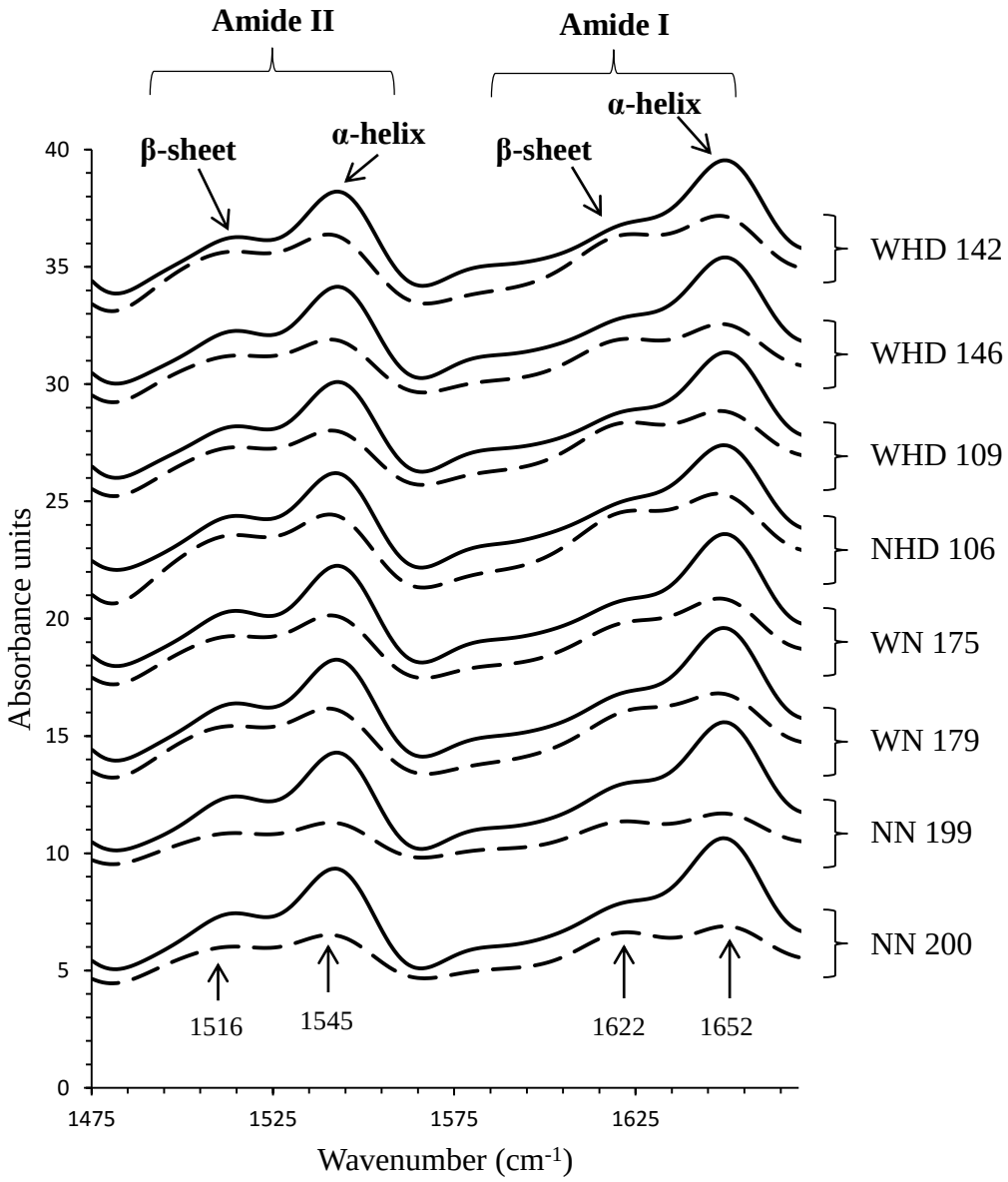

Figure 4.3.5 FTIR spectra of total dry kafirin-1 (solid lines) and dry kafirin-2 (dashed lines) of kafirins extracted (sequential extraction) from waxy and non-waxy HD sorghum lines and waxy and normal kafirin from waxy and non-waxy normal protein digestibility lines

Table 4.3.1 Alpha/beta conformation ratio calculated from the FTIR spectra of the dry total kafirin preparations, dough-like substances formed with glacial acetic acid and true doughs formed by addition of water to kafirin solutions in glacial acetic acid, of HD and GM-HD sorghums and their controls

Kafirin code	Sorghum properties of extracted kafirin		Dry total kafirin preparation		kafirin dough formed by acetic acid		Dough formed by acetic acid and water	
	Starch type	Protein digestibility	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)@	α/β ratio	Relative α-helical conformation (%)*
GM-HD-1	Normal	High	1.27[a] ±0.04	55.9	3.02[b] ±0.11	75.1	1.13[a] ±0.01	53.0
GM-HD-2	Normal	High	0.94[a] ±0.01	48.4	2.55[c] ±0.06	71.8	1.27[b] ±0.06	55.9
N1	Normal	Normal	1.34[b] ±0.02	57.3	2.67[c] ±0.07	72.8	1.11[a] ±0.06	52.6
N2	Normal	Normal	1.17[a] ±0.01	53.9	2.82[b] ±0.16	73.8	1.18[a] ±0.01	54.2
NHD 106	Normal	High	1.14[a] ±0.01	53.3	2.49[c] ±0.01	71.4	1.26[b] ±0.01	54.2
WHD 109	Waxy	High	1.45[a] ±0.02	59.2	2.42[b] ±0.03	70.7	1.27[a] ±0.11	55.9
WHD 142	Waxy	High	2.89[b] ±0.22	74.2	3.01[b] ±0.09	75.1	1.37[a] ±0.05	57.7
WHD 146	Waxy	High	1.45[a] ±0.05	59.2	2.31[b] ±0.01	69.8	1.47[a] ±0.05	59.5
WN 175	Waxy	Normal	2.07[b] ±0.15	67.3	2.27[b] ±0.01	69.4	1.36[a] ±0.01	57.6
WN 179	Waxy	Normal	1.06[a] ±0.01	51.6	2.16[c] ±0.01	68.4	1.10[b] ±0.01	52.3
NN 199	Normal	Normal	1.63[a] ±0.10	61.9	2.51[b] ±0.05	71.5	1.41[a] ±0.04	58.5
NN 200	Normal	Normal	1.57[b] ±0.10	61.0	2.27[c] ±0.01	69.4	1.30[a] ±0.04	56.4

Means with different superscript letters within a row are significantly different ($p < 0.05$). n=3

Wavenumber (cm^{-1}) of α-helix for all samples was 1650 ±2 and for β-sheet was 1620 ±2. * significantly lower than @

Table 4.3.2 Alpha/beta conformation ratio calculated from the FTIR spectra of the dry kafirin-1 preparations and dry kafirin-2 preparations of HD and GM-HD sorghums and their controls

Kafirin code	Sorghum properties of extracted kafirin		Amide I				Amide II			
			Kafirin-1		Kafirin-2		Kafirin-1		Kafirin-2	
	Starch type	Protein digestibility	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)	α/β ratio	Relative α-helical conformation (%)
NHD 106	Normal	High	1.66[b]	62.6	1.17[a]	53.8	1.80[b]	64.3	1.31[a]	56.5
WHD 109	Waxy	High	1.80[b]	64.4	1.17[a]	54.1	1.85[b]	65.2	1.31[a]	56.9
WHD 142	Waxy	High	1.77[b]	64.0	1.23[a]	55.0	1.79[b]	64.3	1.28[a]	56.0
WHD 146	Waxy	High	1.80[b]	64.5	1.23[a]	55.3	1.83[b]	64.9	1.33[a]	57.3
WN 175	Waxy	Normal	1.89[b]	65.3	1.33[a]	57.2	1.81[b]	64.4	1.41[a]	58.3
WN 179	Waxy	Normal	1.83[b]	64.8	1.21[a]	54.6	1.76[b]	63.8	1.36[a]	57.1
NN 199	Normal	Normal	1.80[b]	63.9	1.18[a]	54.1	1.81[b]	64.4	1.34[a]	57.0
NN 200	Normal	Normal	1.92[b]	65.9	1.11[a]	52.6	1.82[b]	64.7	1.32[a]	56.7

Means within different letters superscript within a row for each amide region are significantly different ($p < 0.05$).
kafirin-1 and Kafirin-2 were extracted by sequential extraction (Mazhar and Chandrashekar, 1993)
* Wavenumber (cm^{-1}) of α-helix and β-sheet for all samples were about 1650 ±2 and 1624 ±2 for Amide I and 1542±2 and 1515±2 for Amide II, respectively.

FTIR (Figure 4.3.5 and Table 4.3.2) in the Amide I region of kafirin-1 from WHD, NWHD and their controls showed a higher α/β ratio than for kafirin-2. This indicates that as a result of the reducing agent used to extract the kafirin-2 breaking disulphide crosslinking between the different kafirin sub-classes more kafirin in the form of β-sheet conformation was extracted. An important observation was that the kafirins from the HD lines; WHD 142, WHD 146 and WHD 109 had a somewhat higher α/β ratio higher than the normal lines; NN 199 and NN 200. This can be ascribed to less disulphide crosslinking in the HD lines compared to the normal lines.

4.3.4.3 *Electrophoresis*

SDS-PAGE (Figure 4.3.6) of the total kafirins extracted from all the sorghum lines indicated that both the GM-HD sorghum lines were missing a band of molecular weight ~23 kDa (indicated by Black solid arrows), which was surmised to be γ–kafirin (Da Silva et al., 2011b). The WHD 109, WHD 142 and WHD 146 sorghum lines were missing a band of MW ~18.5 kDa (indicated by Black dashed arrows). The absence of this band indicated a reduction in β-kafirins in these three WHD lines. Bands of this approximate low molecular weight are noted to be β-kafirins (Elkhalifa et al., 2009).

SDS-PAGE of kafirin-1 and kafirin-2 extracted from the non-GM lines, i.e. the line developed by Texas A&M Agrilife (Figure 4.3.7.A) confirmed the reduction of β-kafirin in the WHD sorghums. It appeared that the most effective separation of β-kafirin was achieved by analysis of the kafirin-2 under reducing conditions (Figure 4.3.7.B).

The broad bands of MW approx. 27 kDa of the kafirin monomers resolved by SDS-PAGE (Figure 4.3.6) and indicated by a bracket, can be ascribed to there being more than one kafirin fraction present in this molecular weight range. Thus, 2-D PAGE was performed. This revealed that generally, all kafirin preparations displayed more polypeptide spots within the basic region (pH higher than 7) than the acidic region (Figure 4.3.8). As expected, there were several kafirin fractions that had the same molecular weight but differing in their isoelectric points. GM-HD-1 and GM-HD-2 both had a spot missing of molecular weight about 27 kDa (Figures 4.3.8.K and 4.3.8.L). This missing spot was probably γ-kafirin (Evans et al., 1987; El Nour et al., 1998). The absence of γ-kafirin indicated suppression of γ-kafirin synthesis as is the case in these lines (Da

Silva et al., 2011b). Their null controls, N1 and N2, had a protein spot missing of about 21 kDa as indicated by the arrows when compared to the kafirins from the GM-HD lines (Figures 4.3.8.I and 4.3.8.J). This is possibly as a result of the presence of the γ-kafirin in the N1 and N2 kafirins, so that the particular kafirin protein was cross-linked with γ-kafirin and did not separate. In the GM-HD lines this kafirin was separated and appeared (Figure 4.3.8.K and 4.3.8.L) due to the absence of the γ-kafirin and is indicated by black dashed arrows.

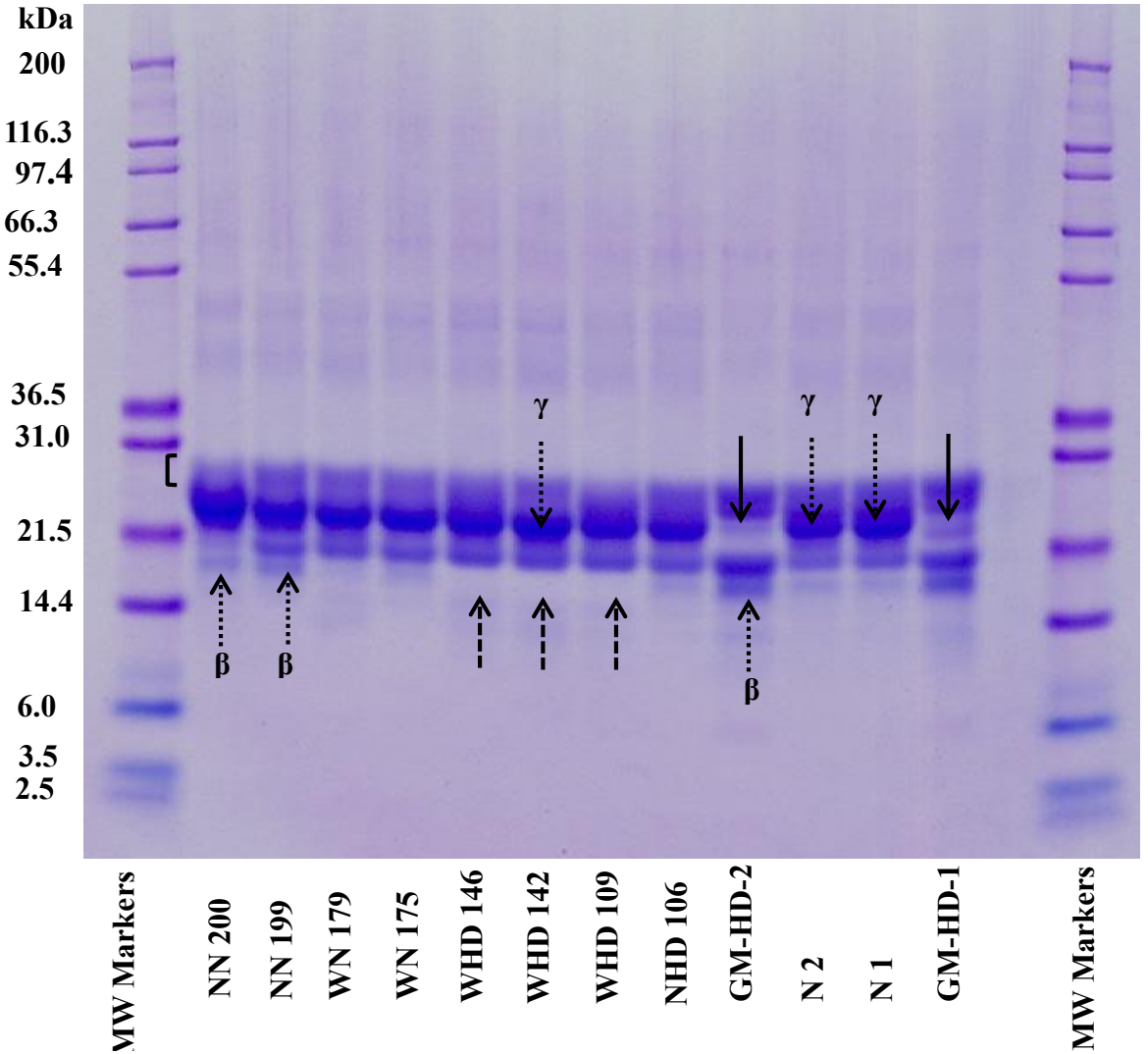

Figure 4.3.6 SDS-PAGE under reducing conditions of total kafirins extracted from conventionally bred WHD and GM-HD sorghums and their controls

Solid arrows indicate the position of missing γ-kafirin bands; dashed arrows indicate missing β-kafirin (β) bands in WHD and GM-HD lines compared to their controls

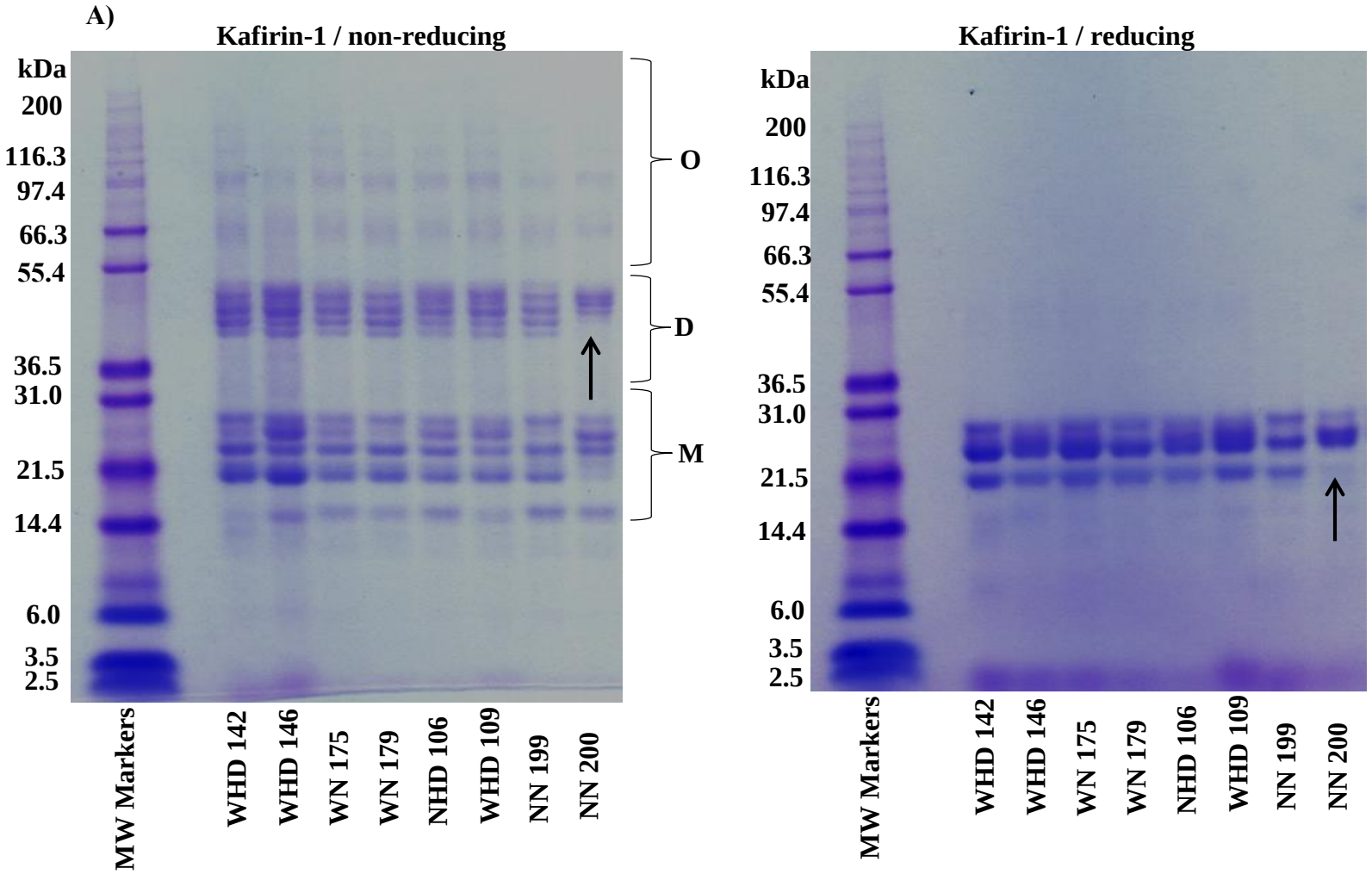

Figure 4.3.7.A SDS-PAGE under non-reducing and reducing conditions of kafirins extracted from conventionally bred sorghums through a sequential extraction. A: kafirin-1

Solid arrows indicate the missing bands. M = monomers, D = dimers, O = oligomers

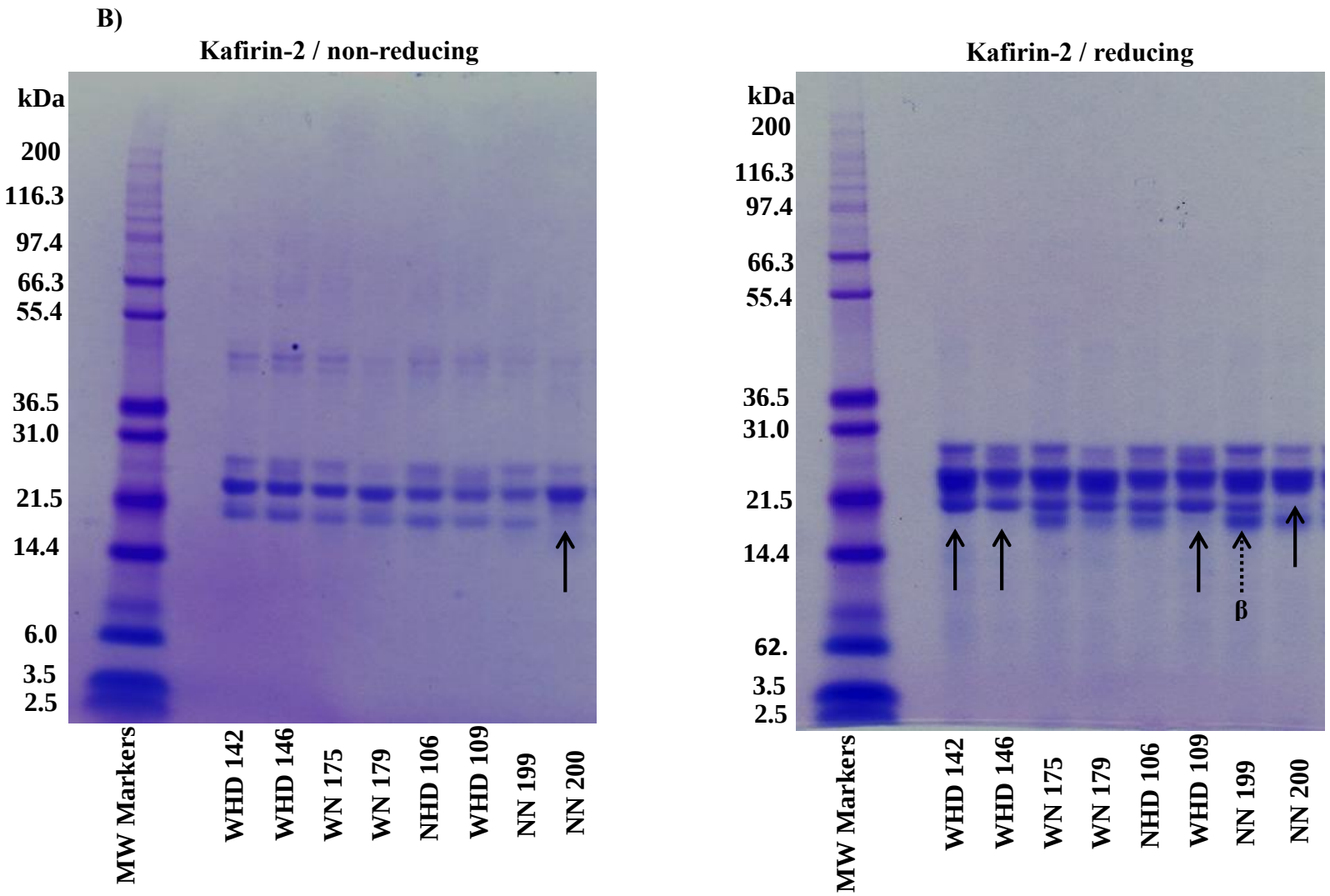

Figure 4.3.7.B SDS-PAGE under non-reducing and reducing conditions of kafirins extracted from conventionally bred sorghums through a sequential extraction. B: kafirin-2

Solid arrows indicate the missing bands. β, β-kafirin

2-D PAGE of the kafirins from the non-GM HD lines did not clearly show the absence of the β-kafirin spot (Figures 4.3.8.F, 4.3.8.G and 4.3.8.H), unlike with SDS-PAGE (Figure 4.3.7.B). El Nour et al. (1998) reported a similar finding that the spot of the β-kafirin in their 2-D PAGE was not clear and could hardly be distinguished. The normal lines NN 199 and NN 200 displayed a protein missing of about 21 kDa. In this current work it was difficult to confirm whether this was related to the presence of β-kafirin or not. However, generally it could be said that there was slight difference in the total 2-D PAGE kafirin patterns of the normal lines and the WHD lines.

Schober et al. (2011) found that total kafirin "dough" containing β- and γ-kafirins rapidly aggregated and became firm, probably due to their presence and lost its extensibility. In this current work, the expression of γ-and β-kafirins from the GM-HD and WHD sorghum lines, respectively, were considerably reduced. Based on this finding, it can be hypothesised that the suppression of both β- and γ-kafirin synthesis improves the viscoelastic properties of kafirin dough. This is consistent with the findings of Schober et al. (2011). These authors also investigated the effect of different zein isolation procedures from maize. These isolation procedures resulted in different zein preparations in terms of proportion of β- and γ-zeins. They found that the proportion of β- plus γ-zein relative to α-zein had a negative effect on the viscoelastic properties of the zein doughs. They further suggested that hydrophobic interactions had more impact than disulphide bonds in terms of improving the zein and kafirin functionality. The current findings that the GM-HD and WHD had more fibril formation than their controls, which was associated with better viscoelasticity is in general agreement with the observations of Schober et al. (2011).

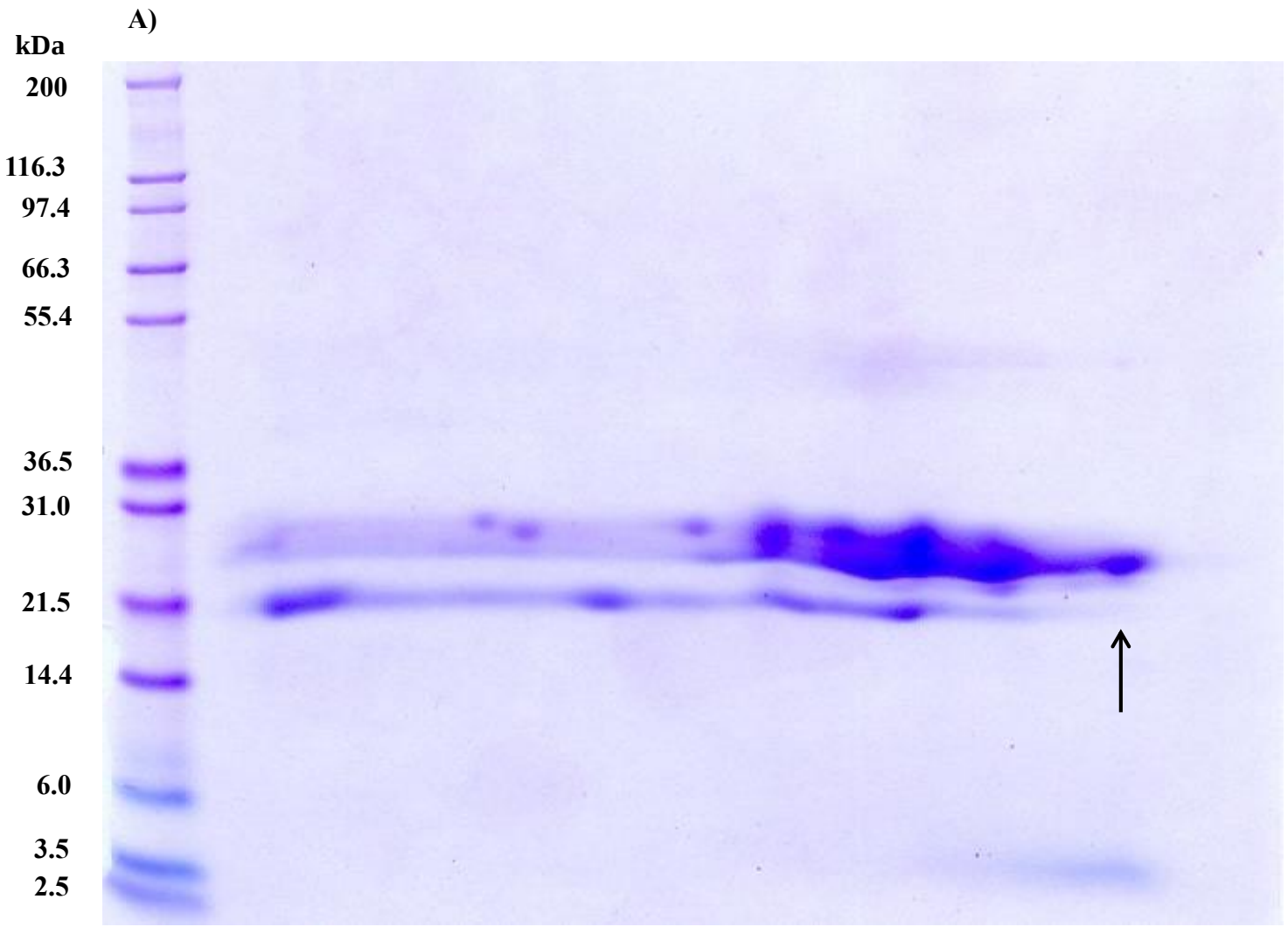

Figure 4.3.8.A 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. A = normal sorghum line NN 199

Arrows indicate the position of the missing spots

B)

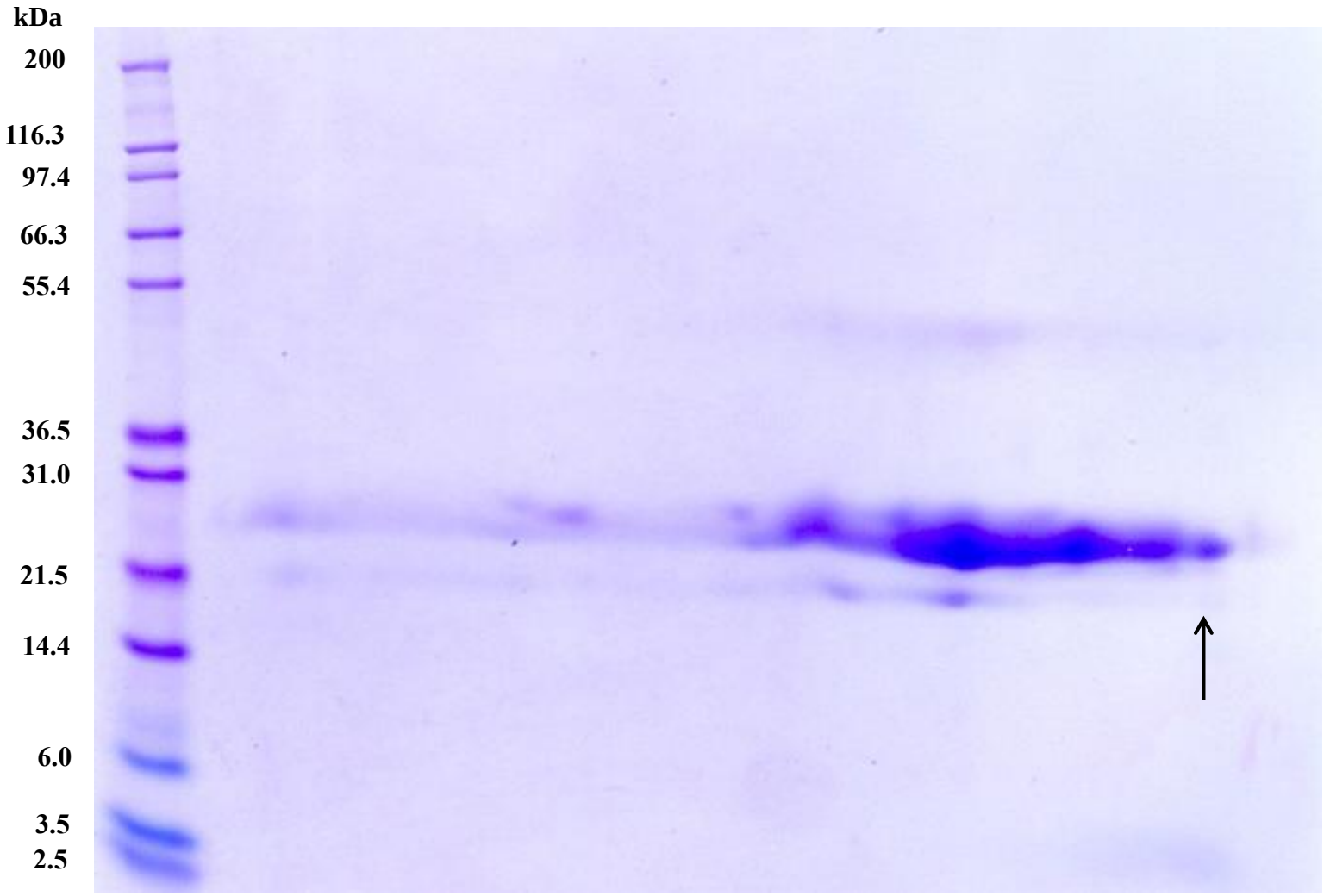

Figure 4.3.8.B 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. B: normal sorghum line NN 200

Arrows indicate the position of the missing spots

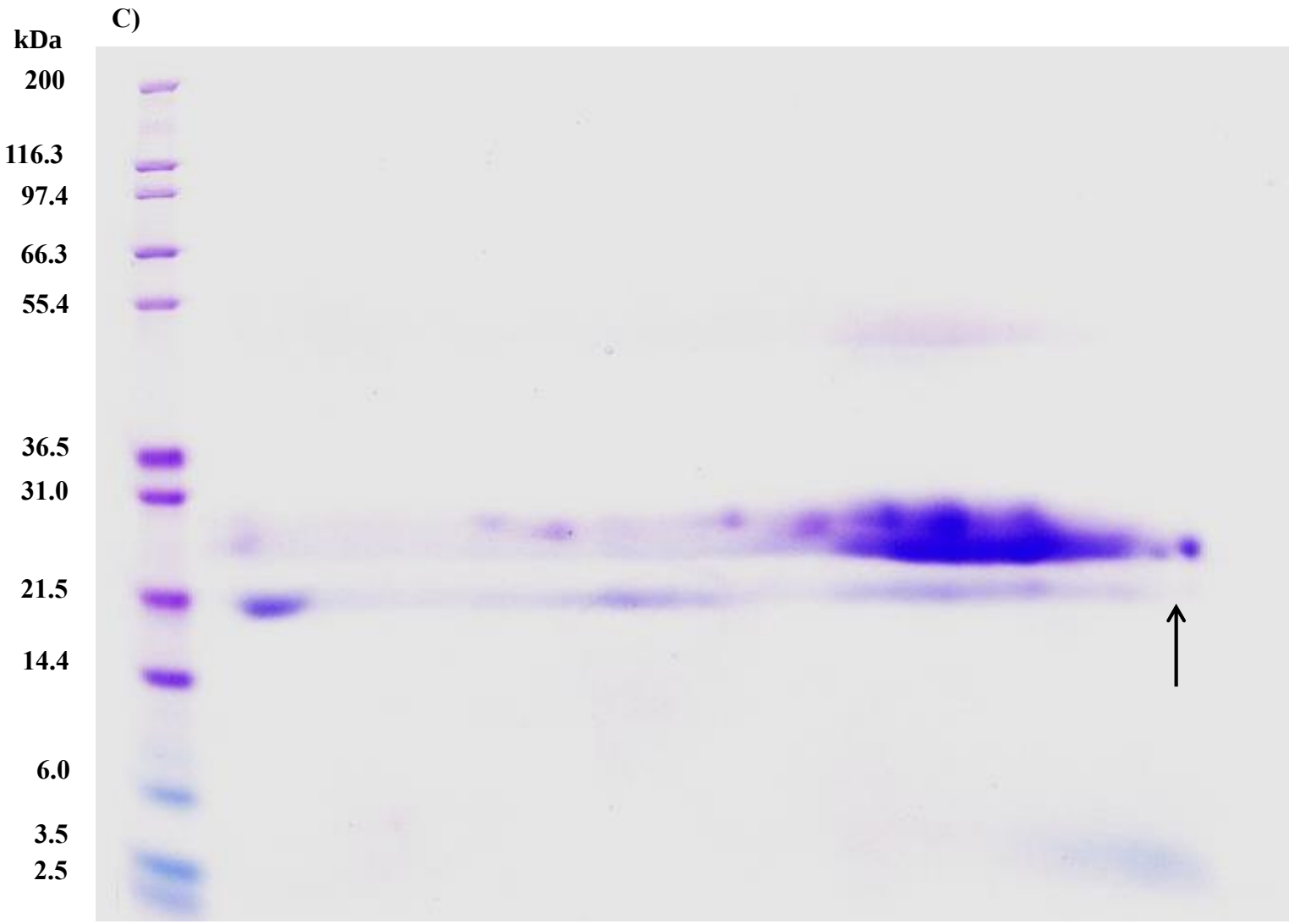

Figure 4.3.8.C 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. C: sorghum line WN 175

Arrows indicate the position of the missing spots

D)

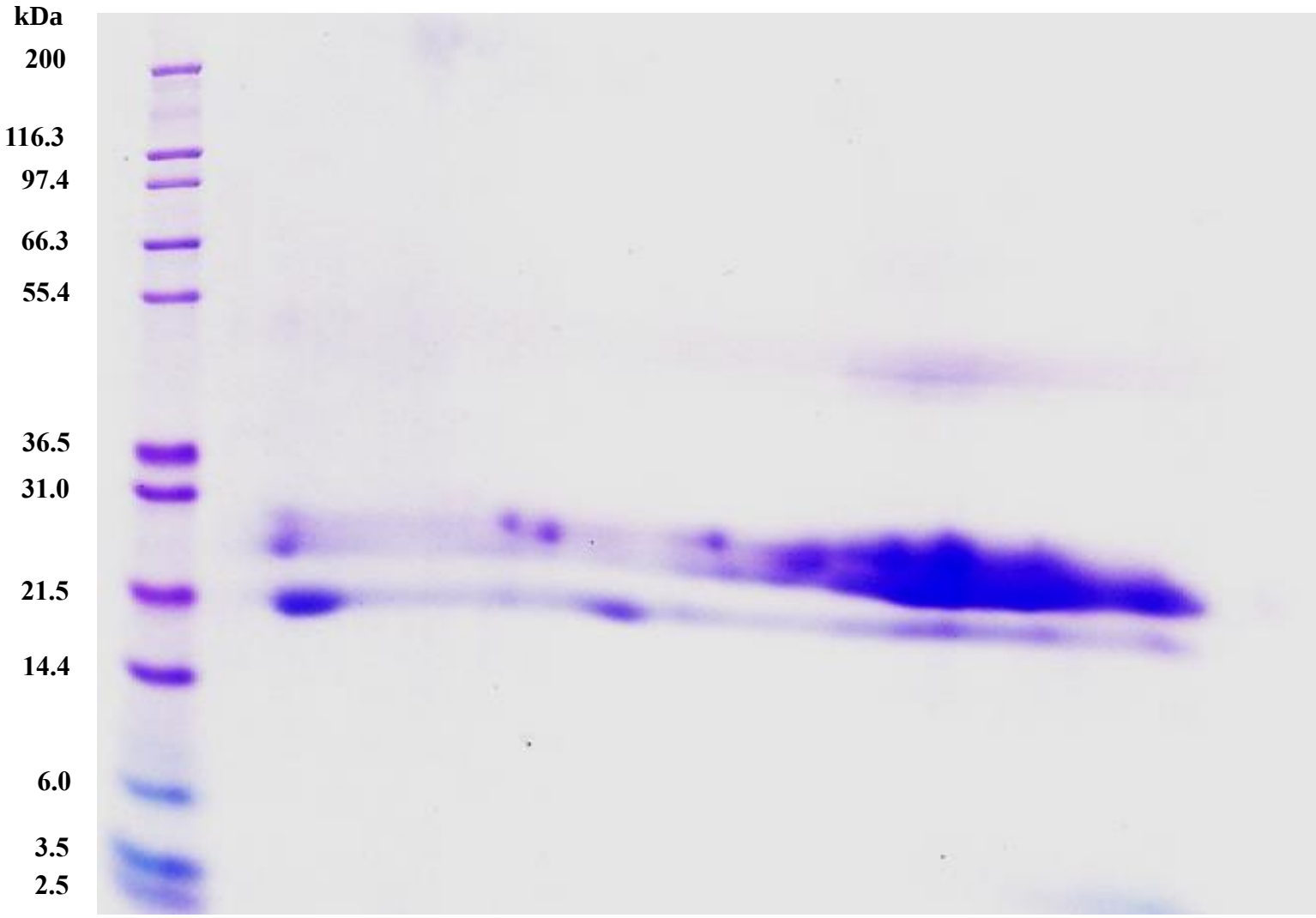

Figure 4.3.8.D 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. D: sorghum line WN 179

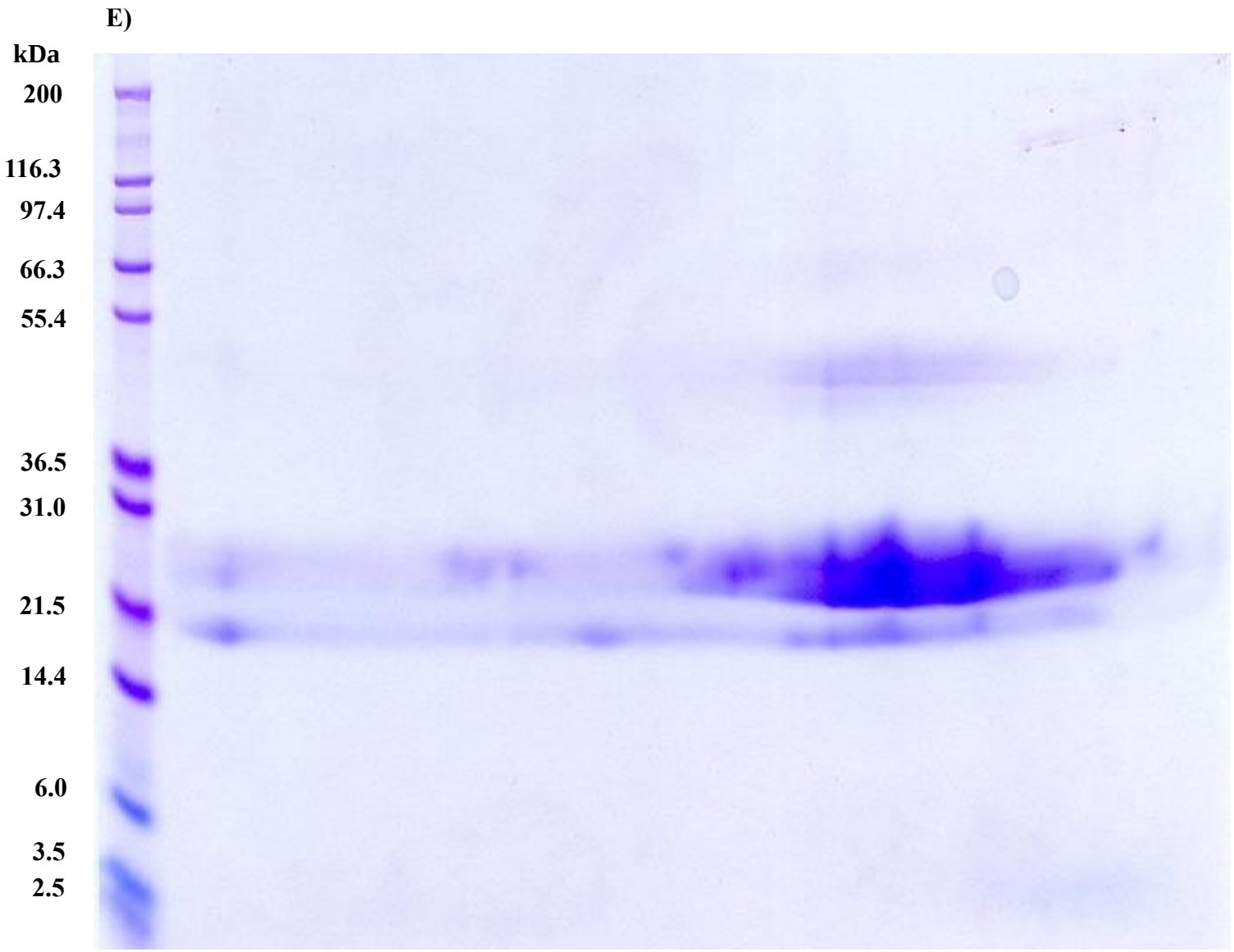

Figure 4.3.8.E 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. E: sorghum line NHD 106

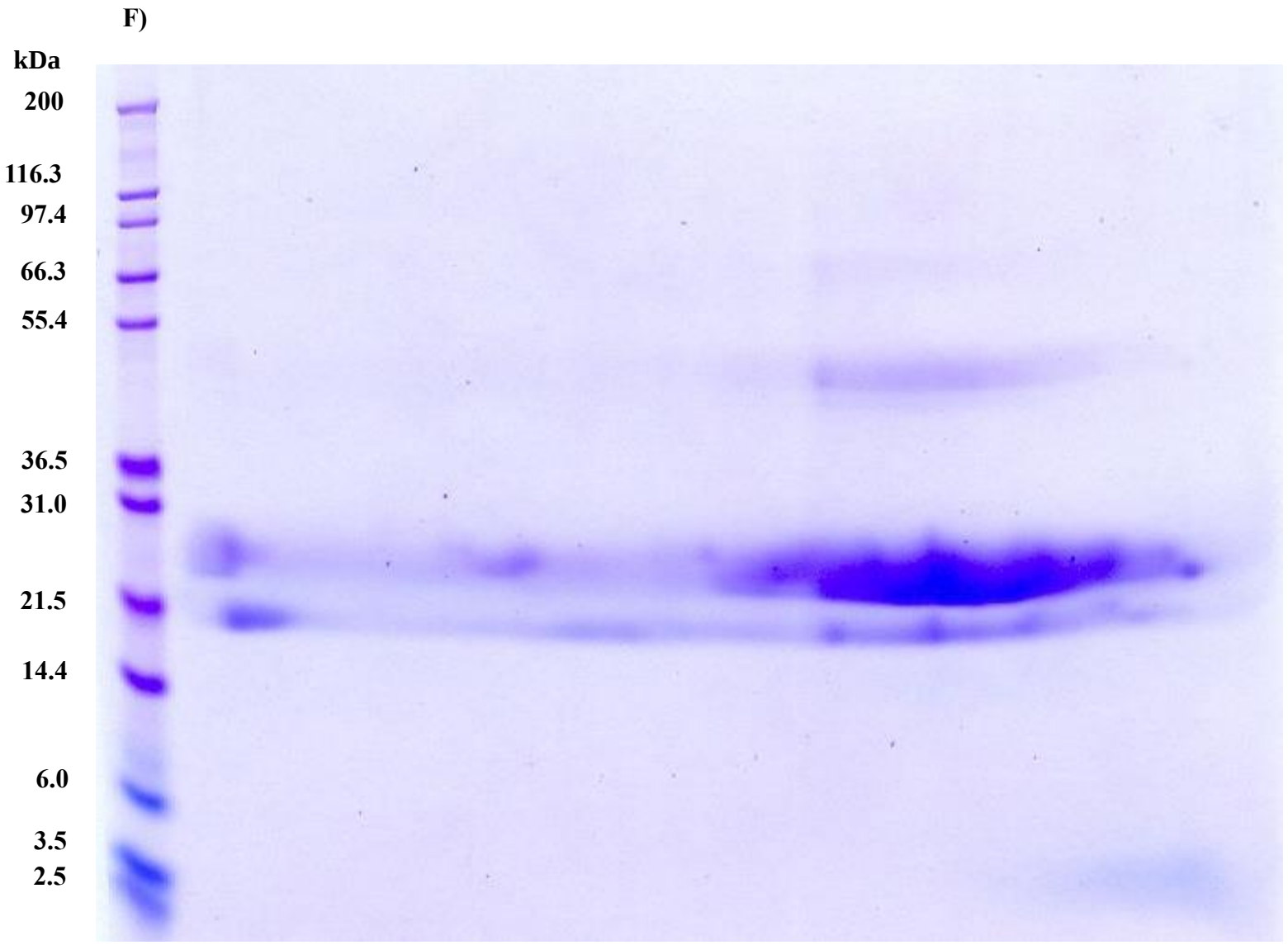

Figure 4.3.8.F 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. F: sorghum line WHD 109

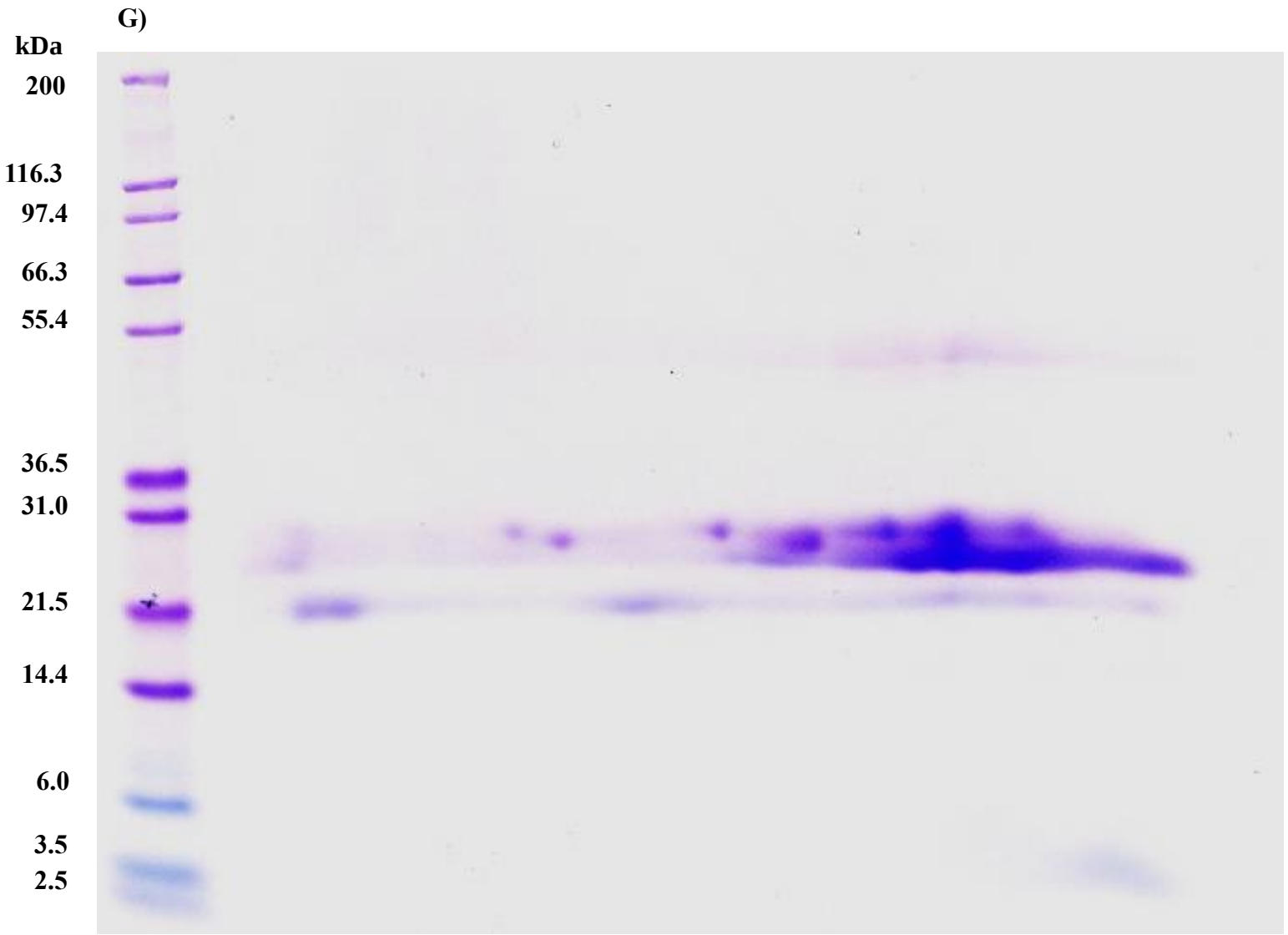

Figure 4.3.8.G 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. G: sorghum line WHD 142

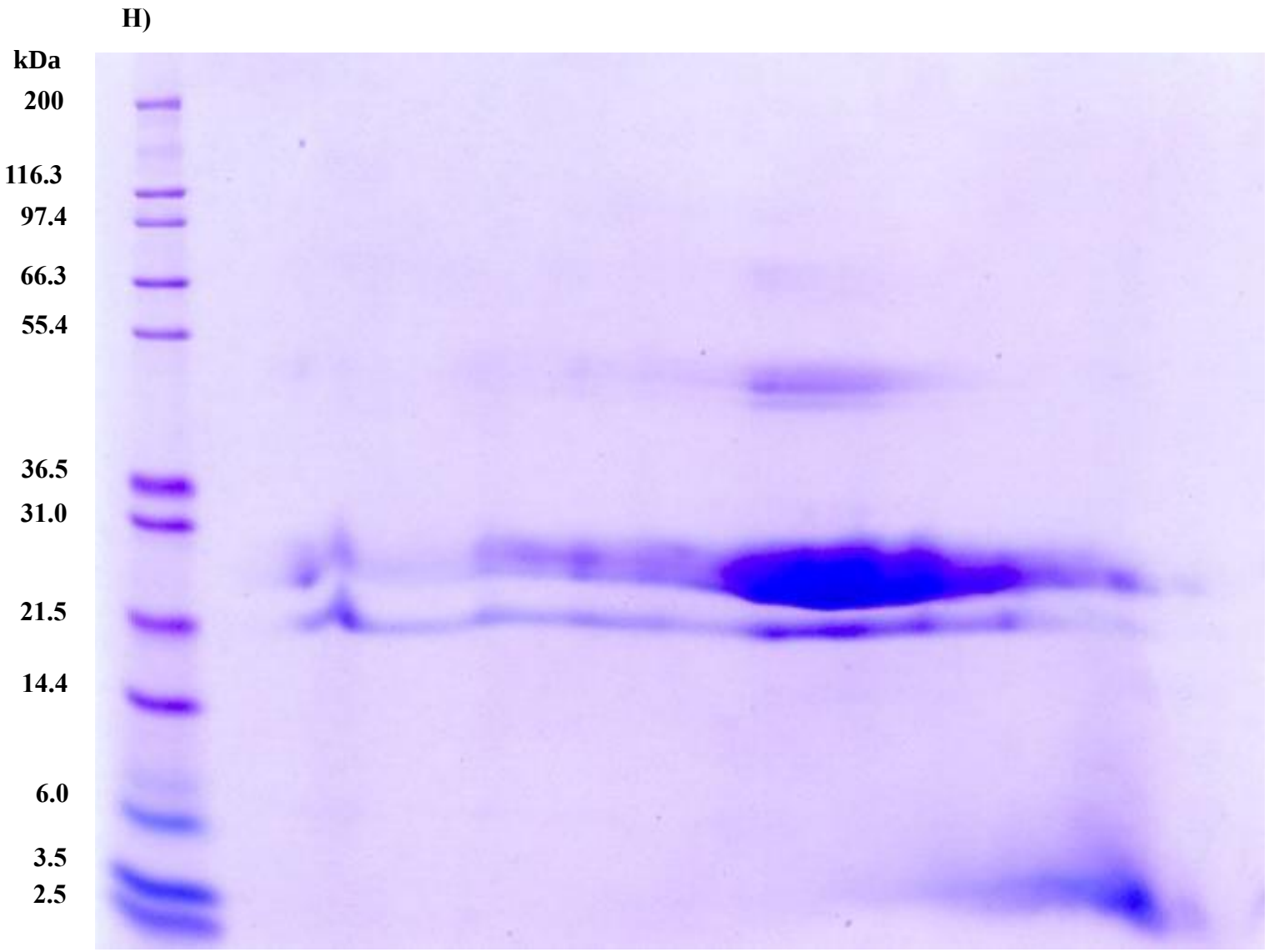

Figure 4.3.8.H 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. H: sorghum line WHD 146

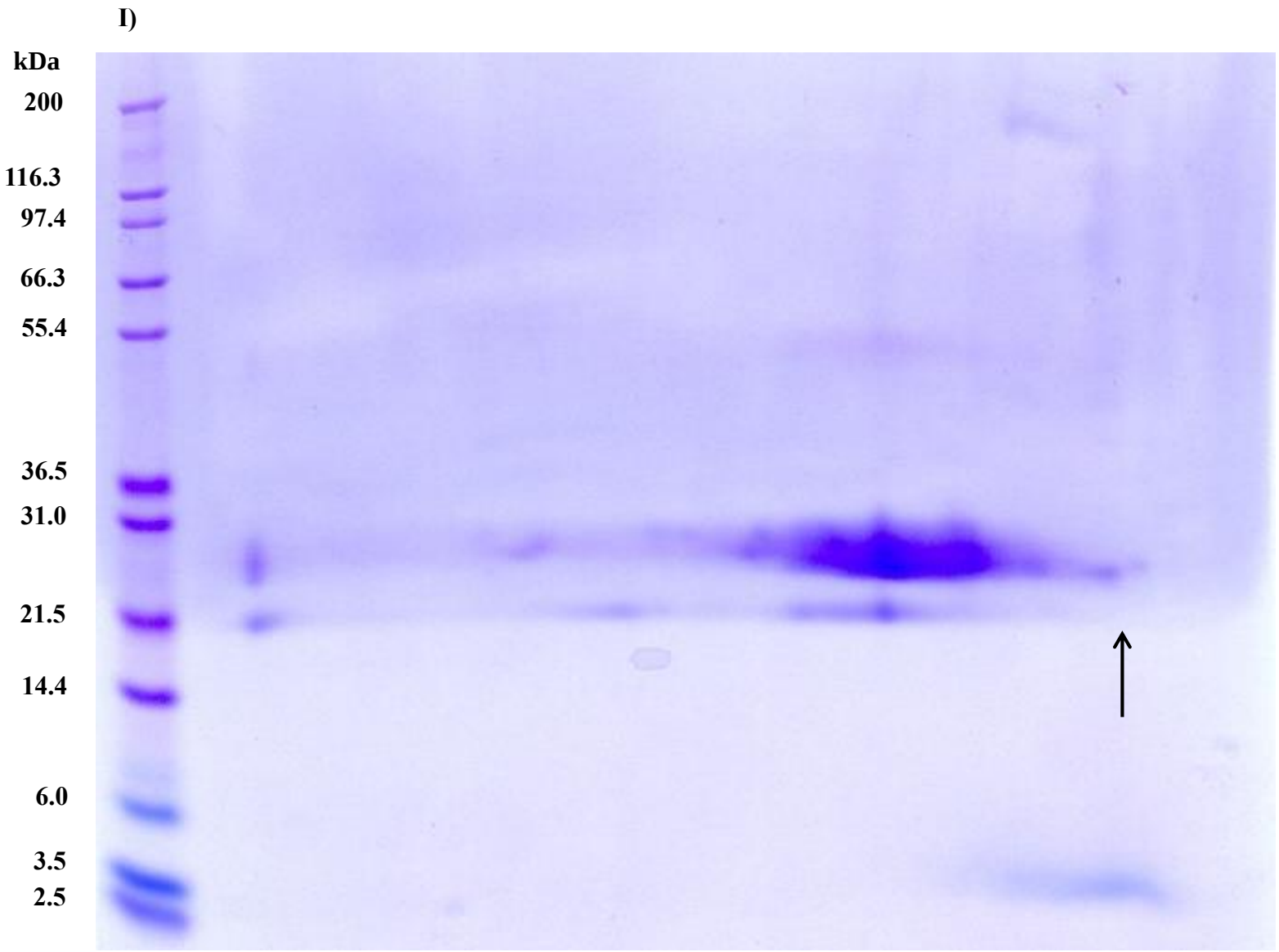

Figure 4.3.8.I 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. I: sorghum line N 1. Arrows indicate the position of the missing spots

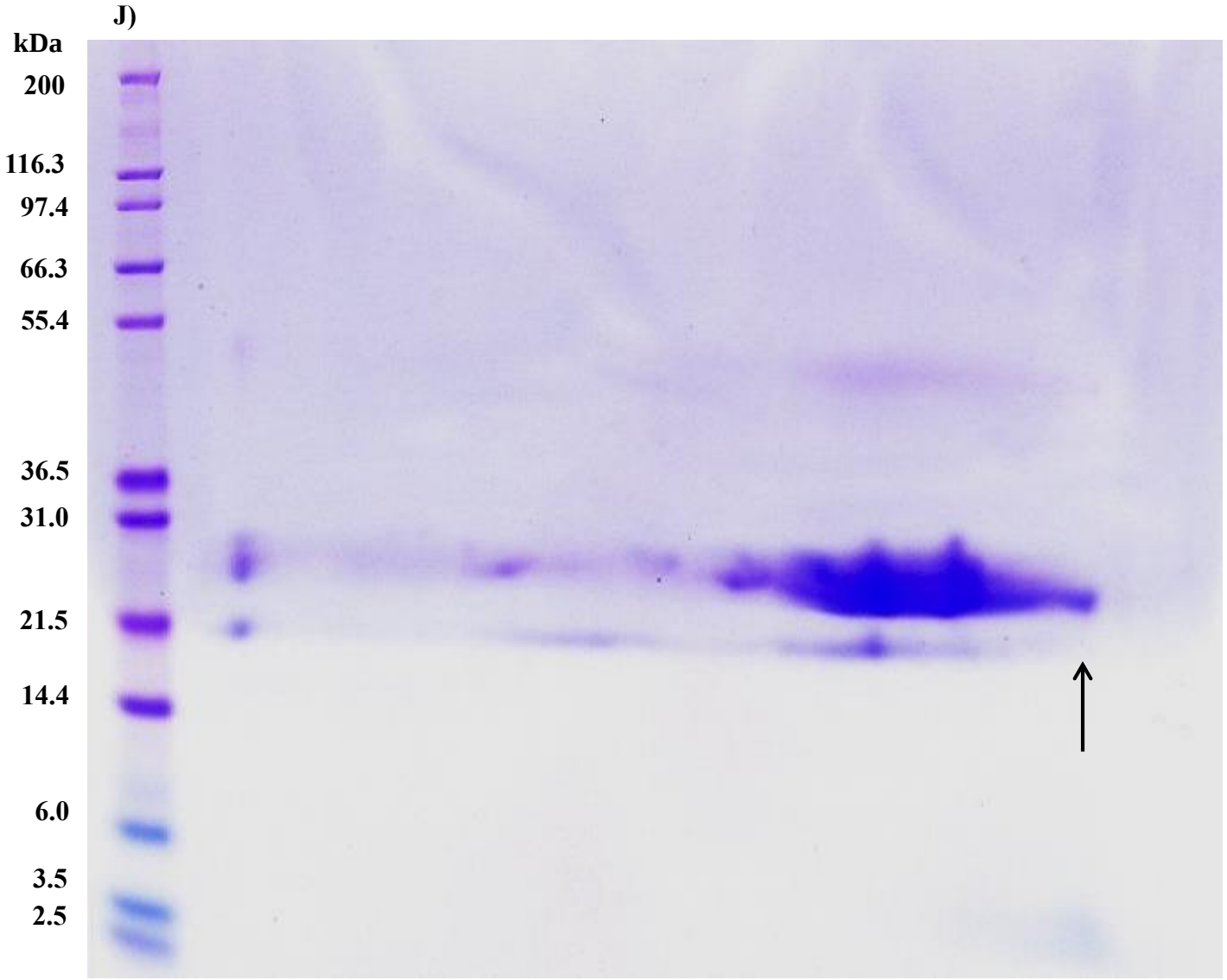

Figure 4.3.8.J 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. J: sorghum line N 2. Arrows indicate the position of the missing spots

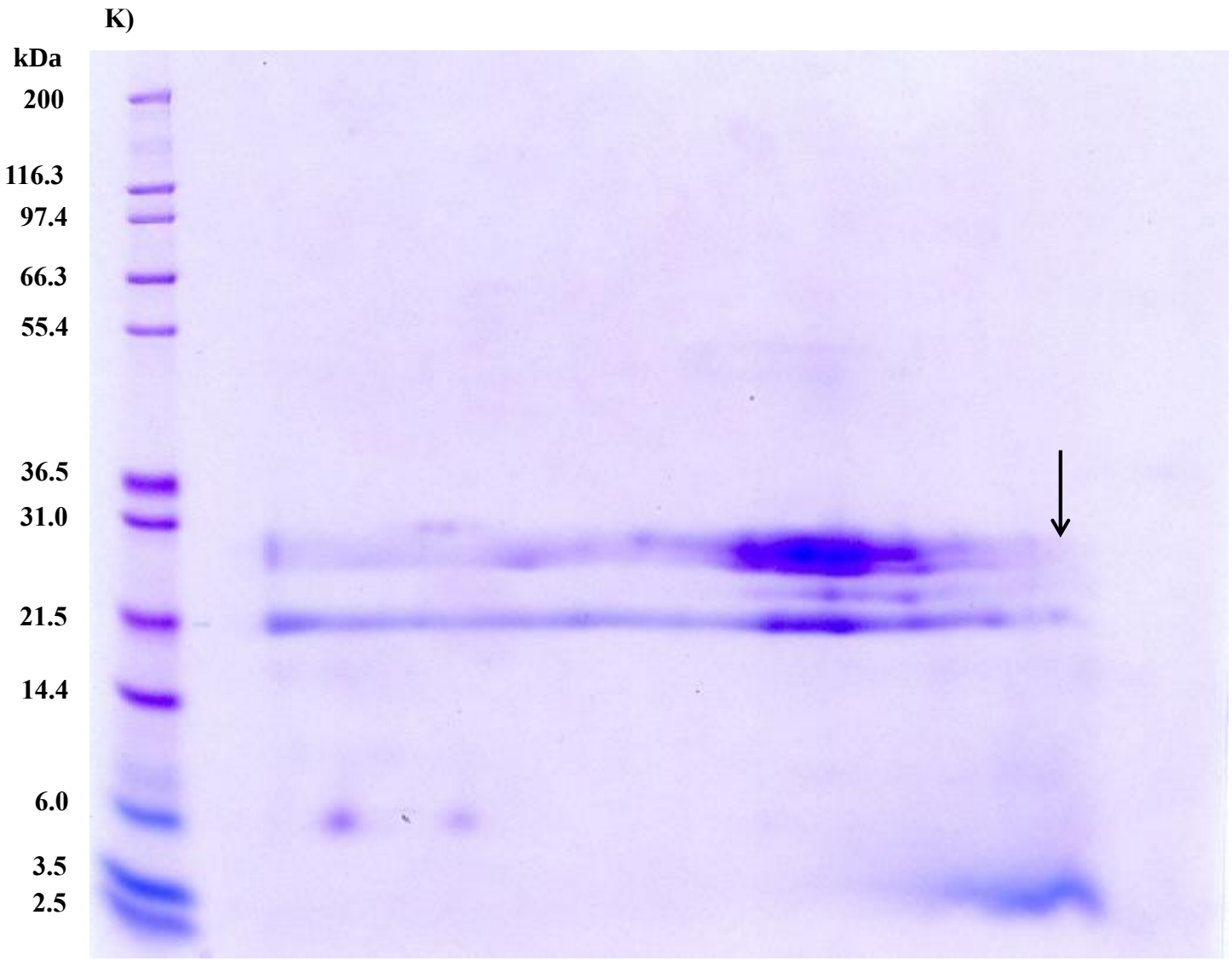

Figure 4.3.8.K 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. K: sorghum line GM-HD-1. Arrows indicate the position of the missing spots

L)

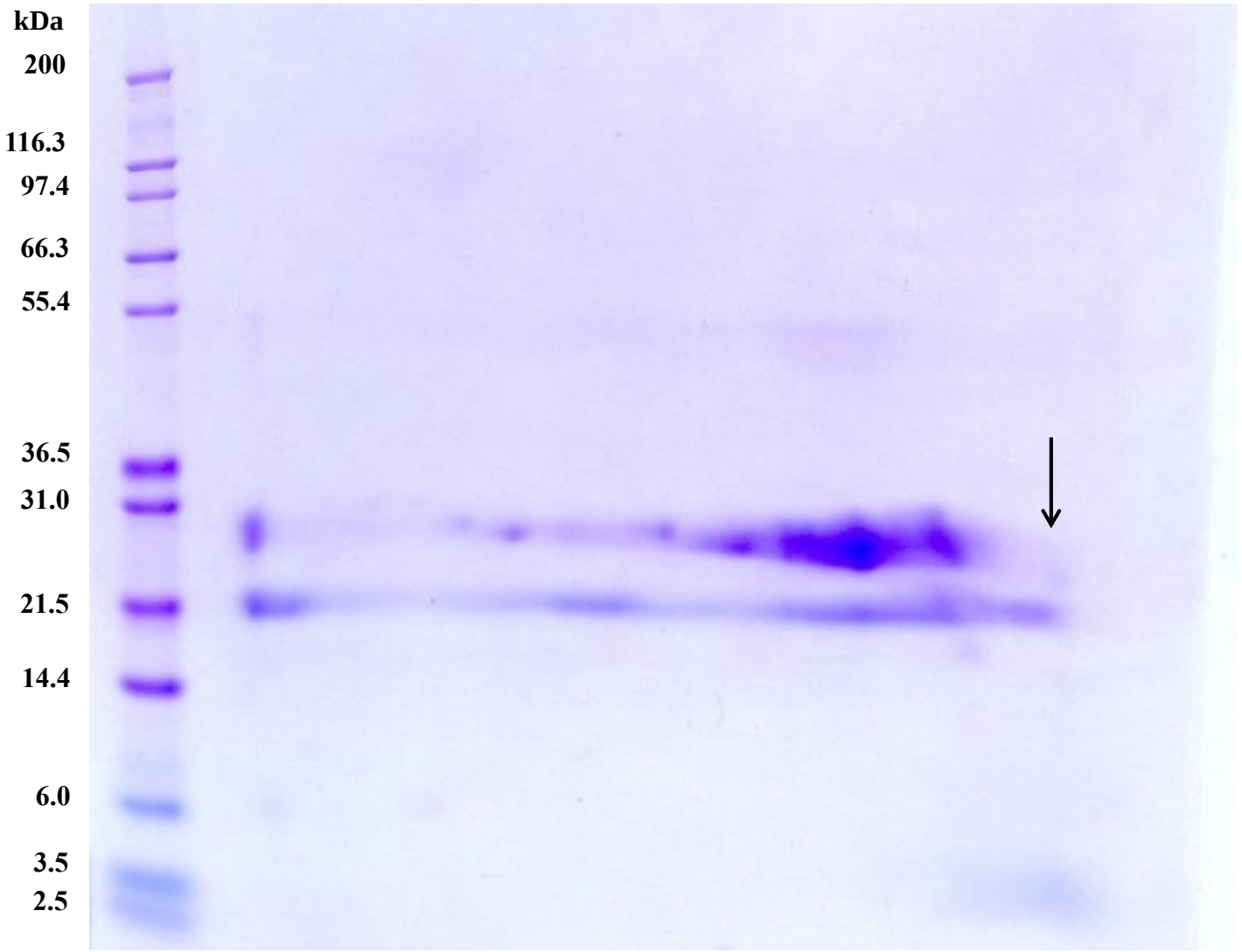

Figure 4.3.8.L 2-D PAGE of total kafirin extracted from conventionally bred WHD and GM-HD sorghums and their controls. L: sorghum line GM-HD-2. Arrows indicate the position of the missing spots

4.3.5 Conclusions

Dough can be formed from total kafirin when it is dissolved in glacial acetic acid and then cold water added rapidly to coacervate it out of solution. This rapid coacervation process seems to enable the kafirin to take up water easily and bind with it to form a stable dough. Reduction in β- and/or γ-kafirin improves the kafirin dough viscosity and this may be as a result of the modified kafirin with less disulphide bonding forming more fibrils than normal kafirin. Reduction in the level of γ-kafirin seems to be more effective than reduction in β-kafirin with regard to improved dough viscoelasticity. As indicated by the findings in research chapters 4.1 and 4.2, these modified kafirins seem to potentially improve sorghum flour properties for bread making.

4.3.6 References

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5 GENERAL DISCUSSION

This general discussion will critically review the experimental methodologies as they were applied in this study. A special focus (but not limited) will be given to the method developed for kafirin dough formation and the mechanisms that are proposed to be responsible for the kafirin dough formation and for viscoelastic properties of the kafirin doughs. The effects of some factors on kafirin dough formation will be discussed, specifically the effect of kafirin modification. As there are many important questions that have arisen from this study, the general discussion will also propose further work that can answer the questions regarding the potential use of glacial acetic acid in sorghum flour dough formation.

5.1 METHODOLOGICAL CONSIDERATIONS

This study aimed in general to investigate the potential to form a viscoelastic dough from sorghum that can be suitable for bread making. The effects of the high protein digestibility trait and/or starch type on the characteristics of the sorghum endosperm, kafirin bodies, flour and dough quality were investigated. The waxy and high protein digestibility sorghum lines and their controls provided by Texas A&M University were not ideal as they were not homologous i.e. the normal (control) lines were just similar lines but not genetically identical and only differing in one specific character compared to the WHD lines. Also, for some traits there was only one sorghum line to represent them such as line NHD 106 which represented the normal (non-waxy) starch and high protein digestibility traits. However, to strengthen the evaluation of how sorghum endosperm expression affected flour and dough quality, two genetically modified sorghum lines that had reduced γ-kafirin expression were studied in comparison with their true null controls. This investigation of the GM lines was considered more rigorous as the null controls differed from the GM lines in the expression of γ-kafirin synthesis but for all other properties they were identical to the GM lines.

A weakness of the study was that although it targeted suitable dough properties for bread making there was no direct evaluation of doughs made from the sorghum flours through common rheological instruments such as in Farinograph or Alveograph as was performed , for example by Goodall et al. (2012) and Sly et al. (2014). This was because the quantity of the grain samples was too little since the lines were genetically modified or conventionally bred on a small research scale. Also, specifically the GM sorghums were provided as crushed whole grain and hence the investigation was limited to analyses that did not require decortication of the sorghum kernels. The

lack of evaluation of sorghum flour dough quality using instruments like the Farinograph was a weakness with regard to evaluation of sorghum lines for bread quality. However, some authors have also used similar techniques that were used in this current study and they evaluated their dough accordingly, for example Schober et al. (2008). These authors used dynamic oscillatory tests and also CLSM for dough investigation to study dough quality in gluten-free bread making from zein strach.

As described in Chapter 4.3, kafirin dough was obtained by dissolution of kafirin powder in glacial acetic acid and then water was added to the mixture and then the kafirin aggregated and was collected then kneaded. This method was applied on the extracted kafirin instead of the whole flour. Such an involved process is unlikely to be workable at the commercial level for bread making. This method was also conducted at very small scale, i.e. a small amount (~ 2 g) of dough was used which was not ideal for characterization. Hence, the rheological properties of the kafirin doughs could not be measured. Further, as the quality of bread is affected by all the components of flour and not just the kafirin protein, the investigation of kafirin can be considered as just an indicator of sorghum flour quality but is not adequate by itself to go ahead and develop the sorghum lines for bread making.

5.2 EFFECT OF THE MODIFIED KAFIRIN EXPRESSION ON ENDOSPERM TEXTURE

Screening of the high protein digestibility sorghum lines developed by Texas A&M University with modified kafirin expression revealed that the endosperm of these sorghum lines tended to have a floury endosperm and that their kafirin was reduced in β-kafirin. This was similar to what Da Silva et al. (2011a) found in genetically modified sorghum lines with high protein digestibility, which had a floury endosperm and had reduced γ-kafirin expression. The degree of the hardness of the sorghum endosperm has an impact on milling and subsequent flour quality (Taylor and Dewar, 2001). In wheat, hard endosperm results in smaller size starch granules (Edwards et al., 2008). It is assumed that the same thing happened in these sorghum lines. As γ- and β-kafirin are responsible for the disulphide crosslinking in kafirin which forms the kafirin polymers, it is assumed that a reduction in the amount of one of these sub-classes of kafirin can weaken the association of the kafirin matrix in the endosperm. The weakening of the protein matrix could

presumably result in a weaker protein matrix around the starch granules. This is probably why the endosperm of the sorghum lines with reduced γ- and β-kafirin was floury.

5.3 PROPOSED MECHANISM FOR KAFIRIN DOUGH FORMATION BY COACERVATION FROM A SOLUTION OF KAFIRIN IN GLACIAL ACETIC ACID

There is some literature on the application of dilute acetic acid in zein dough formation (Sly et al., 2014). However, there is no published research on dough formation from kafirin by simple coacervation from a solution of kafirin in glacial acetic acid using water. From the high extent of homology between kafirin and zein (Belton et al., 2006), it can be expected that the kafirin would behave similarly to the zein in terms of its response to acetic acid treatment. In the work of Sly et al. (2014) acetic acid was added to commercial zein (α-zein) as a dilute solution in water in various percentages. When the method of Sly et al. (2014) was applied on total kafirin in this study (data not presented), it did not form a dough. However, in this current study the kafirin was mixed and dissolved in warm glacial acetic acid only (before the water was added to the solution). This method was used by Taylor and co-workers (Taylor, 2008; Taylor et al., 2009) for kafirin microparticle formation and is patented (Taylor and Taylor, 2010). Thus, it can be expected that essentially the same mechanism was responsible in this current case for kafirin dough formation.

The obvious question here is why in this work the result was dough formation unlike what happened in the work of Taylor (2008) where microparticles resulted. The main difference and assumed reason is that in this study the water was added rapidly, while Taylor (2008) added the water gradually over 5 minutes. Also, in this present work there was no stirring during water addition unlike what Taylor (2008) did. It is considered that the stirring and gradual addition of the water disrupted the formation of the kafirin dough and instead microparticles formed. It is speculated that in this study hydrogen bonding between kafirin molecules and between the kafirin molecules and water molecules kept the kafirin hydrated and due to this the fibrils were formed. During this current work, it was observed that when the kafirin fibrils formed it was very easy to make microparticles by stirring. Further, it was found that kafirin doughs prepared with only glacial acetic acid greatly increased the α-helical structure of the kafirin. Beta-sheet structure can be considered as being involved in kafirin dough elasticity as is the case with wheat glutenin (Shewry et al., 1992; Wieser, 2007) and was proposed for zein elasticity (Erickson et al., 2012).

In this study there was a slight but non-systematic increase in β-sheet in the dough compared to the dry kafirin. Possibly, the α-helical structure that formed due to glacial acetic acid was converted to β-sheet structure when the water was added, which reduced the concentration of the acid. It is speculated that the rapid coacervation with water changed the protein conformation from γ-helical to be partly β-sheet conformation which facilitated kafirin-water molecule hydrogen bonding. Hence, the Loop and Train theory of glutenin elasticity of Belton (1999) can be applied to interpret the formation of a kafirin viscoelastic dough. It is assumed that the kafirin β-sheet conformation gave a loose spiral polymer structure, similar to the glutenin polymer model (Shewry et al., 1992). It is suggested that this change in kafirin secondary structure is a result of the rapid dilution of the glacial acetic acid. The loose β-sheet spiral structure greatly enhanced hydrogen bonding between water molecules, and the kafirin molecules. The loose spiral β-sheet structure is, as is assumed to be the case for glutenin molecules in the hydrated loop region according to the Loop and Train theory. Precipitation of fibrils of kafirin was due to aggregation of kafirin molecules after addition of the water. Because there was no stirring, the aggregation of the kafirin happened suddenly and trapped the water between the kafirin molecules and formed the hydrogen bonds with the water molecules. The collection of these aggregated kafirin fibrils and the formation of a dough through kneading is illustrated in Figure 5.1.

With the exception of the work of Taylor (2008) and Taylor et al. (2009), and papers by Bugusu et al. (2001) and Ezeogu et al. (2008) there is essentially no literature that deals with kafirin aggregation behaviour. However, the aggregation of zein molecules has been examined as the degree of the turbidity using dynamic light scattering (Kim and Xu, 2008). The aggregation size of zein molecules was estimated around 10 molecules at an ethanol concentration of 90%. However, when a 70% concentration of ethanol was used, the aggregation size was estimated as 10 000 approx. zein molecules. Although in this present work glacial acetic acid was used instead of the ethanol, it is hypothesized that the same mechanism took place with kafirin. The temperature of 50°C that was used in kafirin dough preparation played a role in the successful viscoelastic dough preparation. The high temperature of the process kept the kafirin above its glass transition temperature (T_g), the maximum temperature at which the proteins remain in their glassy state before they transform to viscoelastic rubbery state after hydration (Levine and Slade, 1989).

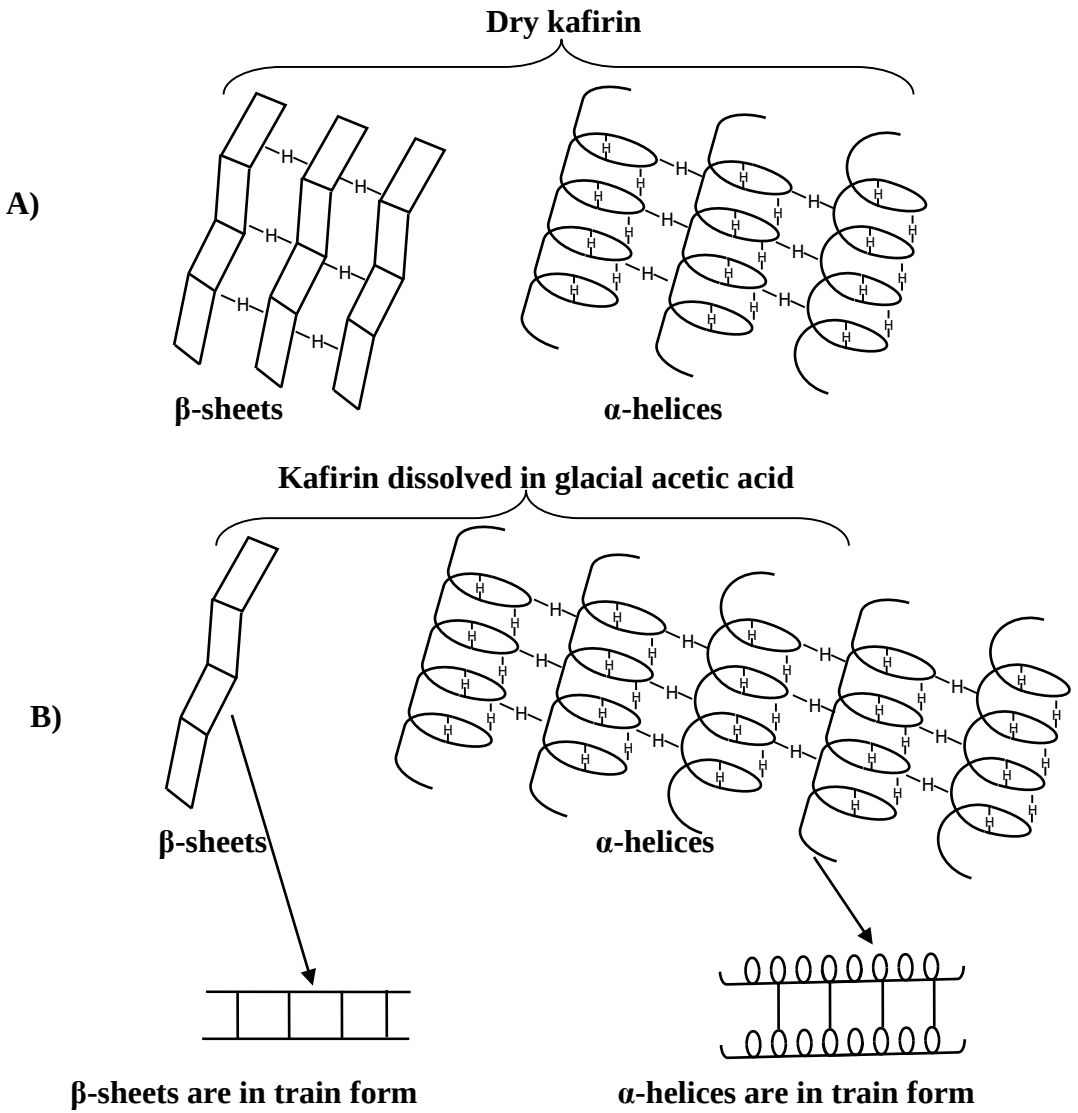
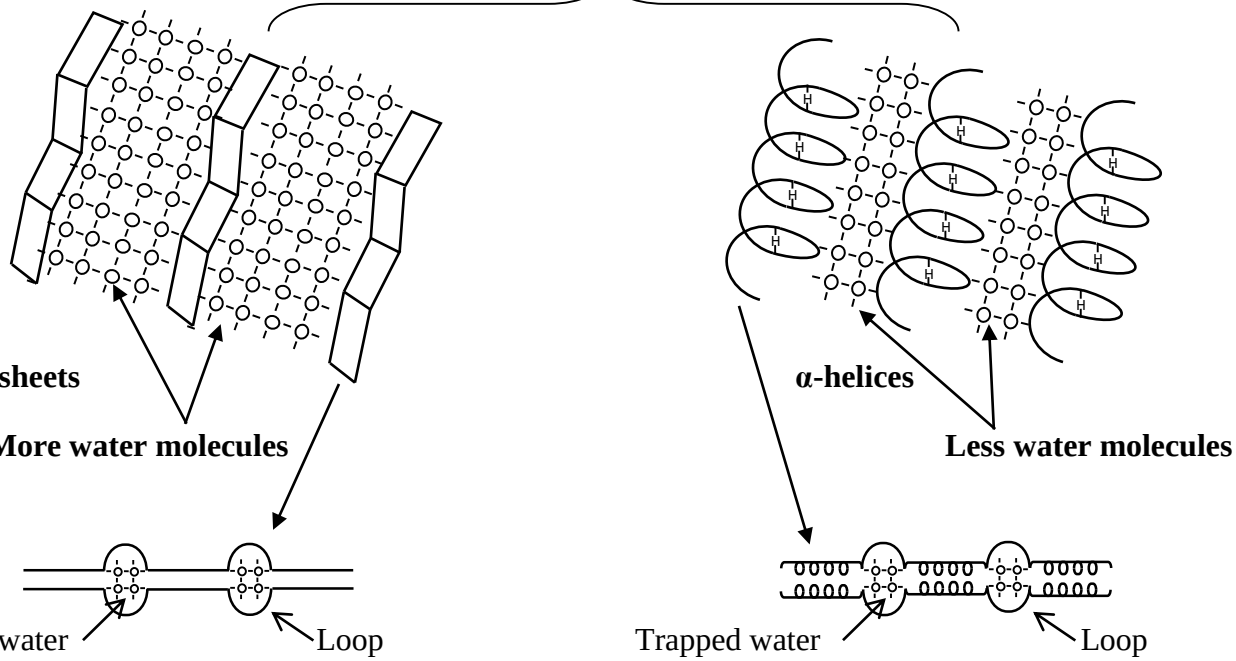


Figure 5.1 Proposed changes in secondary structure of kafirin during viscoelastic dough formation

Kafirin after addition of water

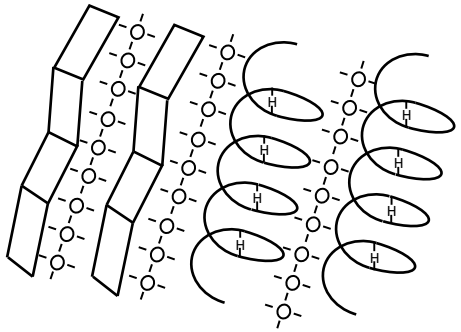
C)



β-sheets form loops

α-helices form loops and some of them as a result of the trapping of water and loop formation return to β-sheet

D)



Kneaded kafirin dough (viscoelastic dough)

In case of wheat flour, the addition of 16% water can act as a plasticizer for gluten so that it has viscoelastic properties at ambient temperature (Hoseney et al., 1986). Similarly, with zein some viscoelastic properties can be observed if water added is above zein's T_g (Lawton, 1992). Although kafirin is always considered as analogous to zein, it does not exhibit the same behaviour when water is added. As stated it is speculated that the mechanism of kafirin fibril formation was a form of protein aggregation similar to what was assumed with kafirin microparticle formation by Taylor (2008) and Taylor et al. (2009). When kafirin microparticles were formed by coacervation with water from a glacial acetic solution, it was found that a low concentration of acetic acid (5.4%) resulted in formation of kafirin microparticles of only 3-4 μm diameter. (Taylor et al., 2009). When the final concentration of acetic acid was higher (21.6%), the size of the microparticles was larger, (6 μm). This means the molecular aggregation number increased due to the increasing acetic acid concentration. This finding by Taylor and co-workers could be an explanation of the finding of the current study that using glacial acetic acid was effective in kafirin aggregation and viscoelastic dough formation. The aggregation of proteins can result in different structures depending on protein monomer interactions (Bolder et al., 2006). These structures can be fibrils as was found in this current study (Chapter 4.3) Microparticles and films can also be formed by kafirin aggregation (Taylor, 2009). In order for zein to form fibrils, protein aggregation was found to be an important factor (Mejia et al., 2007). Some of the conditions that affect protein fragment (amyloid fibril) formation, which are considered analogous to kafirin (Taylor, 2008) and zein structures (Erickson et al., 2012), have been studied, including low pH, temperature and the addition of the organic solvents or denaturants (Gorbenko and Kinnunen (2006). The mutual action of these conditions is formation the β-sheet structure whether through unfolding of the protein structure or refolding and polymerization of the amyloid.

5.4 PROPOSED MECHANISM OF HOW MODIFIED KAFIRINS IMPROVE SORGHUM FLOUR DOUGH RHEOLOGICAL PROPERTIES

In this study the effects of the sorghum high protein digestibility trait on sorghum flour dough rheological properties were studied. The high protein digestibility trait is a consequence of modification of expression of kafirin subclasses. Therefore, in order to be able to understand the mechanisms of the effects of the high protein digestibility trait on the kafirin dough functionality, the concept of the modification of kafirin expression needs to be understood. In Chapters 4.1 and 4.2

it was shown that the three novel combined waxy and high protein digestibility sorghums and the genetically modified sorghums showed higher protein digestibility compared to their controls. The research in Chapter 4.3 revealed that the conventionally bred sorghums showed reduced levels of β-kafirin. It can be assumed that the gene coding for β-kafirins was either missing or its expression was inhibited in these sorghum lines. Beta-kafirin has a single gene (Chamba et al., 2005) unlike α-kafirin which has multigenic family of 23 genes. However, only 19 genes were found to be expressed (Xu and Messing, 2008). Although the proportion of β-kafirin is low in total kafirin , its importance and effect lies in such proteins being rich in methionine and cysteine (Kortt et al., 1991) with 16 and 8 residues, respectively. Cysteine is involved in the disulphide cross-linking of kafirin. The kafirin of the genetically modified sorghums had reduced levels of γ-kafirin (Da Silva et al, 2011b). These two kafirin sub-classes are involved in disulphide cross-linking which results in the formation of kafirin polymers (El Nour et al., 1998). The protein body structure of both types of high protein digestibility sorghums is invaginated, in contrast to the protein body structure of normal sorghums which is not invaginated (Oria et al., 2000; Da Silva et al., 2011b). This invagination is assumed to be one of the reasons for their high protein digestibility (Oria et al., 2000). It has been hypothesized that due to the increase in the surface area of the protein body, the digestive enzymes have more access to the more digestible α-kafirin sub-class. With regard to the lack of kafirin functionality in dough systems, it has been proposed that one of the reasons is that it is encapsulated within the protein bodies (Hamaker and Bugusu, 2003).

The pasting viscosity of both the waxy-high digestibility sorghum and high digestibility GM sorghums was higher than their normal controls. This higher viscosity is indicative of greater starch granule swelling. The high protein digestibility trait resulted in a floury endosperm as described in Chapter 4.1. This is presumed to be because the floury endosperm had a less compact (loose) starch granule arrangement surrounded by weak (not tightly bound) protein matrix. It is assumed that these characteristics of the endosperm of the high protein digestibility sorghums enabled more water to be available (Figure 5.2) to starch granules. The availability of water around the starch granules facilitated greater penetration of the water inside the starch granules. The more open space in the floury endosperm of the high protein digestibility sorghums presumably allowed the starch granules to swell more without retardation, hence the improved rheological properties of the flours of the high digestibility sorghums.

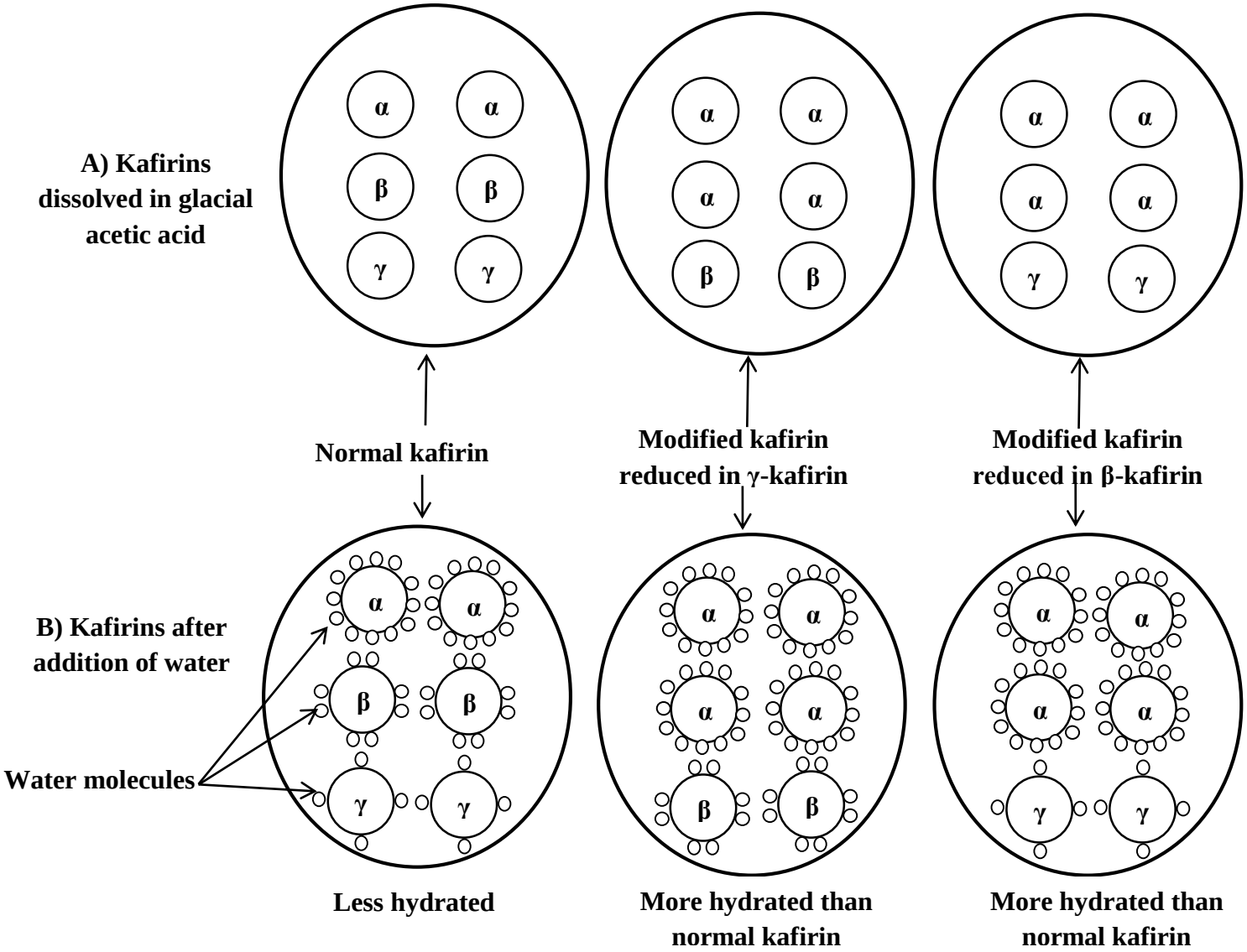


Figure 5.2 Effect of kafirin modification on the hydration of sorghum kafirin

The rheological characteristics of the sorghum dough were affected by the changes in the kafirin. The GM sorghums of high protein digestibility had a stronger dough than their null controls. This property of the GM sorghum doughs was evident from their higher storage modulus (G′) compared to the loss modulus (G″). The mechanism responsible was probably the reduction in γ-kafirin, as γ-kafirin is the most hydrophobic kafirin subclass (Duodu et al., 2003). As normal sorghums contain γ-kafirin, it is assumed that due to the reduction in the level of hydrophobic γ-kafirin in the modified kafirin, the flour bound more water.

Despite the improvements in sorghum flour and kafirin dough rheological properties it is still far from wheat flour, which has the best rheological properties for bread making (Wieser, 2007). Unlike kafirin, wheat prolamins (gliadin and glutenin) can be hydrated easily when water is added to the flour. As a result of the hydration and development of gluten through energy input a three-dimensional network forms that holds carbon dioxide forming a foam-like structure which gives wheat dough its unique viscoelastic properties.

In this current study, as the kafirin dough remained hydrated even after storage for one week in a sealed zip-bag at 5 ºC, this can be considered as a significant achievement that could lead to much more improvement in the sorghum flour properties for bread making. However, there are still some other reasons why wheat flour has the most suitable properties for bread making that are not found in sorghum flour and kafirin. It is assumed that the glutenin is responsible of the elasticity of the wheat flour dough. In this current study, the improvement of the kafirin and sorghum flour properties was attributed to the reduction the levels of β- and γ-kafirin, which means a reduction in polymerization due to disulphide bonding. This is contradictory to some assumptions that are made to interpret the role of the glutenin in wheat flour dough that lead to the dough's viscoelastic properties. Glutenin is a linear polymer made up of high molecular weight subunits linked together at their ends by disulphide bridges and the polymers interact together by hydrogen bonds and disulphide bonds (Belton, 1999). It is assumed that the glutenin due to its disulphide bonding it is responsible for the resistance of the deformation during dough mixing (Abang Zaidel et al., 2009). In case of kafirin because disulphide bonding is associated with the hydrophobic γ-kafirin, the disulphide bonding maybe have negative effect unlike in wheat flour. This is perhaps a major reason that the rheological properties of the sorghum flour of normal kafirin were inferior to the

flour of modified kafirin as shown by rheometry (Chapter 4.2). The hydrophobicity of the kafirin (Taylor and Belton, 2002) can be ascribed to its secondary structure, which is a consequence of its amino acid composition and sequence (Duodu et al., 2003; Belton et al., 2006). Kafirin mainly comprises γ-helical structure, unlike glutenin where the levels of β-sheet and β-turn structure are high (Belton, 1999).

5.5 FUTURE RESEARCH WORK AND DEVELOPMENT OF THE KAFIRIN DOUGH APPROACH

As this study has indicated the main reason for the inferior functional quality of sorghum flour compared to wheat flour lies with the kafirin protein. Hence, it necessary firstly to characterize kafirin more comprehensively.

Thus next research steps to improve the understanding of the effects of kafirin should be to conduct extensive proteomic analysis of the kafirin. LC.MS.MS and MALDITOF MS can be used in combination with SDS-PAGE and two dimensional electrophoresis (Cremer et al., 2014). The sub-classes should be extracted separately and then characterized.

Also, these novel waxy-high protein digestibility sorghum lines and genetically modified sorghums were not actually examined for their bread making quality in this current study. So, bread making using these sorghums should be investigated to determine the actual effects.

The outcome of this study with regard to kafirin dough formation has opened up wide area of research. Although the kafirin dough formation is a totally novel finding, at present the work is little more than an observation. This is because the scientific evidence for the proposed mechanism of the kafirin dough formation is inadequate and mostly still hypotheses. So, in order to further the research in this area more advanced characterization of the kafirin doughs should be conducted. Finding the mechanism of the kafirin dough formation will enable testing treatments other than the glacial aceitic acid method that can be expected to have a similar action on the kafirin and but can be more practicable for use in commercial bread making.

6 CONCLUSIONS AND RECOMMENDATIONS

This study to investigate the modification of kafirin expression through its consequence in the high protein digestibility trait of sorghum has uniquely revealed important factors with respect to sorghum flour functional quality. The combination of the waxy trait with high protein digestibility trait resulting from the reduction of β-kafirin sub-class improves sorghum flour quality. Floury sorghum endosperm is obtained due to the combination of the waxy and high protein digestibility traits which will result in smaller particles of flour upon milling which increase the surface area that available for water binding. The combination of waxy and high protein digestibility traits in sorghums also improves flour functionality as it increases water solubility and dough viscosity during pasting, in particular pasting peak viscosity.

Suppression of γ-kafirin sub-class synthesis in normal starch sorghum through genetic engineering also not only increases protein digestibility, but also improves the flour rheological properties. Genetically modified non-waxy sorghums of altered kafirin (reduced in γ-kafirin) confirms that the high protein digestibility trait due to the kafirin alteration improves the flour functionality. In simulated breadmaking, dough from these GM sorghums exhibit higher elasticity than that of normal sorghum. Also, as observed by CLSM, the dough system in terms of protein-starch interaction seems better in the GM sorghum. Furthermore, flour water solubility increases due to the reduction in γ-kafirin.

A major and novel finding of this study is the preparation of viscoelastic doughs from kafirin through coacervation with water from a glacial acetic acid solution. Kafirin dough formation is generally similar for both kafirin with altered sub-class composition and normal kafirin. This kafirin dough preparation approach may refute all the assumptions as to why sorghum flour quality is not like wheat when the mechanism of dough formation has been fully explained. It seems that the sub-classes of kafirin have an effect on kafirin dough properties, where, absence or reduction of β- and γ-kafirin improve the viscoelastic properties of the dough.

Although modified kafirin expression improves sorghum flour functional quality somewhat these improvements may only be effective if the sorghum flour is used in a composite with wheat flour. Glacial acetic acid coacervation of kafirin cannot be considered as practical dough preparation method. More practical methods need to be developed in order to be used with sorghum flour. Nevertheless, the finding that a viscoelastic dough can be formed with kafirin using the glacial

acetic acid method should be considered as key to solve the secret of the deficiency of kafirin to exhibit appropriate viscoelasticity for bread making. The overall outcome of this study has potential for improving sorghum end-use quality for making dough-based food products if advanced research is continued to explain the mechanism of the glacial acetic acid process and then to look for a workable method with similar action.

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8 PUBLICATIONS, PRESENTATIONS AND POSTERS BASED ON THIS RESEARCH

Elhassan, M. S., Emmambux, M. N., Hays, D. B., Peterson, G. C., Taylor, J. R. N. (2015). Novel biofortified sorghum lines with combined waxy (high amylopectin) starch and high protein digestibility traits: Effects on endosperm and flour properties. J. Cereal Sci., 65, 132-139.

Mohammed S. M Elhassan, M. Naushad Emmambux and John R. N. Taylor. (2013). Effect of starch type and protein digestibility on sorghum quality for bread and beverage making. Poster at the 20th Biennial International SAAFoST Congress and Exhibition, Pretoria.

Mohammed S. M Elhassan, M. Naushad Emmambux and John R. N. Taylor. (2014).Novel biofortified sorghum lines with both waxy and high protein digestibility traits: Effects on flour quality characteristics. Oral presentation in the New Voice Symposium of the Association of Cereal Science and Technology Southern Africa (CST-SA).

Mohammed S. M Elhassan, M. Naushad Emmambux and John R. N. Taylor. (2015). Effects of starch type and protein digestibility on the functional properties of flours from conventionally bred and genetically modified biofortified sorghums. Oral presentation in 21th Biennial International SAAFoST Congress and Exhibition, Durban.