

Characterisation of zein from South African maize of varying endosperm texture

by
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The crest of the University of Stellenbosch is centered behind the text. It features a shield with a blue and white design, topped with a red and white crown. The shield is flanked by two figures, and a banner at the bottom contains the Latin motto "Perfere roborem cultus recti".

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Abstract

Maize is an important crop for both human and animal consumption. Maize kernel texture (kernel hardness) is an important quality trait for many sectors in the South African maize industry, where a harder texture is desired. Both total protein content and the main storage proteins, zein, have been associated with kernel texture. The zein profiles of South African white maize hybrids, from a breeding program, grown at three localities together with their respective inbred parent lines were evaluated to determine the difference in zein expression. For only the hybrids, total protein content, zein content and degree of hardness (kernel texture) was determined to establish possible relationships.

Zein consists of four main classes, α -, β -, γ -, and δ -zein, which can further be divided into sub-classes. Zein was characterised using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) after optimisation of the zein extraction and matrix preparation procedures. Two matrices [2-(4-hydroxyphenylazo)benzoic acid (HABA) and α -cyano-4-hydroxy-cinammic acid (CHCA)] and three pH levels (<1.5, 1.7 and 2.9) for matrix solutions were investigated. Two solvent concentrations (50% and 70% acetonitrile (ACN)) were also investigated. Parallel to this investigation, a zein extraction procedure was optimised. Zein was extracted from non-defatted (NDF) and defatted (DF) maize meal at ambient temperature and 60°C and meal with different particle size distributions. Particle size of maize meal did not influence the quality of spectra. Regardless of matrix conditions used, zein extracted at 60°C from NDF meal produced spectra with a low signal-to-noise (S/N) ratio. When only HABA was included (pH<1.5), not all γ -zein sub-classes were observed. This was also true for zein extracted at ambient temperature from NDF and DF meal. Good S/N ratios for all zein classes were obtained when zein was extracted from NDF meal at ambient temperature using a matrix solution containing both matrices in 70% ACN at pH 2.9. MALDI-TOF MS provided accurate detection and good profile resolution for zein characterisation. Spectral profiles of hybrids were a combination of their respective inbred parent lines.

Zein proteins were quantified using reverse-phase high performance liquid chromatography (RP-HPLC). Total protein content was determined and kernel texture assessed using a particle size (PSI) method. Significant ($P<0.01$) differences were observed for total protein content and degree of hardness between certain hybrids as well as average values for localities. Correlations and principal component analysis indicated that hybrids with a higher protein and total zein content had a harder kernel texture. The β - and γ -zein contents also correlated positively with kernel hardness. However, when expressed as percentage of total zein the opposite was observed. Collectively the α -zeins and most individual α -zeins correlated positively with hardness.

Scanning electron microscopy micrographs showed differences between the floury endosperm of harder and softer maize kernels; illustrating starch types (amylose and amylopectin) should also be analysed in future hardness studies.

The correlations obtained were not strong ($r < 0.6$) and the variation explained by first two principal components was low. Other maize constituents, more hardness measurements should be considered in future studies to improve this.

Uittreksel

Mielies is a belangrike gewas vir beide mens- en dierlike inname. Mieliepittekstuur (pithardheid) is 'n belangrike kwaliteitseienskap vir baie sektore in die Suid Afrikaanse mielieindustrie, waar 'n harder tekstuur verlang word. Beide totale proteïeninhoud en die hoof opbergingsproteïen, zein, is al geassosieer met pittekstuur. Die zein profiele van Suid- Afrikaanse witmielie basters, van 'n teel program, wat by drie lokaliteite verbou is sowel as hul onderskeie ingeteelde ouerlyne is ge-evalueer om verskille in zein uitdrukking te bepaal. Die totale proteïeninhoud, zeininhoud en graad van hardheid is bepaal om verhoudings vas te stel.

Zein bestaan uit vier hoof klasse, α -, β -, γ -, en δ -zein, wat verder onderverdeel word in sub-klasse. Zein is gekarakteriseer met matriks-ondersteunende laser desorpsie ionisasie tyd-van-vlug massa spektrometrie (MBLDI-TVV MS) na die zein ekstraksie en matriks voorbereidingprosedures geoptimaliseer is. Twee matrikse [2-(4-hidroksiephenylazo)benzoë suur (HABA) en α -cyano-4-hidroksie-kaneelsuur (CHCA)] en drie pH vlakke (<1.5, 1.7 and 2.9) vir matriksoplossings was ondersoek. Twee oplossingkonsentrasies [50% and 70% asetonitriël (ACN)] is ook ondersoek. Zein ekstraksie kondisies is ook geoptimiseer. Zein is geëkstraheer van nie-ontvette (NOV) en ontvette (OV) meliemeel by omgewings temperatuur en 60°C. Die partikelgrootte van die meliemeel het nie die kwaliteit van spektra beïnvloed nie. Ongeag watter matrikskondisies gebruik is het zein wat van NGV meel by 60°C ge-ekstraheer is, spektra met 'n lae sein-tot-geraas (S/G) verhouding geproduseer. As die HABA alleenlik gebruik (by pH<1.5) is, is nie alle γ -zein klasse waargeneem nie. Dit was ook waar vir zein wat by omgewings temperatuur van NGV en OV meliemeel ge-ekstraheer is. Goeie S/G verhoudings is waargeneem met zein, ge-ekstraheer van NGV meliemeel by omringende temperatuur, met die gebruik van beide matrikse in 70% ACN by pH 2.9. (MBLDI-TVV MS) het akkurate en goeie resolusie van profiele vir zein karakteriseering verskaf. Spektrale profiele van basters was 'n kombinasie van hul onderskeie ouer lyne.

Zeinproteïene is met omgekeerde-fase hoë prestasie vloeistofchromatografie gekwantifiseer. Totale proteïeninhoud is bepaal en die hardheidsgraad was bepaal met 'n partikelgrootte indeks (PGI) metode. Beduidende verskille tussen proteïeninhoud en hardheidsgraad is waargeneem tussen sekere basters sowel as gemiddelde waardes by die verskeie lokaliteite. Korrelasies en hoofkomponent analise het aangedui dat basters met 'n hoër proteïeninhoud en totale zein 'n harder tekstuur gehad het. Die β - en γ -zeininhoud het ook positief gekorreleer met hardheid. Alhoewel, wanneer die proteïene as persentasie van totale zein uitgedruk is, is die teenoorgestelde waargeneem. Gesamentelik het α -zein asook die meeste individuele α -zeins positief gekorreleer met hardheid.

Skandeer elektron mikroskopie mikrograwe het verskille tussen die meelerige endosperm van harder en sagter pitte aangedui; meer proteïenliggame was aanwesig in die harder pitte en

die stysel was digter gepak. Dus moet stysel tipes ook in ag geneem word in toekomstige hardheidsstudies.

Korrelasies wat verkry is, was nie hoog ($r < 0.6$) nie en die variasie verduidelik deur die eerste twee hoofkomponente was laag. Ander mielie samestellende dele as ook meer hardheidsmetings moet in toekomstige studies in ag geneem word om dit te verbeter.

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Abbreviations

AFM	Atomic force microscopy
ANOVA	Analysis of variance
ASG	Alcohol-soluble reduced glutelin
ACN	Acetonitrile
CE-MS	Capillary electrophoresis mass spectrometry
C/F	Coarse-to-fine
CHCA	α -cyano-hydroxy-cinnamic acid
CI	Chemical ionisation
CIMMYT	International maize and wheat improvement center
CZE	Capillary zone electrophoresis
DAP	Days after pollination
DF	Defatted
DHB	2,5-dihydroxybenzoic acid
DHS	Days of heat stress
DTT	Dithiothreitol
DW	Dry weight
EI	Electron impact
ELISA	Enzyme-linked-immuno-sorbance assay
EST	Expressed sequence tags
ETOH	Ethanol
FA	Formic acid
FAB	Fast atom bombardment
<i>fl2</i>	<i>Floury-2</i>
HABA	2-(4-hydroxyphenylazo)benzoic acid
HMW	High molecular weight
IE	Ion exchange
IEF	Iso-electric focusing
IPA	2-propanol
K_d	Distribution or partition coefficient
LMW	Low molecular weight

LSD	Least significant difference
MALDI-TOF MS	Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry
<i>mo2</i>	<i>Opaque-2</i> modifier
MP	Matrix preparation
m/z	Mass to charge
NaAc	Sodium Acetate
NDF	Non-defatted
PB	Protein body
PCA	Principal component analysis
PSI	Particle size index
QPM	Quality Protein Maize
RP-HPLC	Reverse-phase high performance liquid chromatography
S/N	Signal-to-noise
SAXS	Scattering angle x-ray spectroscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Size exclusion
SEM	Scanning electron microscopy
TFA	Trifluoro acetic acid
2-ME	β -mercaptoethanol

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Chapter 1

Introduction

Chapter 1

Introduction

Maize (*Zea mays*) is an important crop being used in the production of many food products and animal feeds. It is the largest crop produced worldwide, exceeding an annual production of 700 million tonnes and is regarded as a staple food in Africa (Fox & Manley, 2009). Dry milling is predominantly used in South Africa to produce various food products such as samp, maize grits and maize meal. The yield and quality of these products are mainly dependent on the hardness of the kernel. Maize kernels that are harder in texture provide optimum harvest, storage and milling characteristics (Holding & Larkins, 2006). Therefore, maize hardness is an important quality trait to many sectors within the maize industry and breeders aim to develop hybrids that meet the industrial requirements. The endosperm constitutes more than 80% of the maize kernel and consists mainly of starch granules surrounded by a protein network comprising protein bodies. The endosperm is the fraction that is milled into various products and will, thus, determine the degree of hardness. Starch types (amylose and amylopectin) (Dombrink-Kurtzman & Knutson, 1997), total protein content (Mestres & Matencio, 1996; Blandino *et al.*, 2010) and the main storage proteins (Dombrink-Kurtzman & Beitz, 1993; Pratt *et al.*, 1995; Eyherabide *et al.*, 1996; Mestres & Matencio, 1996; Robutti *et al.*, 1997; Landry *et al.*, 2004; Holding & Larkins, 2006; Lee *et al.*, 2006) of maize have been associated with kernel texture (hardness). The main storage proteins of maize, i.e. prolamins (alcohol soluble proteins), is referred to as zein; derived from maize's Latin name *Zea mays*. In South Africa protein content and zein content and/or profiles are not evaluated when assessing maize quality. Thus, there is scope to investigate the possibility of evaluating these constituents.

Zein comprises up to 70% of the total protein in conventional maize (Prasanna *et al.*, 2001). Three classes, namely α -, β - and γ -zein have been classified according to a widely accepted nomenclature (Esen, 1987). A fourth class comprising two proteins, namely 10 kDa δ -zein (Kirihaara *et al.*, 1988) and 18 kDa δ -zein (Woo *et al.*, 2001) was later added to the family of zein proteins. The main classes differ in solubility characteristics, iso-electric point, molecular weights and they have distinctive polypeptide compositions (Esen, 1986; Shewry & Tatham, 1990).

The α -zeins are divided into two classes, 22 kDa and 19 kDa, where each class consists of a family of proteins with similar molecular weights. The α -zeins are expressed by large and complex gene families and uncertainty exists regarding the relative number of functional coding sequences (Holding & Larkins, 2006). Results from cluster analysis of expressed sequence tags (EST's) from endosperm cDNA libraries indicated nine different α -zein genes exist. These genes are divided into three main classes, based on similarities of amino acid sequences; 19 kDa "B" and "D" classes and a 22 kDa "Z" class (Woo *et al.*, 2001). The γ -zeins are subdivided

into three classes, namely 16 kDa, 27 kDa and 50 kDa whereas the β -zein comprises a 15 kDa protein. Characterisation of zein proteins is important to establish the impact of breeding on zein expression and to determine the profiles of purified zein used for certain applications, e.g. the polymer industry.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has conventionally been used to characterise zein proteins according to their molecular weights. However, it has limitations in not being able to distinguish between zein proteins with similar molecular weights. This is especially true for the α -zeins. Over the past decade, mass spectrometry techniques have gained interest to overcome these limitations (Wang *et al.*, 2003; Adams *et al.*, 2004; Huang *et al.*, 2005; Erny *et al.*, 2007; García López *et al.*, 2009). Not only can they distinguish between proteins with similar molecular weights, they also provide more accurate molecular weights. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), capillary electrophoresis mass spectrometry (CE-MS) and reversed-phase high performance liquid chromatography–electrospray mass spectrometry (RP-HPLC-ESI MS) are techniques that have been reported to have been used for zein analyses. Comparisons of zein extraction procedures and zein profiles obtained with these various MS techniques indicated the MALDI-TOF MS method, developed by Adams *et al.* (2004), gave optimal results in terms of simplicity of zein extraction procedure and assignment of zein classes.

It was suggested MALDI-TOF MS can be used to investigate the molecular genetics of zein expression which is usually difficult to study due to the intricacy of their multigene families (Adams *et al.*, 2004). All the main classes were observed and up to seven sub-classes for the α -zeins were obtained. Variation in zein profiles of various inbred lines was observed, demonstrating this technique could distinguish between varieties. Molecular weights of zein classes observed were shown not to deviate by more than 0.43% from their calculated weight; demonstrating this technique's accuracy. Variability was observed for the signal intensity of the 27 kDa γ -zein. This class was the least hydrophobic class and it was suggested a more water soluble matrix could improve its signal variability (Adams *et al.*, 2004). Therefore, there is scope to further optimise this method.

Maize endosperm comprises mainly starch granules surrounded by protein bodies (PBs) that consist of zein proteins. It varies in texture containing both hard and softer endosperm types. The hard regions are referred to as horny, translucent or vitreous, whereas the soft regions are referred to as floury or opaque. Differences exist between these endosperm types in terms of packing of starch granules and protein bodies. In the vitreous endosperm, the PBs are larger and more abundant and the starch granules are more densely packed. In the floury endosperm, the starch granules are less abundant, more spherical and loosely packed and smaller and less abundant PBs are present (Robutti *et al.*, 1974; Dombrink-Kurtzman, 1994). It has been suggested floury endosperm contained immature PBs with less α -zein compared to harder endosperm which contained more mature PBs (Lending & Larkins, 1989).

Comparisons of the zein content [assessed using reverse-phase high performance liquid chromatography (RP-HPLC)] from vitreous and flourey endosperms of maize kernels indicated α -zeins were more abundant (up to 3.3 times) in the vitreous endosperm (Dombrink-Kurtzman & Beitz, 1993). Therefore, α -zeins were positively associated with a harder kernel texture. The γ -zeins and β -zein were negatively associated with a harder kernel texture as they were more abundant in the flourey endosperm. Zein proteins have been correlated with the degree of hardness obtained using a number of hardness measurements (e.g. kernel density, grinding time, particle size index) (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006). In these studies, α -zeins were also positively associated with a harder kernel texture, though not all correlations observed were equally strong. The 27 kDa γ -zein has mostly been positively associated with a harder kernel texture. This was dependent on whether this class was expressed as an absolute value (arbitrary units) or as a percentage of total peak area (Paulis *et al.*, 1993). The 16 kDa γ -zein correlated negatively with kernel hardness. The relationship between the 15 kDa β -zein and kernel texture is uncertain due to contradictory reports (Paulis *et al.*, 1993; Lee *et al.*, 2006). The role zein proteins play in maize kernel texture is apparent; this relationship has, however, not been fully characterised.

A number of methods to assess maize hardness have been described. The most common method is to mill maize and fractionating the meal into coarse and fine material, using a series of test sieves (Fox & Manley, 2009). This is referred to as the particle size index (PSI) method. A course-to-fine ratio (C/F) can be calculated from fractions obtained. This ratio has been shown to be the best predictor of milling quality and total protein was found to correlate the strongest with this ratio (Blandino *et al.*, 2010).

The aim of this study was to characterise zein from a range of South African white maize hybrids and their respective inbred parent lines. The specific objectives of this study were thus to:

- optimise the zein extraction and matrix preparation procedures for MALDI-TOF MS zein characterisation;
- evaluate zein profiles of white maize hybrids and their respective inbred parent lines with MALDI-TOF MS, using the optimised extraction and matrix preparation procedures;
- determine zein content (using RP-HPLC), total protein content (using the Dumas combustion method) and degree of maize kernel hardness (using the PSI method) of the hybrids; and
- establish a relationship between kernel hardness (kernel texture), zein protein- and total protein content.

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Chapter 2

Literature review

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1. Introduction

Cereals are grown worldwide and are cultivated for their edible seeds which are of great importance for human and animal nutrition. Cereals are members of the grass family *Poaceae* and the principal cereal crops include wheat, maize, rice, barley, oats, rye, sorghum and millets. Cereals provide energy, protein, fiber, vitamins and minerals. Protein composition and content are particularly important, not only in terms of nutrition, but also the impact on quality of targeted end-uses of cereals (Shewry & Halford, 2002). The storage proteins of cereals have specifically been shown to be important in this regard (Shewry & Tatham, 1990; Shewry & Halford, 2002).

Maize (*Zea mays*) is the largest produced crop, with an global annual production exceeding 700 million tonnes (Fox & Manley, 2009). In South Africa, maize is an important staple food and is used for both human and animal consumption. Various food products are produced from maize and it is important to have appropriate maize cultivars with desirable characteristics for specific end-uses. In South Africa, dry-milling is primarily used for the production of samp, maize meal and other milled products. The maize milling industry prefers to use large kernelled maize that is hard in texture (Holding & Larkins, 2006). Consequently, maize breeders have to breed suitable material and objectives have become targeted to provide cultivars with optimum quality characteristics. It has been demonstrated that breeding has an impact on the composition of the main storage proteins of maize (zein) and subsequently kernel texture (Gibbon & Larkins, 2005). For breeders, it is important to know the influence of genotype and environment and any interaction thereof, on the storage proteins. This would include maize quality characteristics such as protein content as well as kernel texture (sometimes also referred to as kernel hardness).

In this literature review cereal proteins in general will be discussed briefly, followed by a more detailed review of zein. This will be done in terms of zein formation, characterisation and importance.

2. Cereal proteins

2.1 Classification

Cereal proteins have been studied for many years and, due to their complexity, various classification systems have been developed to distinguish between them. These systems include classifications based on their solubility behaviour, morphology, biological functions, chemical composition (Lasztity, 1984a) and structural and evolutionary relationships (Shewry & Tatham, 1990; Shewry & Halford, 2002). T.B. Osborne, who is regarded as the father of plant protein analysis, developed a classification system which differentiated between proteins based on their solubility. Four main protein classes have been identified; namely, albumins (water soluble), globulins (salt soluble), prolamins (alcohol soluble) and glutelins (soluble in dilute alkali). This system is still widely accepted and used. Other classifications systems such as classification based on biological functions are also used (Fig. 2.1). Morphologically, cereal

proteins can be divided into proteins of the aleurone layer, endosperm and embryo. The protein concentration in each of these morphological parts will vary.

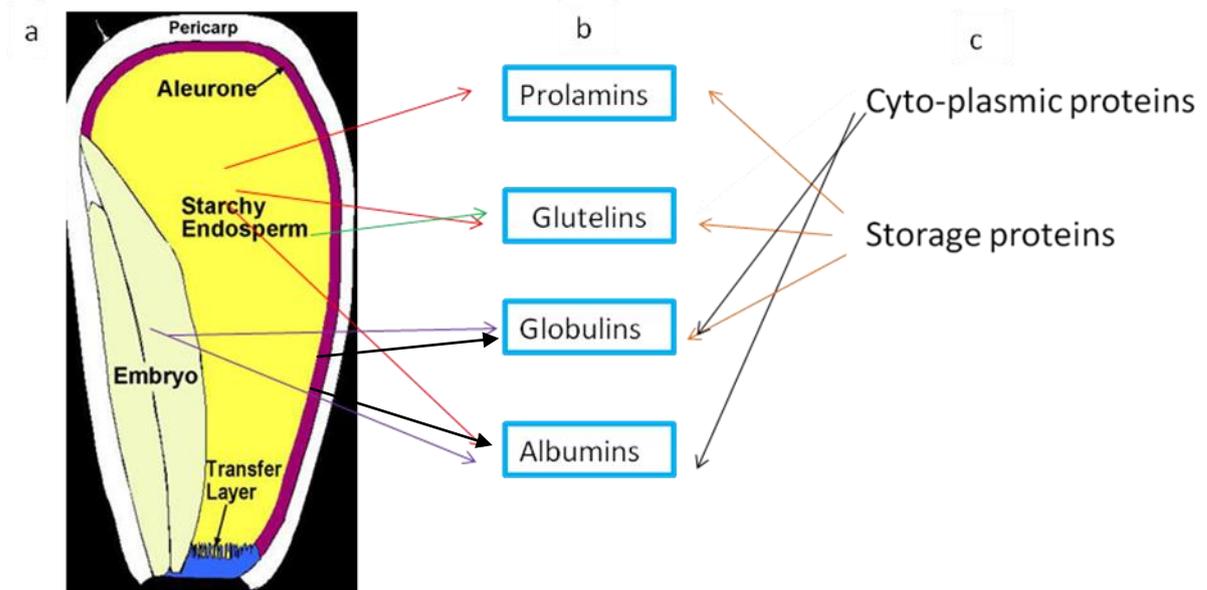


Figure 2.1 Classification of plant proteins with (a) morphological composition of a maize kernel (Anonymous, 2002), (b) Osborne's classification based on solubility and (c) the biological functions of plant proteins.

Proteins can be divided in two major groups in terms of functionality, namely **cytoplasmic** and **storage** proteins (Lasztity, 1984a). The **cytoplasmic proteins** comprise mainly globulins and albumins, and are located in the aleurone layer and embryo. They are synthesised during early stages of kernel development, are relatively low in molecular weight and have a globular form. They are regarded as having a higher nutritional value due to increased levels of lysine and tryptohan. The most important functional proteins in this group include enzymes, membrane proteins, non-enzynamic regulatory proteins and proteins of organelles.

Storage proteins are located in the endosperm and are generally soluble in alcohol (prolamins) and dilute alkali solutions (glutelins). They can be divided into two types of proteins: low molecular weight (LMW), consisting of one polypeptide chain with only intramolecular disulfide bonds; and high molecular weight (HMW), consisting of several polypeptide chains which are crosslinked via intermolecular disulfide bonds (Lasztity, 1984a; Shewry & Tatham, 1990). Storage proteins provide carbon, nitrogen and sulfur resources for growth and development of the germinating seed. Seed storage proteins are among the earliest proteins studied and are the most abundant proteins in cereals, accounting for approximately 50% of the total protein (Shewry & Tatham, 1990; Mu-Forster & Wasserman, 1998; Shewry & Halford, 2002). Their impact on the nutritive value and end-uses of products in the food industry (Shewry & Halford, 2002) as well as the polymer industry (Shukla & Cheryan, 2001), have led to their identification and classification in many cereals. In all cereals, except for rice and oats, the main

storage proteins are the prolamins (Lasztity, 1984b; Shewry & Tatham, 1990). Although globulins are classified as cytoplasmic proteins, they can also be classified as storage proteins. In rice and oats, globulins are the main storage proteins.

2.2 Prolamins of cereals

Prolamins are traditionally classified as proteins that are soluble in aqueous alcohol solutions (Esen, 1986; Shewry *et al.*, 1995; Mestres & Matencio, 1996; Shewry & Halford, 2002). It has been suggested they evolved from amplification of small hydrophobic peptides, rich in proline and glutamine (Herman & Larkins, 1999). The term **prolamin** was derived due to the high content of **proline** and **glutamine**, where combined proportions vary between 30-70% amongst the various cereals (Shewry & Halford, 2002; Holding & Larkins, 2006). For most cereals, except for wheat, prolamins are given names based on their generic Latin names (Shewry & Tatham, 1990). For maize, they are known as zein (from *Zea mays*), barley as hordein (from *Hordeum vulgare*) and rye as secalin (from *Secale cereale*).

Based on amino-acid sequence homologies, the definition for prolamins later expanded. Proteins could also be classified as prolamins in spite of being insoluble in alcoholic solutions in their native state. The prolamins include groups containing interchain disulfide bonds that need to be reduced before being solubilised (Shewry *et al.*, 1995). Subsequently the prolamin super family classification system was developed. This system was based on the complete amino-acid sequences of all the prolamins from the *Triticeae* tribe (wheat, barley and rye) (Shewry & Tatham, 1990; Shewry *et al.*, 1995). This system assigns the prolamins into three groups: sulfur-rich (S-rich), sulfur-poor (S-poor) and high molecular weight (HMW) prolamins. Some minor prolamins from the *Panicoid* tribe (maize, sorghum and millet) are also classified in the prolamin super family (Shewry & Tatham, 1990; Shewry *et al.*, 1995; Shewry & Halford, 2002).

Despite the differences between the prolamins, two common structural characteristics are shared (Shewry & Halford, 2002). The first is the presence of discrete regions, which may have different origins, which take on different structures to one another. The second is repeated blocks consisting of one or more short peptide motifs, or amino acid sequences that are rich in amino acid residues such as methionine. These characteristics are responsible for the high proportions of glutamine and proline as well as other specific amino acids (e.g. histidine, phenylalanine and glycine).

3. Prolamin of maize

Zein is located within protein bodies (PBs) in the starchy endosperm (Lending *et al.*, 1988). In normal maize, zein can account up to 70% of the total protein content (Prasanna *et al.*, 2001). Zein was first isolated in 1821 and has since then become a subject matter of great scientific interest. During the mid 20th century the initial focus was to utilise it as an industrial polymer

(Shukla & Cheryan, 2001). The relationship between zein and endosperm texture (Paiva *et al.*, 1991; Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994; Pratt *et al.*, 1995; Landry *et al.*, 2004) and its nutritional impact due to its amino acid quality (Mertz *et al.*, 1964; Nelson *et al.*, 1965; Gibbon & Larkins, 2005) later also became topics of great interest.

3.1 Brief history zein characterisation

Various protein fractionation schemes have been proposed to characterise zein. The different number of protein fractions identified by various techniques led to confusion. Initially, different terms were given to the various fractions and uncertainty existed as to how these proteins should be classified.

Early zein characterisation studies showed zein could be separated into three distinct precipitated fractions by step-wise addition of water to alcohol solutions (Watson *et al.*, 1936). It was thought these fractions were homogeneous, but when analysed with moving boundary electrophoresis it was clear they were heterogeneous (Scallet, 1947). When analysed by means of isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), these proteins showed charge heterogeneity (Righetti *et al.*, 1977; Vitale *et al.*, 1980; Wilson, 1986; Landry *et al.*, 1987); suggesting several polypeptides existed within each of the molecular weight fractions.

In 1958, two fractions were identified; one being soluble in 95% ethanol (α -zein fraction) and the other in 60% ethanol (β -zein fraction), but not in 95% ethanol (McKinney, 1958). SDS-PAGE results indicated, when proteins were extracted with an aqueous alcohol solvent, two bands migrated in the gel with relative molecular weights of 22000 Da and 24000 Da (Paulis, 1981; Esen, 1986; Esen, 1987; Landry *et al.*, 1987) or 19000 Da and 22000 Da (Lee *et al.*, 1976; Pedersen *et al.*, 1980; Wilson *et al.*, 1981). These two proteins have been collectively referred to as zein-1 (Wilson *et al.*, 1981), the α -fraction (Paulis, 1981) or A (19000 Da)- and B (22000 Da)-zein respectively (Wilson, 1985).

Two fractions, α -zein and β -zein, were obtained when whole-zein was extracted with 70% ethanol and 0.5% sodium acetate, and separated by adding 95% ethanol to the solution (Paulis, 1981). The α -zein fraction stayed in solution and the insoluble β -zein fraction precipitated. SDS-PAGE of unreduced zein revealed two bands for α -zein; 22000 Da and 24000 Da. After reduction with β -mercaptoethanol (2-ME), the β -zein fraction consisted of three bands; 22 000 Da, 24000 Da and 14000 Da. Amino acid analysis of the α - and β -zein fractions revealed the β -zein fraction was higher in glutamine, proline and methionine. This elevated level of amino acids was attributed to the 14000 Da fraction (Paulis, 1981), which was previously suggested to be a LMW alcohol-soluble reduced glutelin (ASG) polypeptide (Paulis & Wall 1977). It has also been referred to as C-zein (Singletary *et al.*, 1990).

Wilson (1985) proposed a nomenclature in which the two major protein classes, 22000 Da and 24000 Da, should be referred to as A- and B-zein, respectively. This study concluded that

the ASG fraction consisted of many polypeptides that were identical to unreduced zein and certain polypeptides existed only in the reduced state. The latter included polypeptides with molecular weights of 15000 Da, 18000 Da and 27000 Da. The 27000 Da protein was previously identified as the reduced soluble protein (RSP) (Wilson *et al.*, 1981) and G₂-glutelins (Landry & Moureaux, 1981). Wilson *et al.* (1981) grouped the 15000 Da and 18000 Da proteins together and termed this group the C-prolamins. The 27000 Da was not considered a prolamin. The zein with a molecular weight of 9000-10000 Da was termed the D-prolamin.

Esen (1987) also proposed a nomenclature where he assigned these fractions, on the basis of their solubility and structural relationship, into three distinct groups: α -, β - and γ -zein. According to his nomenclature, α -zein consisted of the polypeptides with molecular weight of 21000 to 25000 Da plus the low molecular weight fraction of 10000 Da; the β -zeins of 17000 to 18000 Da polypeptides; and the γ -zein class of the 27000 Da polypeptide (Esen, 1987).

This system of Esen (1987) formed the basis of the system widely accepted and used today. It was later modified (Esen, 1990) due to information that became available regarding the primary structure of zein proteins (Prat *et al.*, 1987; Kirihaara *et al.*, 1988). In the modified system, the 18000 Da (also referred to as 16000 Da fraction) polypeptide was removed from the β -zein class and assigned to the γ -zein class due to sequence homology (Prat *et al.*, 1987). The 10000 Da polypeptide was reported to have its own gene encoded sequence (Kirihaara *et al.*, 1988) and was allocated to a fourth class, namely δ -zein. Thus four main classes, namely α - β - γ - and δ -zein, were characterised where α -, γ -, and δ -zeins can further be divided into sub-classes. These sub-classes are described in Table 2.1.

3.2 Development of zein in maize endosperm

3.2.1 Development of zein classes in protein bodies

Zein proteins are assembled into PBs during endosperm development (Fig. 2.2). The structure of PBs has been described in the literature (Lending *et al.*, 1988; Lending & Larkins, 1989). Storage proteins, in general, are synthesised by polyribosomes on the surface of the rough endoplasmic reticulum (ER) whereafter they are transported into the lumen of the ER via a N-terminal signal peptide (Von Heijne, 1984). These proteins can either be directly assembled into PBs alone or be further sequestered as PBs into protein storage vacuoles (Herman & Larkins, 1999; Vitale & Denecke, 1999). For maize, only PBs are formed (Lending *et al.*, 1988; Lending & Larkins, 1989) and formation begins at 10 days after pollination (DAP) with mature protein bodies visible at 40 DAP.

Table 2.1 General classification and characteristics of zein classes

Characteristics	Zein classes													
	α -zein								β -zein	γ -zein		δ -zein		
	19 kDa				22 kDa				15 kDa	16 kDa	27 kDa	50kDa	10 kDa	18kDa
True calculated molecular mass (Da) of zein classes and sub-classes of a well characterised inbred line B73 (Woo <i>et al.</i> , 2001).	B1	B2	B3	D1	Z1	Z3	Z4	Z5						
	23 359	27 128	24 087	24 818	26 359	26 751	26 923	26 701	17 458	17 663	21 882	32 822	14 431	21 220
Solubility (Esen, 1987)	50-95% EtOH*/IPA**/ 4-5 M urea @ 0-1°C, 6-8M urea								30-85% EtOH/IPA + reducing agent/ 1-8M urea	0-80% EtOH/IPA + reducing agent and NaAc***		30-85% EtOH/IPA + reducing agent		
Amino acid composition (Shewry & Tatham. 1990)	High in alanine, leucine								High in methionine	High in proline and cysteine		High in methionine		
Abundance in total zein (Esen, 1987)	75-85%								10-15%	5-10%		<5%		

*EtOH = Ethanol

** IPA = 2-Propanol

*** NaAc = Sodium acetate

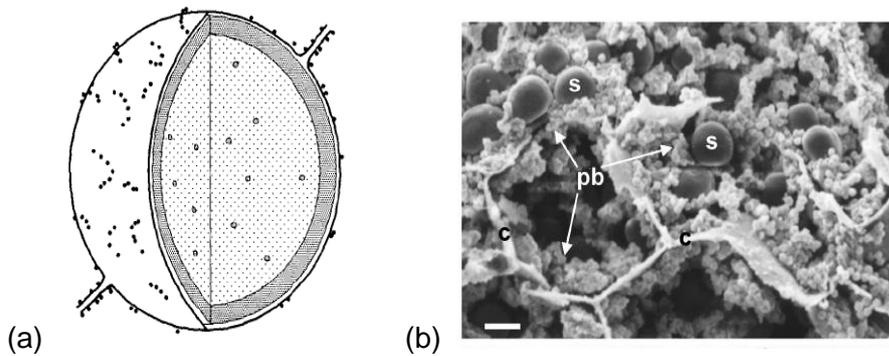


Figure 2.2 (a) Mature protein body in maize endosperm. The inner, lighter stippled region represents the α - and δ -zeins, and the darker outside region and inclusions in the inner region represent the γ - and β -zeins (Lending & Larkins, 1989). (b) Location of protein bodies within maize endosperm (Holding & Larkins, 2006). s=starch granule, pb=protein body, c=cell wall.

PBs do not follow a homogeneous development process throughout the endosperm and zein proteins develop at various stages after pollination (Lending *et al.*, 1988; Lending & Larkins, 1989; Woo *et al.*, 2001). This was demonstrated using immuno-localisation techniques at various stages of endosperm development (Lending & Larkins, 1989). Maize endosperm development was studied at 14 and 18 DAP. Light microscopy revealed that at 14 DAP of endosperm development the majority of PBs were located in the first starchy layers beneath the sub-aleurone layer of the endosperm. The PBs and endosperm cells increased in size with distance from the aleurone layer. Active cell division within sub-aleurone layers often occurred. At 18 DAP the starch granules and PBs were dominant structures in cells with very little cell division within the sub-aleurone layer. In contrast to 14 DAP, PBs were more prevalent in the sub-aleurone cells at 18 DAP. The PBs in the interior endosperm cells were evenly distributed throughout the cytoplasm, whereas the starch granules were distributed more towards the central regions of the cells. As the cells matured, the PBs increased in size and larger protein bodies were observed closer to the aleurone layer

During endosperm development the PBs' zein composition was dependent on the PBs location within the endosperm. During early stages of development, the outer layers of the endosperm had higher amounts of β - and γ -zeins and PBs in these regions were smaller. As the endosperm developed, the PBs increased in size due to increased α -zein content (penetrating the cross-linked β - and γ -zein) in the PBs; with the β - and γ -zeins forming a continuous layer around the α -zein core. This was in accordance with a previous study (Lending *et al.*, 1988). Other immuno-cytochemical studies of isolated maize PBs also indicated the presence of γ -zein surrounding the central α -zein region (Ludevid *et al.*, 1984). The α -zein core also contained β - and γ -zein inclusions that formed either strands or small aggregates throughout the PB (Lending &

Larkins, 1989). Immuno-cytochemical studies of δ -zein distribution indicated at 18 DAP it was only found in the core regions of the protein body (Esen & Stetler, 1992).

3.2.2 Development of zein sub-classes

The development of the zein class sub-families also differ throughout endosperm development. Temporal and spatial expressions of the genes of these sub-families were evaluated using *in-situ* hybridisation (Woo *et al.*, 2001). Three of the gene sub-classes of 19 kDa α -zein, B1, B3 and D1, along with the other zein classes, were examined at various developmental stages in a well characterised inbred line B73. At 10 DAP transcripts encoding for the 19 kDa α -zein sub-families emerged as a narrow vertical stripe on the adgerminal side of endosperm. Temporal and spatial expressions were indistinguishable. The B1 sub-family transcript, however, was expressed at greater levels. The 22 kDa α -zein transcripts were nearly identical. At 15 DAP 22 kDa α -zein transcripts were observed in most of the endosperm except the central and basal regions where weak signals were observed. Expression was greatest for 19 kDa B1 α -zein transcripts in peripheral regions, with some expression observed in the central starchy regions. As development progressed the 22 kDa α -zein remained limited to the more peripheral regions at 20 DAP. At 25 DAP expression was limited to the peripheral region of the lower half of the endosperm. This was also true for the 19 kDa B1 α -zein transcripts. No α -zein gene expression was noted in the very central cells of the starch endosperm.

In contrast to the α -zeins, the 27 kDa γ -zein was highly expressed at 10 and 15 DAP throughout the endosperm, with no expression in some cells of the central starchy endosperm. At 20 and 25 DAP, the 27 kDa γ -zein transcripts occurred more widely in the central starchy regions. No 27 kDa γ -zein expression was observed in the very central starchy region at 20 DAP and at 25 DAP expression was restricted to the lower half of the endosperm. The spatial expression pattern of the 16 kDa γ -zein was similar to the 27 kDa γ -zein but intensity for 16 kDa γ -zein signals were weaker. A higher 16 kDa γ -zein expression in the sub-aleurone layer and crown region at 25 DAP distinguished the spatial pattern of the 16 kDa γ -zein from that of the 27 kDa γ -zein. The 50 kD γ -zein transcripts had similar spatial patterns to other γ -zeins but the signals were notably less abundant. The signal of 50 kD γ -zein was weak at 10 DAP and increased at 15 DAP; at 20 and 25 DAP high levels of 50 kDa γ -zein transcripts were observed the in crown and adgerminal region.

Spatially, the 15 kDa β -zein had a similar pattern to the 27 kDa γ -zein. The level of 15 kDa β -zein expression was similar to that of the 50 kDa γ -zein. However, higher levels of RNA in the peripheral of the adgerminal region differentiated its expression pattern from the 16 and 50 kDa γ -zeins. δ -Zein was only observed at 15 and 20 DAP in the adgerminal and abgerminal regions of the endosperm and was localized in the abgerminal region only at 25 DAP.

3.2.3 Interaction between zein classes

Although the exact mechanisms of how these proteins interact are unknown (Holding & Larkins, 2006), progress has been made to identify interactions between specific zein proteins (Coleman *et al.*, 1996; Hinchliffe & Kemp, 2002; Kim *et al.*, 2002; Coleman *et al.*, 2004; Randall *et al.*, 2004).

Studies using transgenic tobacco plants indicated the importance of certain zein interactions in PB assembly. Genes encoding for different zein classes were expressed in tobacco plants. When α -zein was co-expressed with either 27 kDa γ - (Coleman *et al.*, 1996) or 15 kDa β -zein (Coleman *et al.*, 2004) small PB-like accretions were observed. This suggested the 27 kDa γ -zein and 15 kDa β -zein appeared to stabilise the 22 kDa α -zeins in tobacco plants. This coincided with reports of PB development that indicated the γ - and β -zeins, followed by α -zein, were expressed early in endosperm development (Lending *et al.*, 1988; Lending & Larkins, 1989; Woo *et al.*, 2001).

Co-expression of 15 kDa β -zein and 18 kDa δ -zein in tobacco plants has also been studied (Hinchliffe & Kemp, 2002). Results indicated the 15 kDa β -zein stabilised and increased the accumulation of the 18 kDa δ -zein in both seed and leaf tissues of transgenic tobacco plants. When 15 kDa β - and 10 kDa δ -zein were synthesised individually, small PB-like accretions formed (Bagga *et al.*, 1997). Co-expression resulted in the 15 kDa β - and 10 kDa δ -zeins being co-localised in protein bodies, thus, stabilising each other. These observations are logical considering both 15 kDa β -zein and δ -zein are located within the core of the PB (Esen & Stetler, 1992).

A study conducted where coding regions for zein were cloned in plasmids of a series of yeast hybrid systems, indicated definite interactions between various zein classes (Kim *et al.*, 2002). Some interactions between zein classes were stronger than others. The γ -zein and β -zein interacted strongly with one another. This was consistent with studies indicating their co-localisation in the periphery of the PBs (Lending & Larkins, 1989b). The interactions between the 19 kDa and 22 kDa α -zeins were weak. However, these α -zeins sub-classes each interacted strongly with themselves. This can be problematic as they are the most abundant and need to penetrate to the center, thus interaction prior to PB formation could hinder this process (Holding & Larkins, 2006). Strong interactions existed between the δ -zein and α -zeins, as well as the 16 kDa γ -zein (Kim *et al.*, 2002). It was suggested the δ -zein can force an interaction with α -zeins and 16 kDa γ -zeins in order to target them correctly. In contrast to the 50 kDa and 27 kDa γ -zeins, the 15-kDa β -zein interacted strongly with the α -zeins (especially the 22 kDa α -zein) and the 10 kDa δ -zein. This agreed with data regarding its localisation in the PB, where the 15 kDa β -zein was not restricted to the outer regions (Lending & Larkins, 1989). This allowed for the retention of the α -zeins by linking them to the periphery region of the PB. These interactions were in contrast to the report where the 27 kDa γ -zein stabilised the 22 kDa α -zein in transgenic tobacco plants, thus, interacting strongly (Coleman *et al.*, 1996). It was possible the zeins interacted in a different manner in tobacco plants to the mechanism that occur in maize (Holding & Larkins, 2006).

The 27 kDa γ -zein was the only zein that did not interact strongly with itself. Considering it is expressed at high levels in early stages of endosperm development (Woo *et al.*, 2001), its lack of

interaction with itself would circumvent uncontrolled aggregation from occurring (Holding & Larkins, 2006). This is likely to be responsible for the even distribution of PBs throughout the endosperm cells. The early phases of PB formation were suggested to be driven by distinctive N-terminal signal sequences in the 27-kDa γ -zein (Geli *et al.*, 1994). Via DNA encoding, various deletion-mutants of this class were constructed. It was demonstrated the deletion of a proline-rich domain at the N terminus of γ -zein stop its retention in the ER, resulting in a mutated protein. Repeat regions (PPPVHL) of the 27 kDa γ -zein were also investigated to establish their effect on PB formation (Llop-Tous *et al.*, 2010). Results suggested eight repeat regions (the amount naturally present) were most efficient for self-assembly. Four to six repeats, although not as efficient, formed multimers. It was also concluded, based on site-specific mutagenesis and subsequent analysis of multimer formation, two N-terminal cysteine residues were critical for oligomerisation.

Several types of zein class interactions exist, and all zein classes play an intricate role in PB formation. The lack of a zein class can, thus, disrupt the PB formation.

3.3 Characteristics of zein classes

Native zein proteins are insoluble in water. This behaviour is due to their amino acid composition; they are deficient in acidic and basic amino acids and high in more non-polar amino acids such as leucine, proline and alanine (Shukla & Cheryan, 2001). Zeins are also deficient in the essential amino acids tryptophan and lysine (Lasztity, 1984c; Zarkadas, 1997). Although zein is classically defined as being the alcohol soluble protein of maize, it is also soluble in acetic acid, phenol and dilute alkali solutions (Osborne & Mendel, 1914). Zein is a group of heterogeneous proteins that vary in amino-acid sequence (Woo *et al.*, 2001), surface charge (Zhu *et al.*, 2007) and solubility behaviour (Esen, 1987).

3.3.1 α -zein

The α -zeins include the major bands with molecular weights of 19000 Da and 22000 Da that appear when analysed by SDS-PAGE (Wilson *et al.*, 1981; Wilson, 1985). Hence, these classes were often referred to as 19 kDa and 22 kDa α -zein (Adams *et al.*, 2004; Huang *et al.*, 2004; Erny *et al.*, 2007b). These terms are not a true reflection of their molecular weight when compared to calculated amino acid sequences derived from genes and cloned cDNAs (Shewry & Tatham, 1990; Woo *et al.*, 2001). α -Zeins constitute up to 85% of total zein (Esen, 1987) and comprise a complex group of polypeptides (Righetti *et al.*, 1977; Wilson *et al.*, 1981; Woo *et al.*, 2001). When extracting this class, no reducing agent is needed. This is due to no methionine and few (two) cysteine residues (Shewry & Tatham, 1990) present in this group, resulting in little disulfide bonding. There is no sequence homology between α - β -, γ -zeins when comparing their primary structures. Comparisons of α -zein polypeptides indicated homologies varying from 60 to 97% (Esen, 1987).

The α -zeins were found to be expressed by large and complex gene families, and uncertainty exists for the relative number of functional coding sequences (Holding & Larkins, 2006). Thirty

percent of the zein endosperm-encoding transcripts have been found to come from a small number of genes (Song *et al.*, 2001; Song & Messing, 2002). Results from cluster analysis of expressed sequence tags (EST's) from endosperm cDNA libraries indicated there were nine different α -zein genes divided into three main classes (based on similarities of amino acid sequences): 19 kDa "B" and "D" classes and 22 kDa "Z" class (Table 2.1).

Several structural models have been proposed for α -zein in its native state (Argos *et al.*, 1982; Tatham *et al.*, 1993; Matsushima *et al.*, 1997; Bugs *et al.*, 2004; Guo *et al.*, 2005). A circular dichroic spectrum (191-240 nm) of zein in methanol was measured (Argos *et al.*, 1982). Results indicated the secondary structure of α -zein was mainly α -helical (50-60%) and nine topologically anti-parallel helices, adjacent to each other, grouped within a distorted cylinder (Fig 2.3a). These helices interacted via hydrogen bonding due to glutamic-rich turn regions along the helical surfaces.

Small-angle x-ray scattering (SAXS) has been used to modify the proposed model (Tatham *et al.*, 1993; Matsushima *et al.*, 1997). An extended α -zein structure was reported in both studies. An elongated prism-like structural model was proposed consisting of linear stacks of α -helices that are relatively flexible (Matsushima *et al.*, 1997) (Fig. 2.3b). The authors suggested the hydrogen bonding proposed by Argos *et al.* (1982) contributed to the stability of this structure. Fourier transform infrared spectroscopy (FT-IR), circular dichroism spectroscopy and SAXS have been used to propose a hairpin structural model for α -zein (Bugs *et al.*, 2004). This model consisted of two anti-parallel α -helices and β -sheets that turned and folded on themselves (Fig. 2.3c).

Globular structures of zein have also been observed using atomic force microscopy (AFM) (Guo *et al.*, 2005). Results indicated at a low concentration of zein in 70% ethanol (1 μ g/mL) a uniform globular structure was present (Fig. 2.3d). This could be attributed to the zein proteins aggregating to form a stable network. As the concentration of the zein increased the degree of aggregation increased due to hydrogen and disulfide bonding as well as hydrophobic interactions.

A different three dimensional model has been proposed for the 19 kDa α -zein in a aqueous methanol solution (Momany *et al.*, 2005) (Fig. 2.3e). Probability algorithms and amino acid sequences suggested this class had coiled-coil tendencies. This resulted in a triple super helix containing α -helices with approximately four residues in the central section of their turn. These central sections contained non-polar side chains that formed a hydrophobic layer inside the super helix. The final model contained nine helical sections, divided into three equal interacting groups. Lutein, a carotenoid naturally present in maize, was suggested to fit into the central region of these groups (Momany *et al.*, 2005).

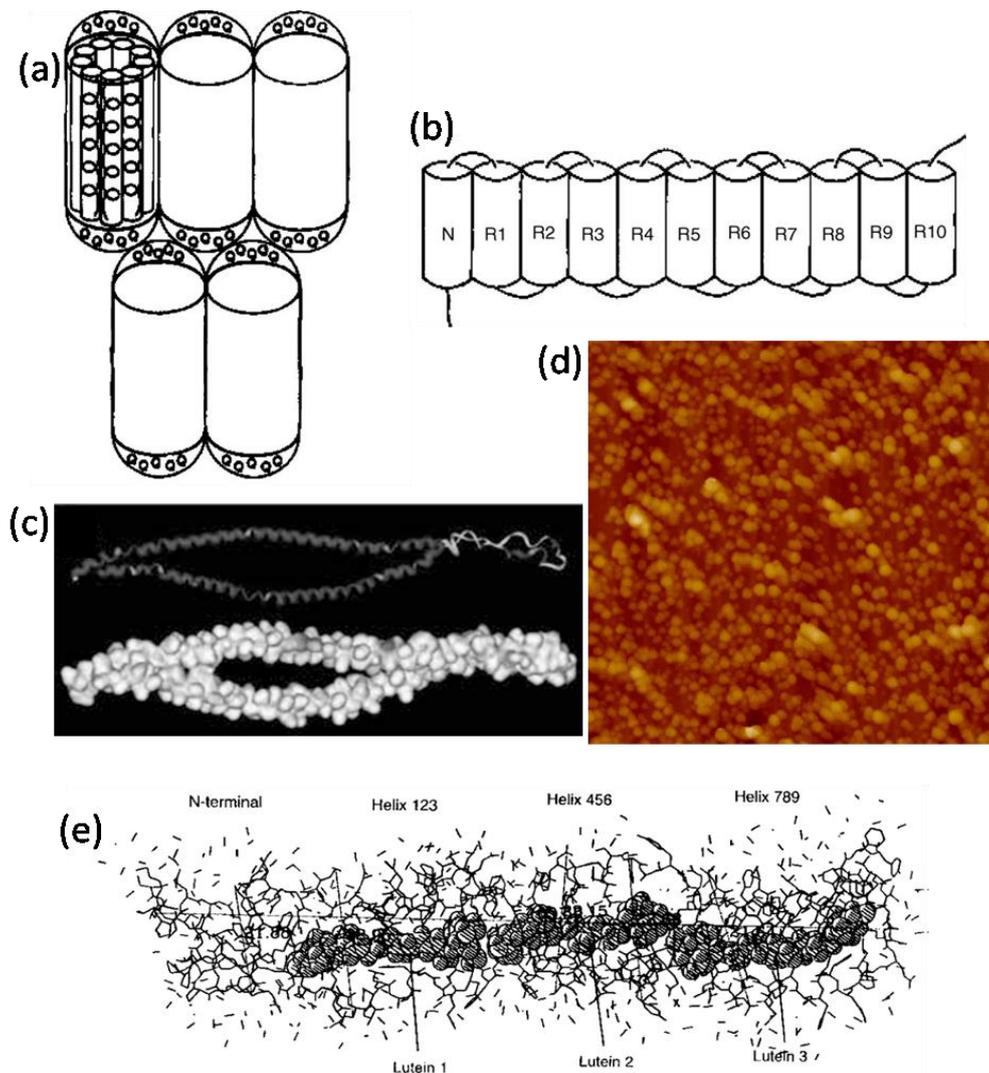


Figure 2.3 Proposed models for α -zeins. (a) Model proposed by Argos *et al.* (1982). The glutamine-rich turns (indicated by Q) are responsible for hydrogen bonding between the anti-parallel α -helices arranged in a distorted cylinder. (b) and (c) Linear models proposed by Matsushima *et al.* (1997) and Bugs *et al.* (2004) respectively. (d) A globular structure, of zein using AFM proposed by Guo *et al.* (2005). (e) A proposed model for 19 kDa α -zein indicating three groups of 9 helices and lutein located in the core region (Momany *et al.*, 2005).

3.3.2 β -zein

The β -zein class comprises a 15000 Da band when analysed by SDS-PAGE (Pedersen *et al.*, 1986). This class is often referred to as 15 kDa β -zein (Adams *et al.*, 2004; Huang *et al.*, 2004; Erny *et al.*, 2007). It has a true molecular weight of 17458 Da (Woo *et al.*, 2001). It is high in methionine (18 residues) and contains seven cysteine residues (Marks *et al.*, 1985; Pedersen *et al.*, 1986). These residues are involved in interchain disulfide bonding and a reducing agent is needed to extract this class (Turner *et al.*, 1965; Paulis, 1981). A limited amount is soluble in 60% ethanol (McKinney, 1958). β -zein is distinguished from the α -zein classes for being insoluble in 95% ethanol. It was suggested this class was more suitably placed with the γ -zein family due to six highly conserved polypeptide cysteine stretches and other conserved polypeptide domains (Woo *et al.*, 2001). Circular dichroism spectroscopy studies indicated the secondary structure of this class was mostly composed of β -sheets and contained very few α -helices (Pedersen *et al.*, 1986).

3.3.3 γ -zein

The γ -zein class consists of two sub-classes, with molecular weights of 16000 Da and 27000 Da, when analysed by SDS-PAGE (Holding & Larkins, 2006) and, as with the other zein classes, these proteins are referred to as 16 kDa and 27 kDa γ -zein (Adams *et al.*, 2004; Huang *et al.*, 2004; Erny *et al.*, 2007a). Their true molecular weights are 17663 Da and 21822 Da respectively (Woo *et al.*, 2001). A third sub-class, 50 kDa γ -zein, with a true molecular weight of 32 882 Da, was later added to this group (Woo *et al.*, 2001). It was previously thought to have been a dimer of the 27 kD γ -zein (Lopes & Larkins, 1991). This class is higher in cysteine and proline compared to the other classes. The 27 kDa γ -zein is separated from the other zein-classes in being soluble in water in its reduced state (Wilson *et al.*, 1981) and is thus the least lipophilic sub-class (Adams *et al.*, 2004). It is insoluble in its native state due to the presence of polymers stabilised by interchain disulfide bonds. Analysis to determine the secondary structure of this class indicated it was composed of a mixture of α -helices and β -sheets (Wu *et al.*, 1983).

3.3.4 δ -zein

In SDS-PAGE the 10 kDa δ -zein appears as a 10000 Da band and is the least abundant zein class. Its true molecular weight is 14431 kDa (Woo *et al.*, 2001). A second class, 18 kDa δ -zein, with a true molecular weight of 21200 Da was later added to this group (Woo *et al.*, 2001). δ -Zein has the highest methionine content of the zein classes and thus is only extractable under reducing conditions.

3.4 Homologies between zein and prolamins of related cereals

Homologies between prolamins of related cereals, maize, teosinte (*Zea mays ssp. parviglumis*), sorghum (*Sorghum bicolor*), finger millet (*Eleusine coracana*) and pearl millet (*Pennisetum americanum*) have been reported.

Teosinte is regarded the ancestor of modern maize (Flint-Garcia *et al.*, 2009). Comparisons of N-terminal amino acid sequences between zein and prolamins of teosinte (teosinte zein) revealed a high degree of homology (Bietz, 1982). Teosinte zein, zein and prolamins of landraces (considered an intermediate between teosinte and modern maize) were evaluated using reverse phase high performance liquid chromatography (RP-HPLC) (Flint-Garcia *et al.*, 2009). Chromatograms were similar in terms of β -, γ - and δ -zein components. However, the teosinte α -zein profile was more complex, containing additional peaks.

The prolamins of sorghum (kafirins), as for maize, are dominated by the α -type, called α -kafirins (Shull *et al.*, 1991). The α -kafirins were reported to be closely related to the α -zeins and two SDS-PAGE bands (23000 Da and 25000 Da) have been reported (Shull *et al.*, 1991).

The number of polypeptides differed between β -zein and β -kafirin, where the latter contained three polypeptides with apparent molecular weights of 20000, 18000 and 16000 Da, based on SDS-PAGE results. Only the 20000 Da protein of the β -kafirin class reacted with β -zein anti-serum when kafirins were tested for immunogenic reactivity with zeins (Shull *et al.*, 1991). Analysis of kafirin genes indicated only one gene is present for β -kafirin (Chamba *et al.*, 2005). This class was homologous with the β -zein and had a similar methionine content. β -Kafirin is more cysteine rich than β -zein with 10 instead of 7 cysteine residues.

γ -Kafirin contains one polypeptide with molecular weight (similar to 27 kDa γ -zein) of 28000 to 30000 Da (Evans *et al.*, 1987; Taylor *et al.*, 1989; Shull *et al.*, 1991). Similar to 27 kDa γ -zein, 28 kDa γ -kafirin was also soluble in water in its reduced state and had also been referred to as the reduced water soluble protein (Evans *et al.*, 1987; Taylor *et al.*, 1989). Amino acid analysis of the 28 kDa γ -kafirin demonstrated a similar amino acid composition compared to the 27-kD γ -zein (99% identical) (Belton *et al.*, 2006). Differences existed in the amount of repeats of a hexapeptide motif (PPPVHL) located in the N-terminal domain; eight repeats were present in 27 kDa γ -zein with only four in 28 kDa γ -kafirin.

High degree of homology also existed between δ -kafirin and the 10 kD δ -zein (Belton *et al.*, 2006). However, the δ -kafirin had less methionine and had not been detected on protein level.

Although little research in terms of homology has been conducted between the prolamins of finger millet and maize, SDS-PAGE and N-terminal amino acid sequences indicated a certain degree of homology (Garratt *et al.*, 1993; Tatham *et al.*, 1995). Pearl millet prolamins (pennisitins) seemed to share little homology with zeins, but α -pennisitins have been reported to have a similar solubility properties to α -zeins (Marcellino *et al.*, 2002).

3.5 Impact of environment on zein accumulation during endosperm development

The grain-fill period begins with successful pollination and subsequent initiation of kernel development. During cereal cultivation, the soil nutrient profile, environmental conditions and available moisture prior and during the grain fill period can influence various constituents.

3.5.1 Effect of temperature on zein accumulation

Temperature is one of the most important environmental factors governing plant growth and development. Cultivation of maize at higher temperatures, which is often the case due to natural environmental fluctuations, can be detrimental for the plant (Monjardino *et al.*, 2005). Reductions in starch, oil and protein content, as well as kernel density were observed when maize was exposed to elevated day and night temperatures in a green house (Wilhelm *et al.*, 1999). Heat stress lengthened the overall grain fill duration and results indicated persistent heat stress during grain-fill restrained seed storage processes i.e. formation of storage proteins.

Zein content at various DAP were studied when maize was subjected to 2 days and 4 days of heat stress (DHS) at 35°C (Monjardino *et al.*, 2005). The effect on other protein classes (globulins, albumins and glutelins) was determined and results showed zein proteins were most affected by heat stress. It was concluded heat stress during early development repressed accumulation of zein at synthesis level. Zein content of maize, subjected to 4 DHS, was significantly ($P<0.01$) lower at 14 and 17 DAP, after which zein concentration recovered. There was no significant difference in zein concentration between 2 DHS and control samples. Individual zeins classes also exhibited differences at various developmental stages. At 14 and 17 DAP all zein-classes content were significantly ($P<0.01$ and $P<0.05$) lower in 4 DHS samples compared to control samples. The δ -zein was the least effected by heat stress.

3.5.2 Effect of nitrogen fertiliszer on zein accumulation

Nitrogen fertilisers have been shown to influence zein accumulation during endosperm development. Generally full season nitrogen (N) availability can be one of the most limiting factors in grain production and high yielding hybrids can have a high carbohydrate:protein content (Tsai *et al.*, 1992). When this occurs, protein accumulation, especially zein, is reduced. The effect of N fertilisation rates [high (201 kg N ha⁻¹) and moderate (134-201 kg N ha⁻¹)] on the rate of total zein accumulation was investigated. (Tsai *et al.*, 1980). A normal maize hybrid line was compared to its homozygous, heterozygous and *opaque-2* (*o2*) mutant (a maize cultivar high in lysine and tryptophan) counterparts. Samples (four ears per developmental stage) were harvested from replicated trials at four developmental stages: 26, 35, 39 and 45 DAP. Zein accumulation in the normal endosperm type of the heterozygote was lower (6.0 mg/endosperm of mature kernel) with a lower fertilisation N rate compared to a high N rate (10.6 mg/endosperm of mature kernel). Endosperm from *o2* hybrids only had a small difference (4.0 mg/endosperm for lower N rate vs 5.3 mg/endosperm for high N fertilisation rate) when grown at high N

fertilisation rates. This was attributed to zein accumulation that was terminated at 35 DAP in *o2*-mutants.

The effect of nitrogen rates on accumulation of various zein sub-classes have been investigated (Tsai *et al.*, 1992). Maize kernel texture of a hybrid was also evaluated in response to N rate application. Increased N rate gave a more translucent and harder kernel as well as higher zein content. Quantitative fractionation of individual zein classes (α -, β - and γ -zein) indicated increases in α - and γ -zein and little increase in β -zein. Unfortunately this study examined only one hybrid grown within a single year.

3.6 Importance of zein

3.6.1 Commercial importance

Since the mid-20th century zein has become a subject of great interest in the polymer industry. Until the mid 30's there was no real use for zein, but when its commercial potential was realised there was a sudden increase in research. The first commercial production of zein from maize gluten meal began in 1939 (Shukla & Cheryan, 2001). Two types of zein are currently produced: white- and yellow-zein (Zhu *et al.*, 2007). Zein production is limited to an annual worldwide production of approximately 500 tonnes. Due to the hydrophobic nature of zein it can form tough, glossy, hydrophobic, greaseproof coatings/films which are resistant to microbial attack. These coatings/films have excellent flexibility and compressibility. More recent applications of zein included adhesives, laminated boards, and solid colour printing (Shukla & Cheryan, 2001). Commercial zein has been analysed using SDS-PAGE (Wilson, 1988; Zhu *et al.*, 2007) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Wang *et al.*, 2003). Commercial zein consisted of primarily α -zein with small amounts of δ -zein (Wang *et al.*, 2003) and β -zeins (Zhu *et al.*, 2007). α -Zeins are preferred as the dominant zein-class in commercial zein due to the other zeins classes (β , γ , and δ) contributing to gelling (Lawton, 2002). Although alcohol mixtures of ethanol or 2-propanol are the most popular choices as solvents, other solvents are being used for commercial zein extraction processes. Numerous patents, differing in temperature, pH control and solvents used, have been granted for extraction of zein (Lawton, 2002).

3.6.2 Impact of breeding on zein

Zein is deficient in the essential amino-acids lysine and tryptophan. Mutants, namely *opaque-2* (*o2*) (Mertz *et al.*, 1964) and *floury2* (*fl2*) (Nelson *et al.*, 1965) were discovered in the 1960's which had a much higher lysine content compared to normal varieties, hence, there was potential to improve the nutritional protein quality of maize. However, it was soon realised these mutants had a soft, chalky endosperm, making it susceptible to insect pests and mechanical damage (Ortega & Bates, 1983). In *o2* and *fl2* mutants it was noted that changes occurred in zein composition of the PBs (Schmidt *et al.*, 1992; Damerval & Devienne, 1993; Dombrink-

Kurtzman, 1994; Robutti *et al.*, 1997; Huang *et al.*, 2004; Gibbon & Larkins, 2005). These changes will be reviewed in more detail in section 3.6.3.3.

Not long after this discovery, modified phenotypes were discovered. These phenotypes had a harder endosperm texture. A modifier gene, *o2* modifier (*mo2*), was responsible for the altered endosperm texture (Paez *et al.*, 1969). The zein and starch composition was altered in these modified varieties (Gibbon *et al.*, 2003; Gibbon & Larkins, 2005). This gene was systematically introgressed into the *o2* germplasm by plant breeders and these modified mutants were designated Quality Protein Maize (QPM). QPM mutants have a similar yield and texture as for normal maize and a similar high lysine content as for *o2* mutants (Gibbon & Larkins, 2005). Various modifiers have been identified which produce a harder endosperm. There are only a few research centres and institutions, such as the international maize and wheat improvement centre (CIMMYT) in Mexico, the University of Kwa-Zulu Natal in South Africa and the Crow's Hybrid Seed Company at Milford in Illinois (USA) that are conducting research to improving the protein quality in QPM (Prasanna *et al.*, 2001).

3.6.3 Link to endosperm texture

Maize kernel texture is important in the agricultural industry due to its role in yield, harvest, storage and milling characteristics. To obtain optimum characteristics for above mentioned roles a hard kernel is needed (Holding & Larkins, 2006).

3.6.3.1 Maize endosperm texture

The endosperm constitutes the largest portion of the total maize kernel (Sofi *et al.*, 2009). It is filled with starch granules which are surrounded by a protein matrix consisting of protein bodies. Maize endosperm varies in texture, containing both hard and soft endosperm. The hard regions are referred to as horny, translucent, glassy or vitreous whereas the soft regions are referred to as floury or opaque (Dombrink-Kurtzman & Beitz, 1993). Variation in the ratio of these endosperm types result in variation in endosperm texture. The vitreous endosperm is located at the sides and the back of the kernel. Starch (Dombrink-Kurtzman, 1994; Dombrink-Kurtzman & Knutson, 1997; Gibbon *et al.*, 2003) and protein composition (Paiva *et al.*, 1991; Dombrink-Kurtzman & Beitz, 1993; Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Robutti *et al.*, 2000; Gibbon & Larkins, 2005; Holding & Larkins, 2006; Lee *et al.*, 2006; Blandino *et al.*, 2010) in these endosperm types have been linked to kernel hardness. When comparing physical differences between these two types of endosperm the following were seen; In the vitreous endosperm, the PBs were larger and more abundant (Dombrink-Kurtzman, 1994) and the starch granules were more compact and polygonal (Robutti *et al.*, 1974). In the floury endosperm, the starch granules were more spherical and loosely packed with less abundant, smaller loosely packed PBs (Robutti *et al.*, 1974; Dombrink-Kurtzman, 1994). It is thus apparent starch and protein play a role in hardness.

3.3.6.2 Impact of starch on endosperm texture

Starch comprises approximately 80% of maize endosperm and exists in two forms, namely amylose and amylopectin. Dombrink-Kurtzman and Knutson (1997) linked amylose to hardness; amylose content was significantly higher (although the variation was low) in harder than softer endosperm. When comparing the impact of the architecture of the starch granules Dombrink-Kurtzman (1994) concluded PBs alone cannot be responsible for hardness. Starch alterations have also been seen in QPM where amorphous, non-crystalline amylopectin molecules at the surface of starch granules interacted to form contacts that linked starch granules together (Gibbon *et al.*, 2003).

3.3.6.3 Impact of zein on endosperm texture

Various studies have been conducted where zein content of vitreous endosperm has been compared to that of flourey endosperm (Dombrink-Kurtzman & Beitz, 1993; Robutti *et al.*, 1997; Landry *et al.*, 2004). Dombrink-Kurtzman and Beitz (1993) and Robutti *et al.* (1997) analysed zein using RP-HPLC. Zeins were extracted from vitreous and flourey endosperms of various inbred lines and hybrid maize kernels. These portions were either hand dissected with a hand-held drill (Dombrink-Kurtzman & Beitz, 1993) or mechanically separated after milling (Robutti *et al.*, 1997). Zein was extracted with 70% ethanol, 2-ME and sodium acetate. Chromatograms (Fig. 2.4) indicated three sets of peaks containing certain zein classes. The first two sets of peaks contained β - (peak 1) and γ - zeins (peak 2 = 27 kDa γ -zein and peak 3 = 16 kDa γ -zein) and the collection of peaks at the end, α -zeins. RP-HPLC analysis showed the complexity of the α -zeins. Robutti *et al.* (1997) grouped β - (peak 1) and γ -zeins (peak 2 and 3) together as zein-2 and the α -zeins as zein-1. Comparisons of integrated peak areas of zein-1 and zein-2 from flourey and vitreous endosperm portions indicated total zein-1 was almost twice as high compared to zein-2 in the vitreous endosperm. Thus, linking α -zein positively with a harder kernel texture. Similar results were also obtained by Dombrink-Kurtzman and Beitz (1993), where the percentage α -zeins were on average 3.3 times higher in vitreous endosperm portions. α -Zein does not contain disulfide bonds and it has been postulated it fills the PBs, giving a higher mechanical stability to the endosperm (Landry *et al.*, 2004). It has been shown vitreous and flourey endosperms differed significantly, being higher in the flourey endosperm, in percentage peak areas of 27 kDa- and 16 kDa γ -zein ($P < 0.05$ and $P < 0.001$ respectively) (Dombrink-Kurtzman & Beitz, 1993). SDS-PAGE was also performed and results indicated higher amounts of δ -zein were present in vitreous endosperms. Lending and Larkins (1989) suggested, when comparing the development of protein bodies (PB) in maize, flourey endosperm contained immature PBs with less α -zein compared to vitreous endosperm which contained more mature PBs.

o2 and *fl2* mutant varieties, which are soft in texture, had lower zein content (especially the α -zeins) than normal varieties. The α -zein reduction was due to effect of the *o2* and *fl2* mutant

genes; both 19 kDa (Huang *et al.*, 2004) and 22 kDa α -zein (Lee *et al.*, 1976; Paulis, 1981; Kodrzycki *et al.*, 1989; Schmidt *et al.*, 1992; Huang *et al.*, 2004) syntheses were reduced. The *o2* mutation influenced the transcriptional activator of a subset of the 22 kDa α -zein genes (Kodrzycki *et al.*, 1989). All zeins (Holding & Larkins, 2006), especially the 22 kDa α -zeins, were reduced (Lee *et al.*, 1976; Damerval & Devienne, 1993). This led to the formation of considerably smaller PBs (Holding & Larkins, 2006). The reduction of zein generally goes together with an increase in other protein classes. In *fl2* kernels the 22 kDa α -zeins were abnormally synthesized resulting in deformed small PBs (Lending & Larkins, 1992). A mutation, previously described (Coleman *et al.*, 1995), of the 22 kDa α -zein gene to a gene encoding for a 24 kDa α -zein was suggested to be responsible, where a soft endosperm texture was observed for transgenic maize lines expressing this mutant gene (Coleman *et al.*, 1997). Thus, confirming its role in causing a softer endosperm.

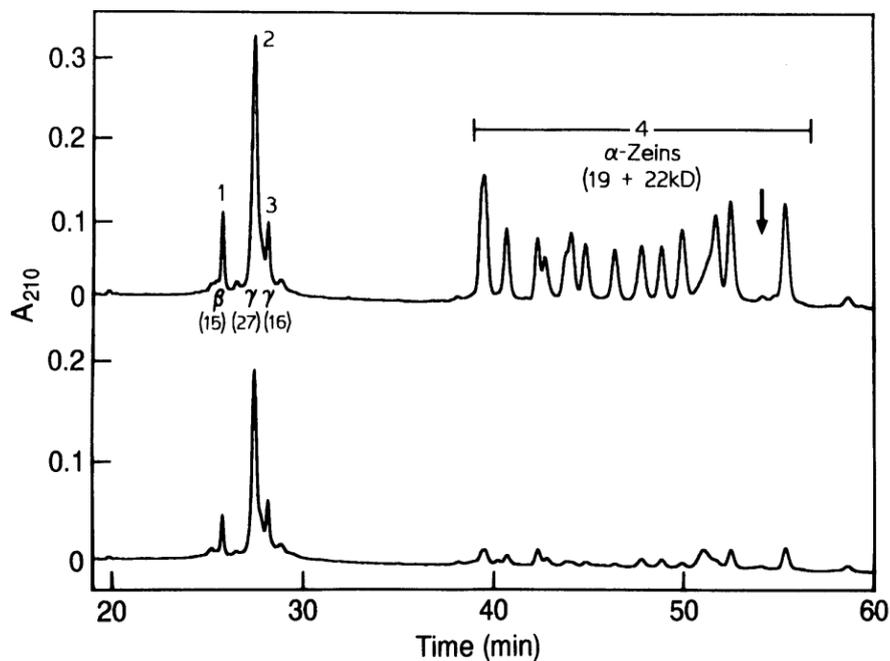


Figure 2.4 RP-HPLC chromatograms of zein from vitreous endosperm (top) and floury endosperm (bottom) (Dombrink-Kurtzman & Beitz, 1993).

The 27 kDa γ -zein has also been positively linked to kernel texture. A comparison of 27 kDa γ -zein content (determined with RP-HPLC) in QPM samples, indicated a higher percentage in harder endosperm portions (Dombrink-Kurtzman, 1994). Wallace *et al.* (1990) also linked 27 kDa γ -zein to endosperm texture. Zein extracted from normal lines and their *o2* and *fl2* counterparts, as well as QPM varieties, were analysed and quantified using enzyme-linked-immuno-sorbance assay (ELISA), Western Blotting and SDS-PAGE with Coomassie blue staining. The 27 kDa γ -zein concentration was higher (2-4 times) in QPM varieties than the *o2* varieties (Wallace *et al.*, 1990). The α - and β -zein contents of the QPM lines were similar to the

o2 varieties but lower than in normal lines. ELISA was used in a similar study and results indicated the same trend; the 27 kDa γ -zein was present in QPM varieties at elevated (2-5 times) concentrations (Paiva *et al.*, 1991). Immuno-staining of zeins in QPM lines indicated intense staining for 27 kDa γ -zein in the sub-aleurone layer of normal, *o2* mutant and QPM varieties (Geetha *et al.*, 1991). In contrast to the other lines, staining was also observed in the central part of the endosperm of QPM varieties. Therefore, indicating an overall higher concentration for this class in QPM lines. When 27 kDa and 16 kDa γ -zein expression in a QPM maize line was inhibited (by introducing a dominant RNAi transgene), irregularities in protein body structure and their interaction with starch granules were observed (Wu *et al.*, 2010). It has also been reported that an altered 16 kDa γ -zein in a *Mucronate* phenotype (maize variety with an opaque kernel mutation) was responsible for a malformed protein body (Kim *et al.*, 2006). Theories regarding 27 kDa γ - and α -zein's role in hardness have been proposed. Due to 27 kDa γ -zein's location in the periphery area of the PB and its higher content of cysteine it forms disulfide bonds that interacts with other components (Robutti *et al.*, 1994). This was seen when the interactions of various zeins were studied (Kim *et al.*, 2002). Also, the 27 kDa γ -zein, being more abundant in QPM lines, increased the amount of disulfide binding, resulting in a covalently linked protein network (Dannenhoffer *et al.*, 1995).

Landry *et al.* (2004) and Zang *et al.* (2003) contradicted 27 kDa γ -zein's link to endosperm texture. SDS-PAGE indicated 27 kDa γ -zein and 10 kDa δ -zein were absent in both types of endosperm of a maize sample (Landry *et al.*, 2004). In comparison to other samples, an opposite trend was observed for another maize sample (normal variety) in the study; 27 kDa γ -zein was more abundant in its vitreous than the floury endosperm.

A cDNA clone of the 27 kDa γ -zein was expressed in developing barley to establish its effect on grain composition and properties (Zhang *et al.*, 2003). Expression of the 27 kDa γ -zein was evaluated by SDS-PAGE, protein dot and western blot analysis. The composition of storage proteins of the barley prolamins, hordein, was evaluated by SDS-PAGE and quantified. Evaluation of transgenic lines (lines where 27 kDa γ -zein was expressed) showed a significant increase ($P < 0.001$), expressed as micrograms per gram grain dry weight (DW), in nitrogen soluble in 55% prop-1-nol with 2% β -mercaptoethanol. This was attributed to the expression of 27 kDa γ -zein. Quantification revealed it accounted for 4% of the total alcohol soluble proteins whereas D- and B-hordein (sub-classes of hordein) were reduced, with no significant changes in C-hordein (depending if expressed as percentage total grain N or micrograms per gram grain DW). Comparisons of gel protein fractions established 27 kDa γ -zein was incorporated in protein polymers. When comparing grain textures (determined by a Perten Single Kernel Characterisation System) results showed, although not statistically significant, hardness of transgenic lines was slightly lower than that of non-transgenic lines. Visual inspection did not indicate differences in degree of vitreousness. Although no difference in texture was seen, it can not be ruled out the 27 kDa γ -zein did not influence texture, due to the lower amount (4% of total

prolamins) found than in QPM and normal maize varieties (Zhang *et al.*, 2003). Higher expressions of this sub-class should also be investigated. It should also be considered that if expressed in other grains, interactions with their prolamins compared to other zein classes may not be the same. Thus, this sub-class may not influence the texture of other grains as it does for maize.

Zein classes have also been quantified, using RP-HPLC, and correlated to degree of hardness assessed with various hardness measurements (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006). All the studies indicated α -zeins, collectively, were positively associated with a harder kernel texture but, as for the other zein classes, not all correlations observed were equally strong. The 27 kDa γ -zein has mostly been positively associated with a harder kernel texture. This was dependent on if this class was expressed as an integrated peak area given by arbitrary units (AU) or a percentage of total peak area (Paulis *et al.*, 1993). The relationship between 15 kDa beta-zein and kernel texture is uncertain due to inconsistent (negative or positive) correlations that have been reported (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Lee *et al.*, 2006). The 16 kDa γ -zein, correlated negatively to kernel texture assessed with hardness measurements (Pratt *et al.*, 1995).

Although the relationship between zein and kernel texture appeared to be genotype specific (Pratt *et al.*, 1995) and not always strict (Landry *et al.*, 2004), studies indicate zein plays a role in maize hardness.

4. Chromatography

Chromatography is a separation process where a sample mixture is distributed between two phases; a stationary and mobile phase. The stationary phase is either a solid, porous, surface active material or a thin film of liquid coated on a solid support. The mobile phase is a gas or liquid. Separation is based on the affinity of the sample to the two phases (Meyer, 2004).

4.1 Chromatographic analysis of zein

Several chromatographic methods are used to separate proteins: adsorption, affinity, hydrophobic phase, normal phase, ion exchange (IE), size exclusion (SE), RP-HPLC and gel filtration chromatography. The latter four have been used for analysis of zein. For the purposes of this thesis, the focus will be on RP-HPLC and SE and IE chromatography will be briefly reviewed.

SE and IE chromatography have been used to separate individual zeins for further analysis. A protocol for SE chromatography (separates analytes according to size) was developed to attempt to separate and purify the zein proteins from other ethanol soluble components of maize (e.g. zein from xanthophylls) (Zhu *et al.*, 2007). The authors were not successful in completely separating the zein classes due to contaminants of other classes present in the purified fractions.

IE chromatography was applied to separate the 19 kDa and 22 kDa α -zeins. 19 kDa α -Zein was further analysed by circular dichroism to provide more information on its structure. It was concluded the α -helix content was lower, and the β -sheet content higher than the mixture of the two α -zeins.

4.2 Reverse phase high performance liquid chromatography (RP-HPLC)

RP-HPLC methods for zein separation are well established. Parameters (capacity and selectivity factors, plate height and resolution) that were investigated during method development will, thus, not be reviewed and only an overview of this technique will be given. RP-HPLC is a form of column chromatography where a mobile separation, with the aid of polar mobile solvents (usually two), is based on the extent of how the analyte interacts with the non-polar packing material (stationary phase) of the column. In RP-HPLC the stationary material is inert and only non-polar (hydrophobic) interactions are possible (Wilson, 2005). The stationary phase in RP-HPLC consists of silica that has been treated with, RME_2SiC ; the R is a straight alkyl group such as $\text{C}_{18}\text{H}_{37}$ and C_8H_{17} . The analytes continuously pass back and forth between the two phases until they separate. The principle of this separation is based on the distribution or partition coefficient (K_d) which describes how the analyte distributes between two immiscible mobile phases.

A chromatogram is a graphical record of the detector response indicating retention times of the separated analytes. The analytes are indicated by peaks and will have characteristic retention times. RP-HPLC is used to quantitatively and qualitatively analyse substances. Concentration of an analyte can be determined by relating its retention time and integrated peak areas to a reference standard (Wilson, 2005). Integrated peak areas can also be compared with a higher area indicating a higher amount of analyte. This is done when there is no reference standard available. Zein proteins have been quantified in this manner (Dombrink-Kurtzman & Beitz, 1993).

The RP-HPLC operation can be summarised as follows; a small amount of analyte is injected into the column and two (A and B), sometimes three (C), polar solvents (mobile phase) are pumped through the column under high pressure. One of the solvents is usually a strong polar solvent and the other a more aqueous solvent. When analysing hydrophobic proteins such as zein the two solvent concentrations are varied. The more hydrophobic molecules are retained in the column when the concentration of the aqueous solvent is greater in the beginning. A gradient elution is applied by varying the mobile phase composition during the analysis. Two types of gradients can be used, namely linear and non-linear. Various detectors can be used and detectors monitoring the eluate in the ultra-violet range are most commonly used (Wilson, 2005). Analytes are monitored at a specific or a range of wavelengths depending on the capability of the instrument.

4.3 RP-HPLC analysis of zein

The first RP-HPLC analyses separated zein, using a linear gradient, into several fractions (Paulis & Beitz, 1986). The first three peaks contained the alcohol soluble glutelins; now referred to as β - and γ -zein. The major zeins (α -zeins) eluted last as a collection of peaks. The column that was used was a C18, 5 μm (pore size), 250 x 4.6 (length x diameter) mm. Columns with these specifications were used in most zein analysis studies (Wilson, 1991; Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994; Pratt *et al.*, 1995; Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Lee *et al.*, 2006). The eluate was monitored at 210 nm, which was also the case for most other studies. UV absorption at 210 nm is largely due to peptide bonds and therefore is affected by the amount of amino acids present in proteins (Buck *et al.*, 1989). Operating temperatures reported for columns varied; ambient temperature (Flint-Garcia *et al.*, 2009), 55°C (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994) and 60°C (Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Robutti *et al.*, 2000) have been used. Different A and B solvents have also been reported. Solvent A being ultrapure water when using a linear gradient (Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Robutti *et al.*, 2000) or 15% acetonitrile (ACN) when using a non-linear gradient (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994). Solvent B being 100% ACN when using linear and 80% when using a non-linear gradient. Both solvents contained 0.1% trifluoroacetic acid to maintain a low pH to ensure the hydrophobicity of the analyte was not influenced. Profiles obtained from linear and non-linear gradients were very similar, with β -zein and the γ -zein eluting between 25 and 30 minutes and the α -zeins between 40 to 60 minutes. In both gradients the ACN concentration increased over time thus retaining the more hydrophobic α -zeins due to their low affinity to the initial higher aqueous concentrations of the solvent. A detailed review of RP-HPLC analyses of zein and other maize proteins has been published (Rodriguez-Nogales *et al.*, 2006).

RP-HPLC fractions of zein have been subjected to 2D gel electrophoresis to identify zein classes (Wilson, 1990). Various inbred lines and hybrids were compared. Zeins were extracted with 55% 2-propanol and 5% 2-ME, with or without 0.5% sodium diacetate. Elution was monitored at 210 nm for quantification and 280 nm for identification. Several fractions were observed and identified. Similar grouping of peaks were observed as observed by Paulis and Beitz (1986). Comparisons of hybrids and their respective inbred parent lines revealed this is a powerful tool to study inheritance of zeins. Similarities between α -zein patterns of hybrids and their inbred parent lines were observed. Closely related inbred lines also had similar patterns. Serial analysis revealed a total of 40 different zein proteins present. RP-HPLC could identify 34 of these compared to IEF.

5. Mass spectrometry

Mass spectrometry (MS) is a technique widely used in proteomics for accurate mass measurement of proteins/polypeptides, protein identification i.e. amino acid sequencing,

reaction monitoring i.e. monitoring enzyme reactions, chemical modification and structural analysis (Caprioli & Gross, 2005; Watson & Sparkman, 2008a). Fig. 2.5 illustrates the main processes involved in MS.

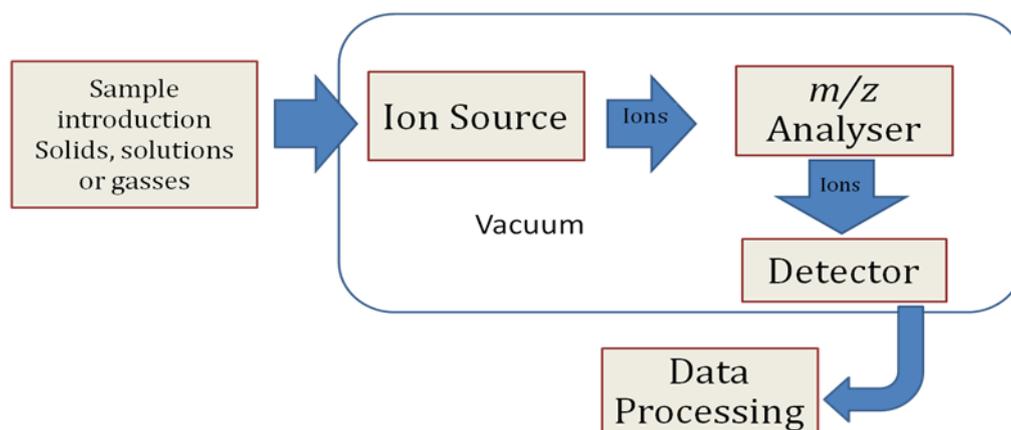


Figure 2.5 Conceptual illustration of a mass spectrometer.

Sample introduction can vary depending on the compound (analyte) is analysed. Analytes can either be separated prior to MS analyses by chromatographic methods [HPLC or capillary electrophoresis (CE)] or be a product of protein digestion or be directly extracted, i.e. zein extraction from maize. Many types of ionisation sources exist including, matrix assisted laser desorption (MALDI) (analyte introduced as solid), electron spray ionisation (ESI) (analyte introduced as liquid), fast atom bombardment (FAB), chemical ionisation (CI) and electron impact (EI) (samples introduced as fixed gasses). The ions pass through to a mass analyser where they are separated according to their mass-to-charge ratio. In mass spectrometry the molecular weight of the compound is not directly measured but rather the mass-to-charge ratio of ions produced in a gaseous phase is given. Ions reach the detector and are processed in the form of spectra (Watson & Sparkman, 2008a).

Mass spectrometry techniques that have been used for zein analysis include: matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) (Wang *et al.*, 2003; Adams *et al.*, 2004; Huang *et al.*, 2004), CE-MS (Erny *et al.*, 2007a; Erny *et al.*, 2007b) and RP-HPLC-ESI MS (García López *et al.*, 2009).

5.1 MALDI-TOF MS

MALDI-TOF MS is a powerful technique widely used in proteomics. It is a soft ionisation technique that allows for the sensitive detection of bio-molecules and large organic molecules. It can detect a wide mass range and can be used for protein detection and identification. This technique consists of various components (matrix, laser, mass analyser, detector) and processes (desorption/ionisation, ion separation, detection of ions).

5.1.1 Matrix

The matrix is an organic compound of low molecular weight, containing functional groups (e.g. aromatic rings) that absorb photons emitted from the laser. The matrix is thought to serve two major functions (1) to absorb energy from the laser light and (2) to isolate biopolymer molecules from one another (Hillenkamp *et al.*, 1991). Various matrices are used depending on the analyte. In protein analysis nicotinic acid, 2,5-dihydroxybenzoic acid (DHB), sinipinic acid (SA), α -cyano-4-hydroxycinnamic acid and 2-(4-hydroxyphenylazo)benzoic acid (HABA) are widely used. A near-saturated concentration of the matrix is usually prepared with a similar or compatible solvent solution as used for the analyte. A solution with a low pH was shown to be required to promote sufficient ionisation (Adams *et al.*, 2004). An amount of 0.5 to 1.0 μL is generally applied. An important feature is the way the matrix interacts with the analyte. After the matrix and analyte are applied to the target plate, the solvent evaporates and the matrix crystallises to form a bed of small crystals that range in size from a few microns to few hundred microns (Hillenkamp *et al.*, 1991). This size variance is dependent on the matrix and preparation. It is important the matrix co-crystallise/precipitate with the analyte. A typical molar analyte to matrix ratio ranges from 1:100 to 1:50 000 (Karas & Hillenkamp, 1988). Various analyte and matrix preparation procedures exist, with variation existing in each procedure. These include dried droplet (Karas & Hillenkamp, 1988), fast evaporation (Vorm *et al.*, 1994) and matrix layer (Garaguso & Borlak, 2009) techniques.

5.1.2 Laser and desorption/ionisation

Ionisation is triggered by a pulsed laser beam. Various types of lasers are used which include, N_2 , and CO_2 . N_2 lasers are commonly used (Hillenkamp *et al.*, 1991). It is difficult to separate desorption from the ionisation process because both processes are intertwined and both take place on a micrometer geometrical and nanosecond scale (Hillenkamp & Peter-Katalinic, 2007a). The mechanisms of desorption and ionisation are not well understood and various theories/models have been suggested. A general overview of the concepts will be discussed.

When the laser is triggered and reaches the target plate, energy is absorbed by the matrix ions and monolayers of matrix molecules are removed and desorption occurs, i.e. transformation from the solid state to gaseous phase. The energy transferred is equal or close to the sum of all bound energies in the solid phase (matrix and analyte crystal formation). Once in a gaseous phase ions are generated. Two models for the formation of ions have been proposed. In the first model it is assumed that the analyte molecules are neutral in the matrix crystal formation. An initial step of photo-ionisation of the matrix molecules initiates a charge transfer to the analyte. A more recent model, suggested analytes are incorporated in the matrix as charged species where after they become re-neutralized within clusters that form during desorption of matrix and analyte (Hillenkamp & Peter-Katalinic, 2007a). This model is known as the lucky survivor model.

During ionisation, two modes of ionisation occur: positive and negative ionisation. In positive ionisation mode singly protonated molecular ions ($M+H^+$) are the dominant species. Positive ionisation is generally used for analyses of protein and peptides. In negative ionisation mode the opposite occurs, with ($M-H^-$) species dominating. This mode is usually applied for oligosaccharides and oligonucleotides.

5.1.3 Time-of flight mass analyser

Once ions are generated they are accelerated by an applied voltage (ranging from 20 kV to 25kV) at the interface of the target plate to the TOF mass analyser, where they are separated according to their mass-to-charge (m/z) ratio. The analyser consists of a drift tube which is a field free drift region. Two types of TOF mass spectrometers can be used, namely linear/axial or reflectron mass spectrometer (Hillenkamp & Peter-Katalinic, 2007b). In linear mass spectrometers ions fly down the flight tube and hit a detector. The most common detector used is the micro channel plate (MCP). It is specially fabricated to enhance the signal of the ions. The time it takes for ions to arrive at the detector is recorded and plotted against the ion current signal with a digital storage oscilloscope. The m/z is determined by the derived equation:

$$\frac{m}{z} = 2eU \left(\frac{t}{L} \right)^2$$

Where m is the mass, z is the charge of the ion, e the elementary charge, U the potential (voltage) applied, t the time of arrival and L the length of the drift tube. If an ion is doubly charged ($z=2$) it arrives in half the time it takes singly charged ion, thus resulting in a peak with half the m/z of the singly charged counterpart. These peaks are known as the doubly charged signals/peaks ($M+H^{2+}$) and, along with dimeric species, accompany the singly charged peaks. In certain applications linear mass spectrometers are not appropriate, i.e when ions with similar masses cannot be individually detected due to similar velocities or ions with the same mass have different energy distributions and are detected as individual ions. Reflectron or non-linear mass spectrometers are used to overcome this by refocusing the ions onto a detector via an ion mirror. The ions generated fly through the first field free region and reach the first detector where a constant electric field is applied to compensate for the initial velocity and slow down ions. Ions are then focused to reach the second detector. Limitation exists for the size of the molecule that can be measured. Generally, this mode is applied for molecules with low molecular weights. Both linear and non-linear mass spectrometers can be operated within the same instrument.

5.2 MALDI-TOF MS analysis of zein

MALDI-TOF MS analysis has been used to examine prolamins from other cereals (wheat, maize) due to their importance in food-processing and because they are possible allergens. Studies have been conducted to optimise protocols for identification of the various zein classes by using proteases to digest zein proteins for further analysis with MALDI-TOF/TOF (MS/MS technique used for protein identification) (Sergeant *et al.*, 2009).

Analyses of zein directly extracted from maize meal have been also reported (Wang *et al.*, 2003; Adams *et al.*, 2004; Huang *et al.*, 2005). Wang *et al.* (2003) first described a protocol for general analysis of zein extracted from maize. Zein was extracted from defatted maize with 55% aqueous 2-propanol (1:4.5 endosperm-to-solvent ratio) and then precipitated. Another extraction was made at 60°C with 75% ethanol with or without a reducing agent dithiothreitol (DTT). Various matrices were tested and it was found that 2,5-DHB gave optimum results. A Voyager-DE STR system (Applied Biosystems, Inc., Foster City, CA) was used and measurements were performed in a positive ion linear mode. Ions were accelerated at 25 kV. Extracted/precipitated zein was compared to commercial zein with SDS-PAGE and MALDI-TOF MS analysis. SDS-PAGE analysis of the commercial zein revealed three bands which appeared at 10000 Da (δ -zein), 22000 Da (19 kDa α -zein) and 24000 Da (24 kDa α -zein), whereas, the zein extracted with 75% ethanol only showed the latter two bands. The mass spectrum obtained for commercial zein had three singularly charged species (peaks) with molecular weights of 23362, 24097 (19 kDa α -zeins), 26838 Da (22 kDa α -zein) and 14446 Da (10 kDa δ -zein) along with dimer and doubly charged peaks. A species with molecular weight of 20386 Da was noted and this could possibly be 27 kDa γ -zein or 18 kDa δ -zein. Three main species with molecular weight of ca. 23000, 24100 (19 kDa α -zein), and 26900 (22 kDa α -zein) were observed in spectra of extracted zein without reducing agents. Minor species with molecular weight ca. 17700 (β -zein) and 14440 (δ -zein) appeared with extraction at 60°C with a reducing agent.

The width of the peaks that appeared in the spectra was broad and the authors suggested each peak contained multiple components (Wang *et al.*, 2003). This agrees with the large number of proteins in the α -zein family having similar molecular weights. Although peak masses correlated well with gene sequencing data (Woo *et al.*, 2001), differences were seen between molecular weights of gene sequencing data and that obtained in spectra. Differences also appeared between repeated measurements. The authors suggested several factors could influence this; the measurement accuracy of mass decreases for broad peaks, which may have been due to peak shifts caused software smoothing (Wang *et al.*, 2003). In addition, the minor components determined by the gene sequencing will contribute to the MS peaks, resulting in some shift in peak position. However, no calibration was performed with an external standard in this study which might have improved differences between molecular weights observed in repeat measurements as well as comparisons to gene sequencing data.

MALDI-TOF MS analyses of zein from various inbred and mutant lines have been conducted (Adams *et al.*, 2004). The extraction procedure used was more simplistic than the one used by Wang *et al.* (2003). Zein proteins were directly extracted from defatted maize meal (from a single maize kernel), with 60% ACN, 25 mM ammonium hydroxide (NH₄OH), and 10 mM of DTT for an hour at 60°C (1:5 endosperm-to-solvent). The NH₄OH was added to increase the pH needed for sufficient extraction of 27 kDa γ -zein. The mixture was shaken every 15 minutes and centrifuged after incubation. After testing various concentrations (10, 20, 30 and 40 mM) of the matrix, HABA, a near-saturated matrix mixture (40 mM) in 70% ACN containing 0.03% TFA gave optimum results. TFA was added to lower the pH to below 1.5 to ensure sufficient ionisation of proteins (Adams *et al.*, 2004). An internal standard was used to calibrate molecular weights and normalise spectra. Idoacetamide (IAA) derivatisation was also performed to identify zein classes. Fig. 2.6 is an example of a spectrum obtained.

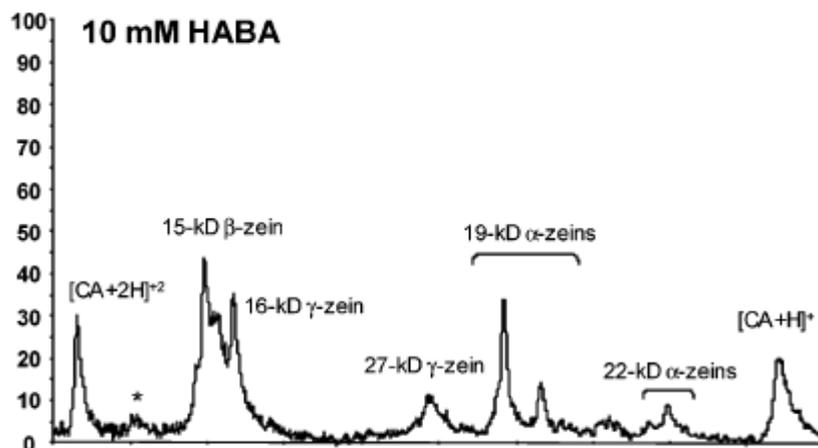


Figure 2.6 MALDI-TOF MS spectrum of zein from an inbred parent line acquired with 10 mM HABA (Adams *et al.*, 2004).

The identification of peaks was initially based on gene sequencing data and masses differed only by 0.43% (Woo *et al.*, 2001). IAA derivatisation was used to identify the species observed by determining the number of cysteine residues present per zein class and comparing it to known amino acid sequences of the classes. In contrast to the previous study (Wang *et al.*, 2003), all main zein classes were observed in spectra of various samples. This can be attributed to the extraction procedure and possibly the matrix used. Several of the low abundant zeins, i.e. 10 kDa and 18 kDa δ -zein (although not identified using IAA derivatisation), as well as the major 22 kDa α -zeins (Z5 and Z4) and the 19 kDa α -zein (B2) were also detected. The relative abundance of zein classes, from a well characterised inbred line (B73) were also measured (by weighing peaks cut from printed spectra) and compared to their percentage abundance of transcript levels (Woo *et al.*, 2001). In general the zein expressed at higher levels correlated with larger peak areas. The 27 kDa γ -zein did not correlate well with its expression and this

could have been due to matrix conditions used. This class is more water soluble than the other zein classes and inconsistency in solvent evaporation could have resulted in variability in peak height. A more water soluble matrix might improve this (Adams *et al.*, 2004).

Inbred and mutant line comparisons showed variation in α - and β -zeins (Adams *et al.*, 2004). *Opaque-2* mutants were compared to normal lines. Reduction of 22 kDa α -zein was observed in spectra, consistent with reports mentioned earlier (section 3.6.3.3). Spectra also showed reduction in 27 kD γ -zein and 15 kDa β -zein. Variation in the 15 kDa β -zein and 16-kDa γ -zein was also observed between opaque mutant lines and normal lines.

MALDI-TOF MS is an important technique and has superior properties over SDS-PAGE in terms of resolution of zein sub-classes and mass accuracy. Thus, it can aid in the characterisation and comparison of zein profiles from various hybrids and parent lines.

5.3 Capillary electrophoresis mass spectrometry analysis of zein (CE-MS)

CE-MS, also known as capillary zone electrophoresis (CZE), is a technique widely applied for the electrophoretic separation of molecules where liquid separation via a column is combined with subsequent mass analysis using an appropriate mass analyser.

5.3.1 Sample introduction and analyte separation

Electrophoresis is the forced flow of charged particles in an electric field, where separation is based on the differential migration of charged analytes through a certain medium (electrophoretic mobility) (Walker, 2005). Biological compounds such as amino acids, peptides and protein possess ionisable groups and at a certain pH they will be negatively or positively charged. By applying an electric field these charged species will migrate to the cathode or anode. Capillaries/columns used in CE have inner diameters less than a 100 μm with a high surface area to volume. The latter feature allows dissipation of heat energy generated by the applied field and thus a greater field can be applied to enhance separation (Watson & Sparkman, 2008b)

Similar mechanical features are shared with HPLC, where a fluid flows through a column with migration of distinct concentration zones of analytes through a detector at specific times. Samples are injected under pressure via two mechanisms (electrokinetic and displacement injection) into the capillary. Electrokinetic injection allows only species of similar charge to enter the capillary. In displacement injection the capillary inlet is directly placed into the sample solution resulting in a pressure gradient across the capillary. This forces an aliquot of the larger sample into the inlet of the capillary. Displacement injection is preferred because analyte ions present, are in direct proportion to their concentration in the bulk sample (Watson & Sparkman, 2008b).

5.3.2. Ionisation source

ESI is an ionisation source commonly coupled to CE (Watson & Sparkman, 2008c). Ionisation is accomplished by forcing a solution of analyte through a small capillary so that the fluid is sprayed into an electric field. The electric field is necessary to promote effective spraying action by keeping formed droplets from freezing and to protect charged droplets to tolerate collisions. The fluid is forced through the tip and moves through the electric field to the counter anode. Ions become concentrated as the droplet shrinks due to evaporation of the solvent. This increases the repulsive forces between excess charges in the droplets, promoting dispersion to form smaller droplets. When the macroscopic solvent finally evaporates the analyte molecule has residual charges attached. In positive ionisation protons attach to site with high Lewis basicity. Similar to MALDI the exact source of ionisation is still poorly understood. It has been suggested that protons either come directly from the sample solution or enter the solution during the ESI process. Ions that are formed then go through to a mass analyser (Watson & Sparkman, 2008c). Various mass analysers have been coupled to CE (Ding & Vouros, 1999).

5.4 CE-MS analysis of zeins

CE-MS or CZE-MS analyses of zein have been reported (Erny *et al.*, 2007a). A method for separation of zein was optimised to find appropriate background electrolytes (BGE) compatible with ESI-MS (Erny *et al.*, 2007b). Various parameters for the optimisation of zein separation with ESI-MS were investigated, but will not be described here in further detail. The optimised method was used to compare zein from various samples of transgenic and normal lines (Erny *et al.*, 2007a). Zein proteins were directly extracted from 50 mg maize meal with 1 ml of 60% ACN containing 5% 2-ME and 120 mM NH₄OH. Mixtures were vigorously shaken for 5 minutes and centrifuged. This was repeated three times. Zein proteins were then precipitated with acetone, the mixture was centrifuged, and the pellet was redissolved in a ACN/formic acid/water solution prior to analysis. No major differences were seen between transgenic and corresponding non-transgenic lines. Small differences were detected for 22 kDa and 19 kDa α -zeins between normal lines. Variation of 15 kDa β -zein and 16 kDa γ -zein was also detected, in accordance with Adams *et al.* (2004). CE-MS detected more α -zeins proteins compared to MALDI-TOF MS (19 vs. 7) (Adams *et al.*, 2004). This was due to their separation that was based on differences in their electrophoretic mobility. The 10 kDa δ -zein and 27 kDa γ -zein, however, were not detected; even though 10 kDa and 27 kDa γ -zein have been observed in other studies using MALDI-TOF MS (Wang *et al.*, 2003; Adams *et al.*, 2004). A possible reason for this could be the temperature of extraction as extraction took place at room temperature. Wang *et al.* (2003) observed 10 kDa δ -zein only after zein extraction at 60°C. Adams *et al.* (2004) also extracted at 60°C and observed both classes. In addition, the duration for zein extraction was shorter (15 min vs. an hour) than reported by Adams *et al.* (2004).

5.5 RP-HPLC-electron spray ionisation (ESI) MS

RP-HPLC-ESI MS has also been applied to analysis zein proteins together with other maize protein classes (López *et al.*, 2009). The principles of RP-HPLC and ESI have previously been described (4.3 and 4.5.2). An ion-trap mass spectrometer was used in this study. ACN (45%) containing 0.5% (v/v) 2-ME and 0.5% (m/v) ammonium acetate was used for extracting maize proteins. Two columns, a perfusion column (100 mm×2.1 mm ID and 10 µm particle size) and a C18 column (75 mm×1 mm ID), were used. No conclusive assignments of zein classes to peaks were made and thus this method should be optimised.

6. Conclusion

Maize endosperm texture is an important characteristic in the industry as it influences yield, harvest, storage and milling properties. Zein has been linked to endosperm texture. Zein consists of a complex group of proteins, each performing an important role in the formation of the final PB. It is thus important to characterise and quantify these proteins to be able to establish the impact they have on kernel texture. Conventionally SDS-PAGE has been used to characterise zein but mass spectrometry techniques have been used to improve characterisation of these proteins in terms of resolution and mass accuracy. Therefore, it should be used to investigate zein profiles of maize varieties.

7. References

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Chapter 3

Characterisation of zein with matrix-assisted laser
desorption/ionisation time-of-flight mass spectrometry
(MALDI-TOF MS): method optimisation

Chapter 3

Characterisation of zein with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS): method optimisation

Abstract

An optimised zein procedure for matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analyses of zein was established by investigating extraction and matrix preparation conditions. The matrix, 2-(4-hydroxyphenylazo)benzoic acid (HABA), was used at near saturated concentration in all matrix preparation procedures. Acetonitrile (ACN) was used as a matrix solvent at concentrations of 70% and 50% (v/v). Three pH levels (<1.5, 1.7 and 2.9) and two concentrations (6.0 and 7.5 mg/mL) of an additional more water soluble matrix, in combination with HABA, α -cyano-4-hydroxy-cinammic acid (CHCA) were also investigated. Parallel to this investigation, a simplified extraction procedure for zein was attempted. Evaluation of the extraction procedure from maize meal included two extraction temperatures (ambient temperature vs. 60°C), extraction from non-defatted (NDF) vs. defatted (DF) meal and meal with different particle size distributions (milled through 1 and 0.5 mm sieves). Particle size of maize meal did not influence the quality of the spectra and using the 1mm size sieve was suitable for zein extraction. Zein extracted at 60°C from NDF maize meal, produced spectra with lower signal-to-noise (S/N) ratios than zein extracted from DF meal. The 27 kDa γ -zein, using the HABA matrix at pH <1.5, was only present when extracting zein from DF maize meal at 60°C. Optimisation of the matrix preparation procedure was based on the behaviour of the more water soluble 27 kDa γ -zein with respect to the two matrices (HABA and CHCA) and the pH of the matrix solution. Good S/N ratios were obtained for all zein classes, extracted from NDF maize meal at ambient temperature, using both HABA and CHCA (6.0 mg/mL) dissolved in 70% ACN at pH 2.9. Zein profiles of ten hybrids and their respective parents were evaluated using the optimised procedure. Hybrid profiles were a combination of both parent profiles, demonstrating this method can be used to establish the impact of breeding on zein expression.

Introduction

The prolamins (alcohol soluble proteins) of maize are known as zein. The name prolamins was derived due to the high content (30-70%) of **proline** and **glutamine** within these plant proteins. Zein appeared to have evolved by the amplification of small hydrophobic proline and glutamine rich peptides (Herman & Larkins, 1999). Zein is the major storage protein in maize and is

located in protein bodies in the starchy endosperm. Its insolubility in water and salt solutions is an important attribute for its classification and successful function as the storage protein (Shewry & Tatham, 1990). Zein comprises a family of proteins which differ in solubility characteristics, pI values and molecular weights. It can constitute up to 70% of total protein in maize. Zein is divided into four main classes namely α -, β -, γ - (Esen, 1987) and δ -zein (Prat *et al.*, 1987; Kirihaara *et al.*, 1988). α -Zein comprises two proteins with molecular weights of 19000 Da and 22000 Da α -zeins. γ -Zein comprises three proteins with molecular weights of 16000 Da, 27000 Da and 50000 Da and δ -zein two; 10000 Da and 18000 Da (Woo *et al.*, 2001). The molecular weights assigned to the various classes were based on results obtained from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In literature zeins' names are given based on these molecular weights e.g. 27 kDa γ -zein (Adams *et al.*, 2004; Huang *et al.*, 2004; Erny *et al.*, 2007)

Characterisation of cereal proteins is important due to their role in food processing (Shewry & Tatham, 1990), for cereal variety identification (Dworschak *et al.*, 1998) and for breeding purposes. SDS-PAGE has conventionally been used for characterisation zein proteins (Esen, 1986; Esen, 1987; Shull *et al.*, 1991b; Shewry & Halford, 2002). However, the use of SDS-PAGE is limited by resolution, which inhibits the differentiation of proteins with similar masses, and mass accuracy. Mass spectrometry techniques are widely used today in proteomics to overcome these problems.

Three mass spectrometry techniques namely matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Wang *et al.*, 2003a; Adams *et al.*, 2004), capillary electrophoresis mass spectrometry (CE-MS) (Erny *et al.*, 2007) and reversed-phase high-performance liquid chromatography–electrospray mass spectrometry (RP-HPLC-ESI-MS) have been used for to analyse zein (García López *et al.*, 2009). In contrast to results obtained from MALDI-TOF MS and CE-MS, zein classes were not assigned using RP-HPLC-ESI-MS.

MALDI-TOF MS is a soft ionisation technique where MALDI (ionisation step) is combined with TOF MS. The ions generated are separated according to their mass/charge ratio (m/z) via the TOF mass analyser. Due to this technique's increased accuracy and resolving power, improved zein characterisation profiles can be obtained compared to SDS-PAGE. This is particularly true for the α -zeins which are a family of proteins with similar molecular weights. A MALDI-TOF MS profile of zein extracted from a maize sample at 60°C, had three peaks corresponding to α -zein, two peaks for 19 kDa α -zein and one for 22 kDa α -zein (Wang *et al.*, 2003a). Poor signals for 15 kDa β - and 10 kDa δ -zeins were also present. SDS-PAGE indicated bands corresponding to 19 kDa and 22 kDa α -, 15 kDa β - and 10 kDa δ -zeins. The protocol for zein extraction in this study was rather time consuming (2-3 days) due to precipitating the zein prior to analysis. Although only one additional class (belonging to the 19 kDa α -zeins family) was observed, the molecular weights were more accurate than using SDS-PAGE. A simplified, more efficient extraction procedure generated a more complete profile of zein (Adams *et al.*

2004). In this procedure, zein was extracted from multiple samples (milled to a fine powder of maize meal); a reducing agent was introduced and pH and temperature adjusted. Extraction at 60°C was needed to obtain this more complete profile. It was not possible to obtain a comparable profile using conventional SDS-PAGE. Extraction at room temperature was sufficient to observe all zein classes when using RP-HPLC (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994). It is, thus, unclear if a higher extraction temperature is needed and should be investigated. This zein extraction and matrix procedure (Adams *et al.*, 2004) was applied in a study where zein from high lysine transgenic lines were analysed (Huang *et al.*, 2005). However, the maize samples were not defatted before extraction, suggesting defatting was unnecessary. Both studies emphasized the need to extract zein from a fine powder meal and it would be beneficial to investigate if particle size of maize meal influenced zein analysis using MALDI-TOF MS.

In this study, the MALDI-TOF MS procedure (matrix preparation and extraction of zein) was optimised and simplified by investigating:

- extraction temperature (ambient vs, 60°C)
- influence of particle size of maize meal (1 mm vs 0.5 mm sieve for milling);
- defatted (DF) vs. non-defatted (NDF) maize meal;
- different matrices, i.e. 2-(4-hydroxyphenylazo)benzoic acid (HABA) and α -cyano-4-hydroxy-cinammic acid (CHCA) and
- different matrix solvent concentrations and pH levels.

Finally, zein profiles of ten maize hybrids (grown at three localities) and their respective inbred parents were evaluated using the optimised extraction and matrix preparation procedures. The prolamins of two unrelated sorghum hybrids were also analysed.

Materials and Methods

Samples

Maize samples comprising three field replicates of 10 white maize hybrids (H1-10) grown at three localities [A (Greytown), B (Klerksdorp), C (Delmas)] and their respective inbred parent lines (P1-13) were used in this study. Sample numbers were assigned by referring to the hybrid, locality and replicate, e.g. H4C1 would be the first replicate of hybrid 4, obtained at locality C. Four maize samples (H4B2, H4C1, H9B2 and P7) were used during optimisation of the MALDI-TOF MS procedure (zein extraction and matrix preparation). The optimised procedure was subsequently used to analyse the remaining hybrids and their respective parent lines. Two unrelated sorghum hybrids were also evaluated. All the samples were supplied by PANNAR (Greytown, South Africa).

Sample preparation

Maize samples were milled using a hammer mill fitted with 1 mm sieve (Scientific, Cape Town South Africa). Subsamples of equal portions were milled using a Cyclone mill; fitted with a 0.5 mm sieve (Foss Tecater, Höganäs, Sweden). Subsequently portions of maize meal were milled four times using the Cyclone mill to obtain a fine powder of maize meal. Zein was extracted from maize meal samples before (non-defatted; NDF) and after being defatted (DF). The maize meal samples were defatted with two hexane (HPLC grade, Sigma-Aldrich, Johannesburg, South Africa) washes (100 mg/mL per wash), centrifuged between each wash and air dried over night.

Zein extraction

Zein was extracted in duplicate from 40 mg DF and NDF maize meal samples with a 1 mL 70% (v/v) aqueous ethanol solution containing, 10 mM dithiothreitol (DTT) and 25 mM ammonium hydroxide (NH₄OH). DTT was added immediately prior to extraction. An hour was allowed for extraction at ambient temperature and 60°C. At ambient temperature, zein was extracted with continuous agitation using a vortex; at 60°C samples were shaken every 10 min. Samples were centrifuged at 6000 g for 10 min and the supernatants kept for analysis.

Preparation of internal standard

The calibration standard was prepared by adding 5 mg of bovine erythrocyte carbonic anhydrase (Sigma-Aldrich, Johannesburg, South Africa) to 1 mL of water containing 0.03% (v/v) trifluoroacetic acid (TFA) to obtain a 200 µM concentration.

Matrix preparation

Three matrix preparation (MP) procedures were investigated using DF and NDF maize meal extracted at both ambient temperature and 60°C. All chemicals were supplied by Sigma-Aldrich, Johannesburg, South Africa

Procedure 1 (MP1)

A single matrix, 10.7 mg 2-(4-hydroxyphenylazo)benzoic acid (HABA), was dissolved in 1 mL 70% (v/v) acetonitrile (ACN) containing 0.03% (v/v) trifluoroacetic acid (TFA) (adapted from Adams *et al.*, 2004; Huang *et al.*, 2005). A 10 µL aliquot of the internal standard solution was added to matrix solution and briefly mixed using a vortex.

Procedure 2 (MP2)

HABA alone (10.7 mg/mL), and in combination with α-cyano-4-hydroxy-cinnamic acid (CHCA) (7.5 mg/mL HABA and 6.0 mg/mL), was dissolved in 50% (v/v) ACN containing 0.01% (v/v) formic acid (FA).

Procedure 3 (MP3)

HABA alone (10.7 mg/mL), and in combination with CHCA (6.0 mg/mL), was dissolved in 70% (v/v) ACN containing 0.01% FA or 0.01% TFA.

The pH of MP2 and 3 matrix solutions (made up to 10 mL) were measured using a pH meter (HANNA instruments, Cape Town, South Africa).

Analyte preparation

The analyte (zein dissolved in supernatant) obtained from the extraction was diluted ten-fold with the matrix solution and briefly mixed on a vortex. A dried-droplet (DD) sample-matrix crystallisation method was used; a 0.5 μL aliquot of analyte/matrix solution was applied to a Voyager-100 well sample plate with a hydrophobic surface and left to dry in a fume cabinet. Samples were applied and analysed in duplicate.

MALDI-TOF MS conditions

MALDI-TOF MS analyses were performed using a Voyager-DE STR (Applied Biosystems, Inc., Foster City, CA). The instrument was operated in positive ion linear mode with a delayed extraction of 1050 ns. Ions were generated by a pulsed UV laser (nitrogen laser, 337 nm) and accelerated with 25 kV. Spectra were obtained from 100-200 shots per random selection of five positions on a sample-matrix spot and averaged for further data analyses.

Data analysis

Data Explorer v.5.0 software was used for data analyses. Peaks were smoothed using a mass peak resolution of 0.7 and baseline correction was applied to the spectra.

Results and Discussion

Matrix preparation procedure 1

A typical MALDI-TOF MS spectrum of zein is depicted in Fig. 3.1. All the major zein classes were observed as singly charged $(M+H)^+$ species along with less abundant doubly charged $(M+2H)^{2+}$ species. Signal due to a dimer (commonly formed during the ionisation process) of low abundance, was also observed. Spectra of zein from samples H4B2, H9B2 and P7 are depicted in Fig. 3.2. These samples were used during the optimisation of zein extraction and matrix preparation procedures. Zein classes were assigned to peaks according to molecular weights that have been calculated (Woo *et al.*, 2001) and peaks that were previously positively identified using iodoacetamide (IAA) derivatisation (Adams *et al.*, 2004). Only samples H4B2 and P7 had a peak corresponding to 27 kDa γ -zein (X) whereas hybrids H4C1 and H9B2 had peaks corresponding to unknown molecular weights (Y and Z). The 27 kDa γ -zein only appeared with the addition of NH_4OH to the extraction solution. It was added to increase the pH to level (pH 10) required to extract this class. In RP-HPLC analysis of zein, sodium acetate was used to increase the pH (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994) but if used for MALDI-TOF MS analyses, sodium cations will be produced during ionisation. These cations will form adducts with proteins resulting in less accurate molecular weights. The NH_4OH

is a volatile organic amine and when used at concentrations <50 mM, ammonia adducts are likely to decay into ammonia gas (Adams *et al.*, 2004).

A number of observations were made when comparing spectra of zein extracted from NDF and DF maize meal at both temperatures. Firstly, the signal-to-noise (S/N) ratio of zein extracted at 60°C from DF samples was higher (Fig. 3.1 & 3.2), compared to spectra of zein extracted from NDF samples (Fig. 3.3). Although a good S/N ratio was occasionally observed with zein extracted at 60°C from NDF maize meal (data not shown), it appeared a more reproducible optimal S/N ratio was observed with zein extracted from DF meal. Zein extracted from NDF samples at ambient temperature (Fig. 3.4) gave an overall better S/N ratio compared to extraction at 60°C (Fig. 3.3). When using 70% ethanol to extract zein in the presence of fat, some fatty acids were also extracted (Wang *et al.*, 2003b). It is possible at elevated extraction temperatures, enzyme activity was triggered and fat was hydrolyzed into simpler units which were more extractable. This could inhibit proper analyte/matrix co-precipitation and/or fat slightly inhibited desorption or ionisation due to interaction with the hydrophobic zeins.

Secondly, the 27 kDa γ -zein peak was absent or weak in the spectra of zein extracted from NDF maize meal at 60°C. The 27 kDa γ -zein has been referred to as the reduced soluble protein (Wilson *et al.*, 1981) due to its solubility in water when reduced, and is thus the least lipophilic protein. It was possible, when extracting zein from NDF maize meal, the more hydrophobic zeins and/or matrix interacted with the extracted fat and the 27 kDa γ -zein did not co-precipitate sufficiently with the matrix.

Thirdly, when extracted at ambient temperature the 27 kDa γ -zein was absent or had a weak signal when NDF maize meal was used (Fig. 3.4). This was also true when using DF maize meal (data not shown). The β -, γ - and δ -zeins all contain a higher amount of cysteine residues relative to α -zein; cysteine residues are responsible for disulfide bonding within the protein body. The 27 kDa γ -zein protein contained the highest amount and thus a higher extraction temperature (at least 60°C) is necessary to increase the extractability of this class (Adams *et al.*, 2004).

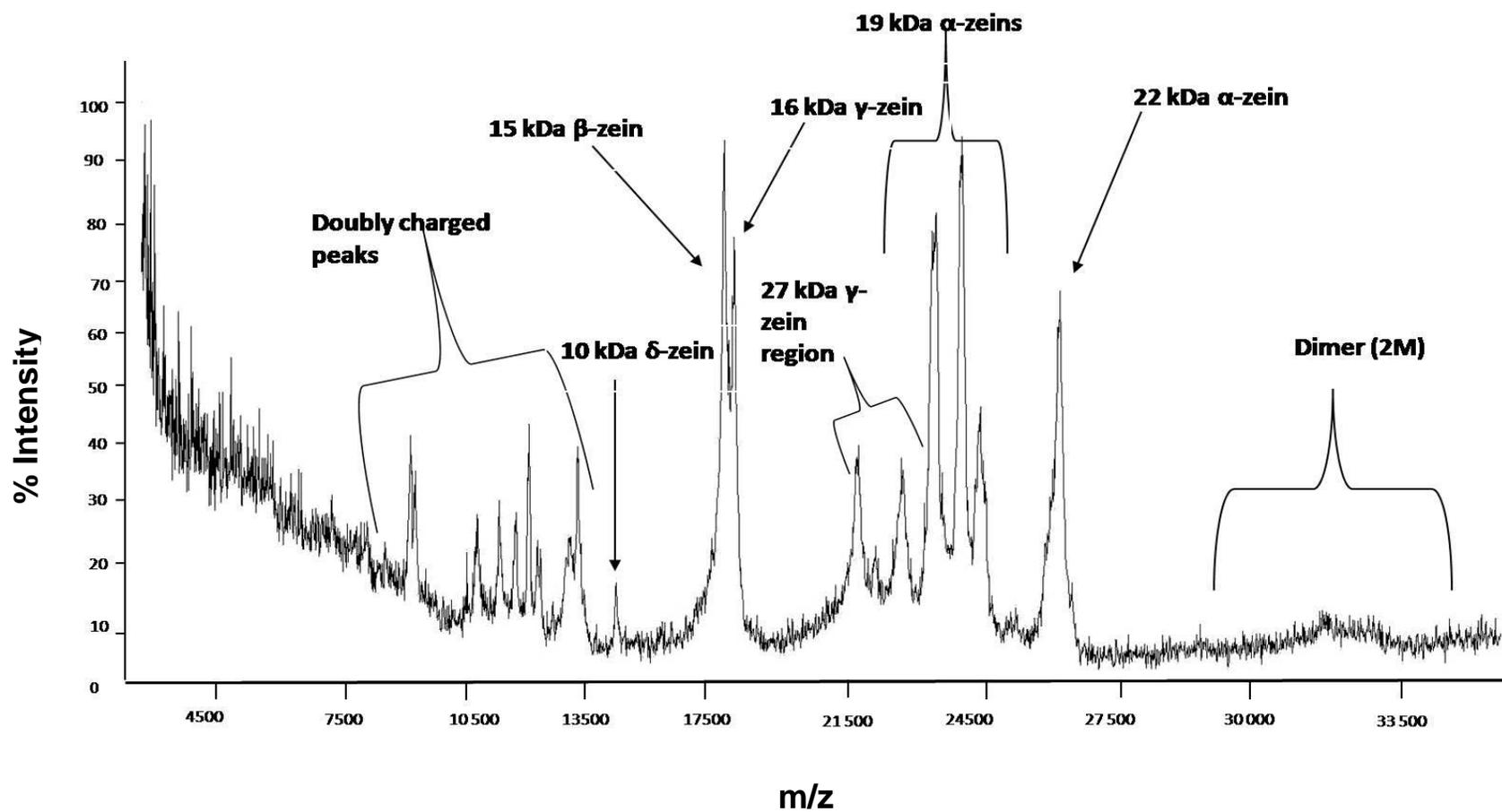


Figure 3.1 MALDI-TOF MS spectrum of zein, extracted from DF sample H4C1 at 60°C, obtained using MP1.

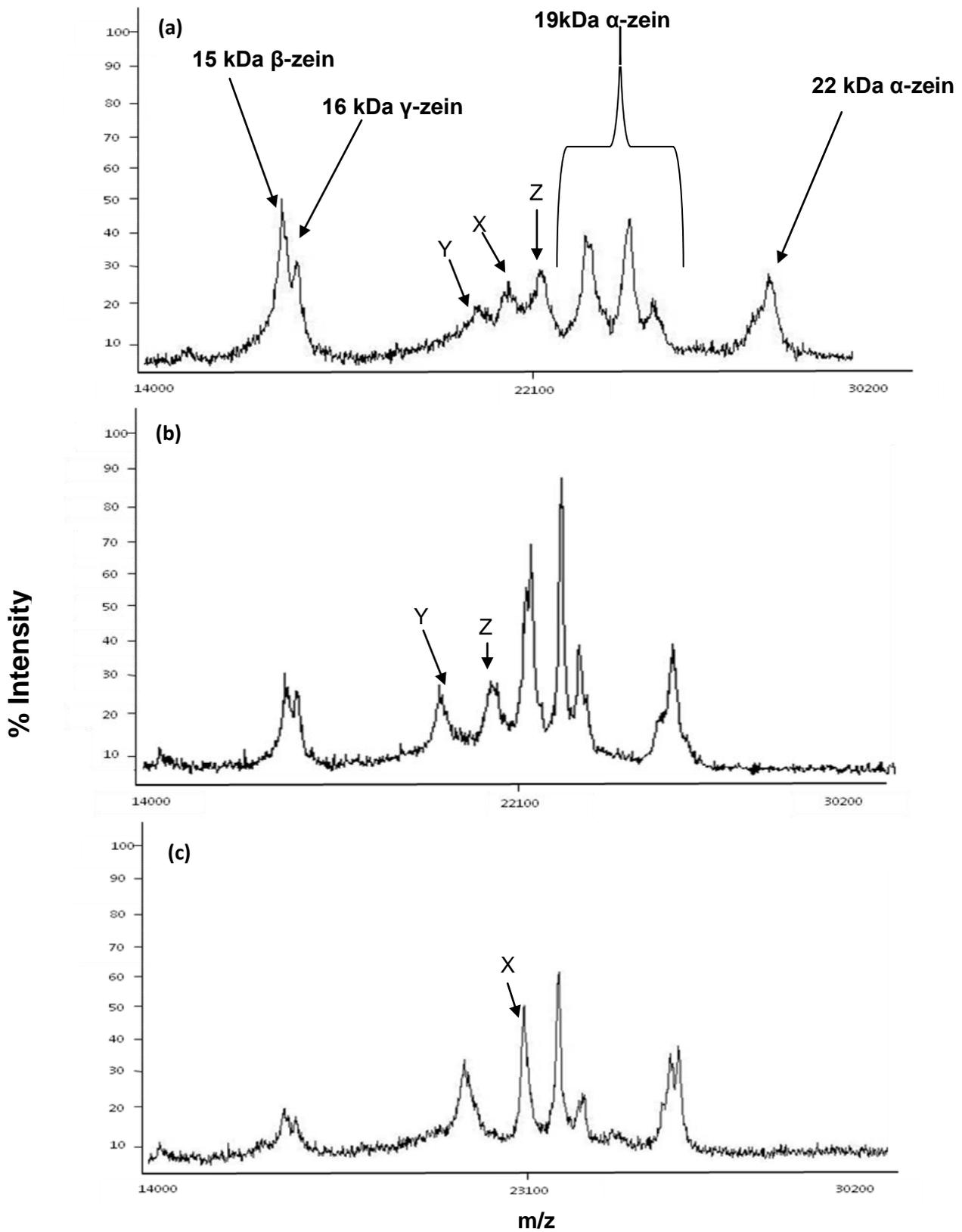


Figure 3.2 MALDI-TOF MS spectra, obtained using MP1, of zein extracted at 60°C from DF maize meal of samples (a) H4B2, (b) H9B2 and (c) P7 (X=27 kDa γ -zein, Y~21200 Da and Z~22400 Da).

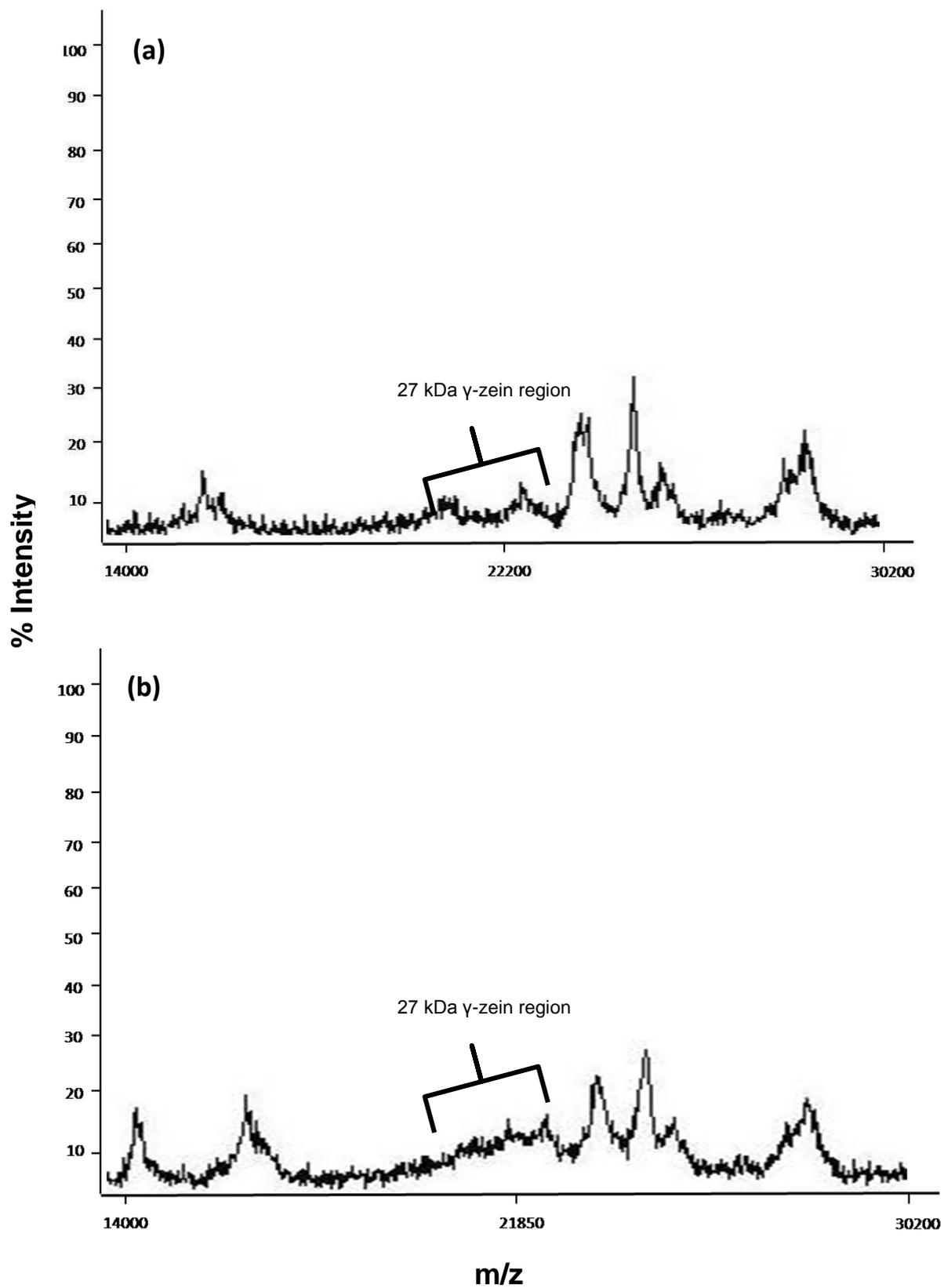


Figure 3.3 MALDI-TOF MS spectra of zein extracted from NDF maize meal at 60°C of (a) sample H4C1 and (b) sample H4B2 where a lower S/N ratio is observed and poor signal for 27 kDa γ -zein.

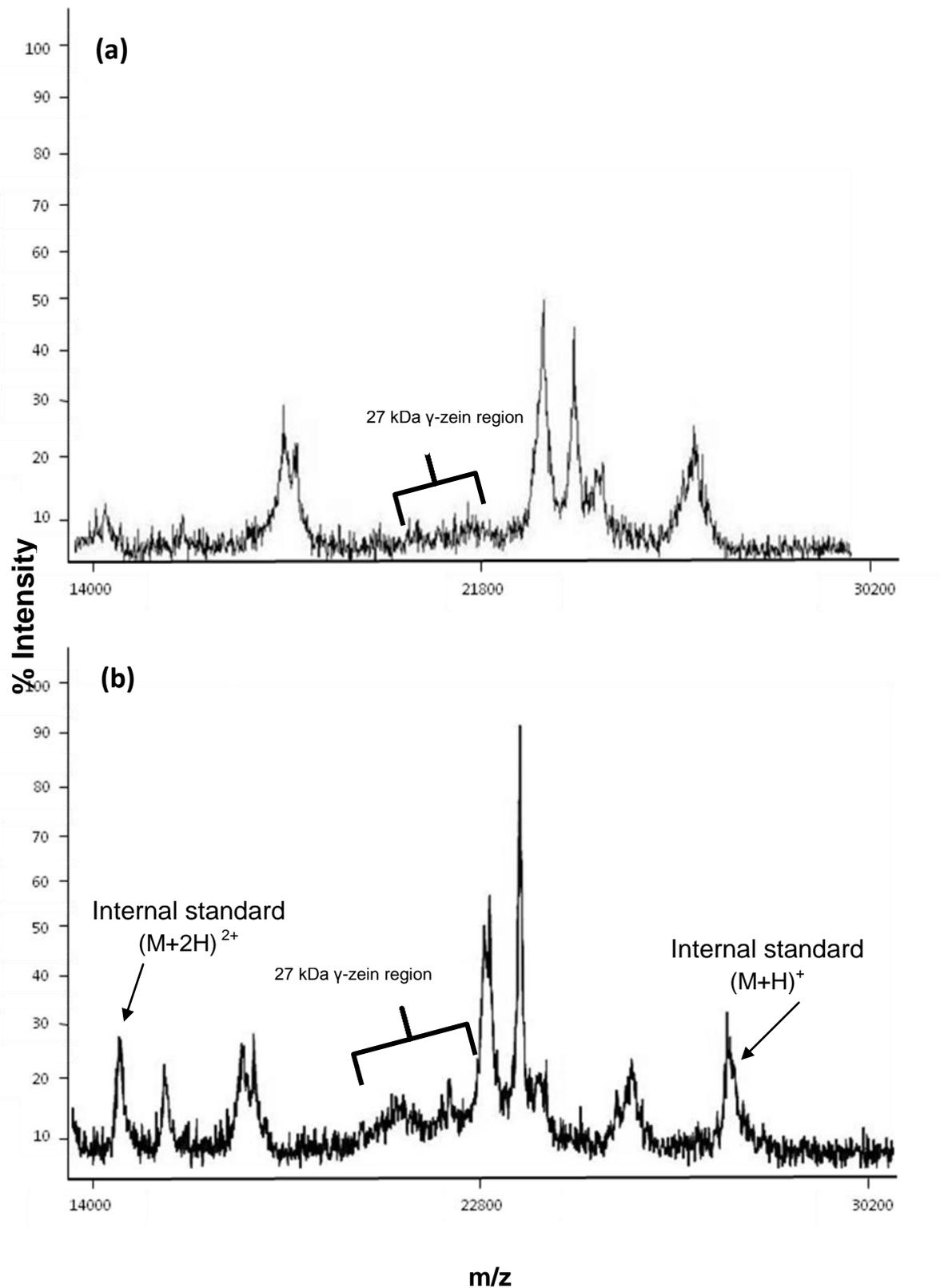


Figure 3.4 MALDI-TOF MS spectra of zein extracted from NDF maize meal of sample H9B2 at ambient temperature where (a) the 27 kDa γ -zein region was absent and (b) a weak signal for this region was present.

Comparisons of zein profiles using MP1

The 10 kDa δ -zein was absent in spectra where the internal standard had been added to the matrix solution. An internal standard was used in MALDI-TOF MS analyses to calibrate molecular weights. After calibration, the molecular weights did not shift more than ~20-40 Da. Thus, the calibration did not influence the mass accuracy as much. Ideally, a standard would have a singly charged ion $[M+H]^+$ above, and a doubly charged ion $[M+2H]^{2+}$ below, the mass range of the proteins analysed (Adams *et al.*, 2004). Bovine erythrocyte carbonic anhydrase (CA) was used as an internal standard due to the singularly charged and doubly charged peaks being above and below the mass distribution of all the zein classes except the 10 kDa δ -zein. The $[M+H]^+$ of CA is 29024 Da, thus $[M+2H]^{2+}$ is 14515 Da which is only slightly above the mass of the 10 kDa δ -zein (14431 Da). The 10 kDa delta-zein class could therefore not be observed due to obstruction by the internal standard's doubly charged ion (Adams *et al.*, 2004).

Broad peaks arise during MALDI-TOF MS analysis when operating in linear mode. For example, the 19 kDa α -zein peaks consist of a broad base where three peaks split from it (Figs. 3.1 & 3.2). Wang *et al.* (2003) suggested each peak included multiple components and lead to peak broadening due to similarities in molecular weights. Non-linear mode is used to overcome this problem in MALDI-TOF MS. However, the molecular weights of the zeins are too high to apply this mode. α -Zein peaks obtained from the four samples in this study were similar to peaks reported previously (Adams *et al.*, 2004), with the exception of additional 19 kDa and 22 kDa α -zein peaks observed by Adams *et al.* (2004). The molecular weights observed in MALDI-TOF MS spectra of an inbred line (B73) and its calculated molecular weights (obtained from cDNAs sequenced from endosperm cDNA libraries (Woo *et al.*, 2001)), were compared to assess the accuracy of this MS technique (Adams *et al.*, 2004). Molecular weights observed in the current study, without using the internal standard, were compared to the calculated molecular weights of B73 and molecular weights obtained by Adams *et al.* 2004 and Wang *et al.* 2003 (Table 3.1). Larger differences between the observed and calculated molecular weights were recorded for 19 kDa α -zein B1 sub-class in this study. The peaks corresponding to the 19 kDa α -zein B1 sub-class differed up to ~100 Da in this study, whereas peaks observed by Adams *et al.* (2004) and Wang *et al.* (2003) were closer to the calculated molecular weight of this class. An observed-calculated molecular weight difference of ~100-150 Da was obtained for the peak corresponding to the 19 kDa α -zein D2 sub-class in this study. A larger difference (191 Da; 2-3 amino acids) was noted by Adams *et al.* (2004) who positively identified this class using IAA derivatisation.

Variation for the 22 kDa α -zeins was observed for inbred line P7. Two distinct peaks corresponding to the 22 kDa α -zein Z3 or Z5 and Z1 sub-classes were present (Fig. 3.2c). Hybrids H4B2, H4C1 and H9B2 had only one peak corresponding to the Z3 or Z5 sub-class (Figs 3.1 & 3.2 a & b).

Table 3.1 Comparisons of molecular weights observed in MALDI-TOF MS spectra

Zein class	Calculated weight (Da) ^a	Molecular weights observed in MALDI-TOF MS spectra					
		P7	H4B2	H4C1	H9B2	Observed by Adams <i>et al.</i> , (2004)	Observed by Wang <i>et al.</i> , (2003)
10 kDa δ -zein	14431	14440	14449	14450	14447	14432	14466
15 kDa β -zein	17458	17453	17449	17450	17437	17125	17792
16 kDa γ -zein	17663	17652	17650	17649	17687	17714	N.O.
18 kDa δ -zein	21220	N.O.	21160	21116	21133	21220 ^b	N.O.
19 kDa α -zein B1	23359	23258	23262	23253	23245	23318	23362
19 kDa α -zein B2	27128	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
19 kDa α -zein B3	24087	24096	24083	23996	24071	24069	24097
19 kDa α -zein D1	24818	N.O.	N.O.	N.O.	N.O.	24644	N.O.
19 kDa α -zein D2	24706	24561	24565	24670	24567	24515	N.O.
22 kDa α -zein Z1	26359	26406	N.O.	N.O.	N.O.	26318	26838
22 kDa α -zein Z3	26751	26745 ^c	26651 ^c	26696 ^c	26719 ^c	26741	N.O.
22 kDa α -zein Z4	26923	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
22 kDa α -zein Z5	26701	26745 ^c	26651 ^c	26696 ^c	26719 ^c	N.O.	N.O.
27 kDa γ -zein	21822	21824	21798	N.O.	N.O.	21793	N.O.
50 kDa γ -zein	32882	N.O.	N.O.	N.O.	N.O.	N.O.	N.O.
Unknown	No data	N.O.	22401	22382	22374	N.O.	N.O.

^aMolecular weights based on data from Woo *et al.* (2001). ^bNot identified by IAA derivatisation Adams *et al.* (2004). ^cDifficult to assign classes due to small differences in molecular weights between sub-classes Z3 and Z5. N.O.= Not observed.

It was difficult to distinguish between the Z3 and Z5 classes due to a small difference, 50 Da, in calculated molecular weight. A peak with a molecular weight of 26741 Da in this region was positively identified with IAA derivatisation as the Z3 class (Adams *et al.*, 2004).

The minor zeins, 15 kDa β -, 16 kDa γ - and 10 kDa δ -zein, were observed in all samples. Peak broadening was observed for the 15 kDa β - and 16 kDa γ -zein species, which can be attributed to the two proteins having similar molecular weights (Fig. 3.1). The signal for the peak corresponding to the 10 kDa δ -zein was much weaker compared to the other classes. This was also observed in spectra obtained by Adams *et al.* (2004). This class was the least abundant zein and was expected to form fewer ions compared to the other zein classes. In addition, this zein class may not co-precipitate well with the matrix. Samples P7 and H4B2 were the only samples with peaks corresponding to the molecular weight of 27 kDa γ -zein. Adams *et al.* (2004) identified this peak positively with IAA derivatisation. Samples H4B2, H9B2 and H4C1 had two additional zein peaks in the 21000 – 22500 Da region, one having a molecular weight of ~21220 Da (Y) and the other ~22400 Da (Z). The molecular weight of Y corresponded to the 18 kDa δ -zein. This peak (Y), although having a much poorer intensity, was observed by Adams *et al.* (2004) but not identified with IAA derivatisation. These peaks could initially not be assigned. The identities of these zein classes are discussed in results of matrix preparation procedure 2.

Matrix preparation procedure 2 (MP2)

The intensity of the 27 kDa zein (extracted from DF maize meal) varied more at various positions within a spot than other zein classes (data not shown). Adams *et al.* (2004) noted the peak height of this class to be highly variable depending on extraction and matrix conditions. The relative abundance of zeins from B73 was determined by measuring the peak areas from the spectra obtained (Adams *et al.*, 2004). These areas were compared to the percentage expression of their cDNAs sequenced from endosperm cDNA libraries (Woo *et al.*, 2001). Peaks with a larger area corresponded to zeins that were highly expressed. However, the 27 kDa γ -zein's peak area was too high compared to its expression. For a protein to be detected, it must co-precipitate with the matrix so it can undergo desorption with the matrix after excitation with the laser. For this, a near-saturated solution of HABA was necessary for all zeins to be observed (Adams *et al.*, 2004). At this matrix concentration, the α -zeins will precipitate first, followed by the less lipophilic β and γ -zeins, as the solvent (ACN) evaporates. The 27 kDa γ -zein which has the highest solubility in water, will co-precipitate last. This may explain the peak intensity variability observed and it was important to keep solvent evaporating conditions constant. The addition of a more water soluble matrix can also improve the signal of the 27 kDa γ -zein (Adams *et al.*, 2004).

Two concentrations of a more water soluble matrix (CHCA) were tested in MP2. The higher concentration of this matrix (7.5 mg/mL) generated intense 27 kDa γ -zein peaks, indicating this

class co-precipitated well with the CHCA matrix (Fig. 3.5a). The lower CHCA concentration (6.0 mg/mL) resulted in a more consistent intensity of the 27 kDa γ -zein peak (Fig. 3.5b). The concentration of ACN was lowered to 50% (v/v) due to the less hydrophobic character of the CHCA matrix. HABA was used at the same concentration as in MP1 to ensure the other zein classes co-precipitated.

When excluding the CHCA matrix, the 27 kDa γ -zein peak was almost completely absent in the spectra and more intense signals were observed for the other zeins, especially in the α -zein region (Fig. 3.6). This was true for zein extracted from NDF and DF maize meal at ambient temperature. The 27 kDa γ -zein peaks, using MP2, only had appreciable intensity in spectra when the CHCA matrix was used (Fig. 3.7). The signals of the other classes decreased when adding the CHCA matrix. This reduction could be due the matrix co-precipitating better with the 27 kDa γ -zein than the other classes. When using the MP1, it was originally thought the 27 kDa γ -zein was absent due to inefficient extraction at ambient temperature. Considering the extraction conditions at ambient temperature, it was likely a small amount of this zein was extracted but insufficient to co-precipitate efficiently with the HABA matrix. As described earlier, the 27 kDa γ -zein co-precipitated well with the CHCA matrix and the amount extracted at ambient temperature was adequate to produce a good signal. Similar to spectra obtained using MP1, the signals of zein extracted at 60°C from NDF maize was weaker than zein extracted from DF maize meal (data not shown). The unknown peaks, ~21220 Da (Z) and ~22400 Da (Y), behaved in a similar manner as the 27 kDa γ -zein peak. These peaks were originally thought to be post translational modifications of the 27 kDa γ -zein but analysis of the parents and remaining hybrids suggested otherwise and will be discussed later.

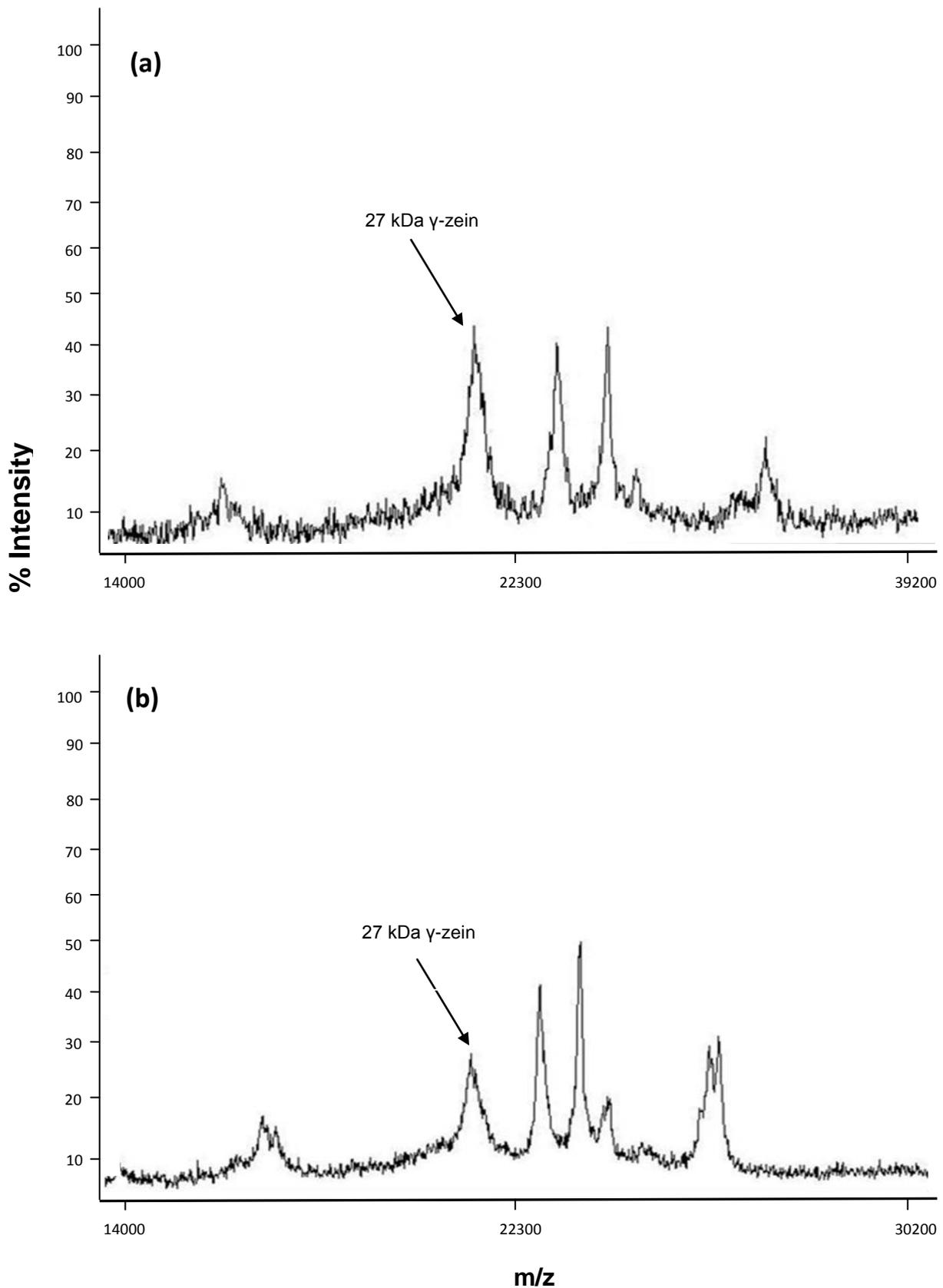


Figure 3.5 MALDI-TOF MS of spectra of zein extracted at ambient temperature from parent line P7 with (a) 7.5 mg CHCA and (b) with 6 mg of CHCA in 50% ACN containing HABA and 0.01% FA. The higher concentration of CHCA generated more intense ion signals for 27 kDa γ -zein compared to the lower concentration.

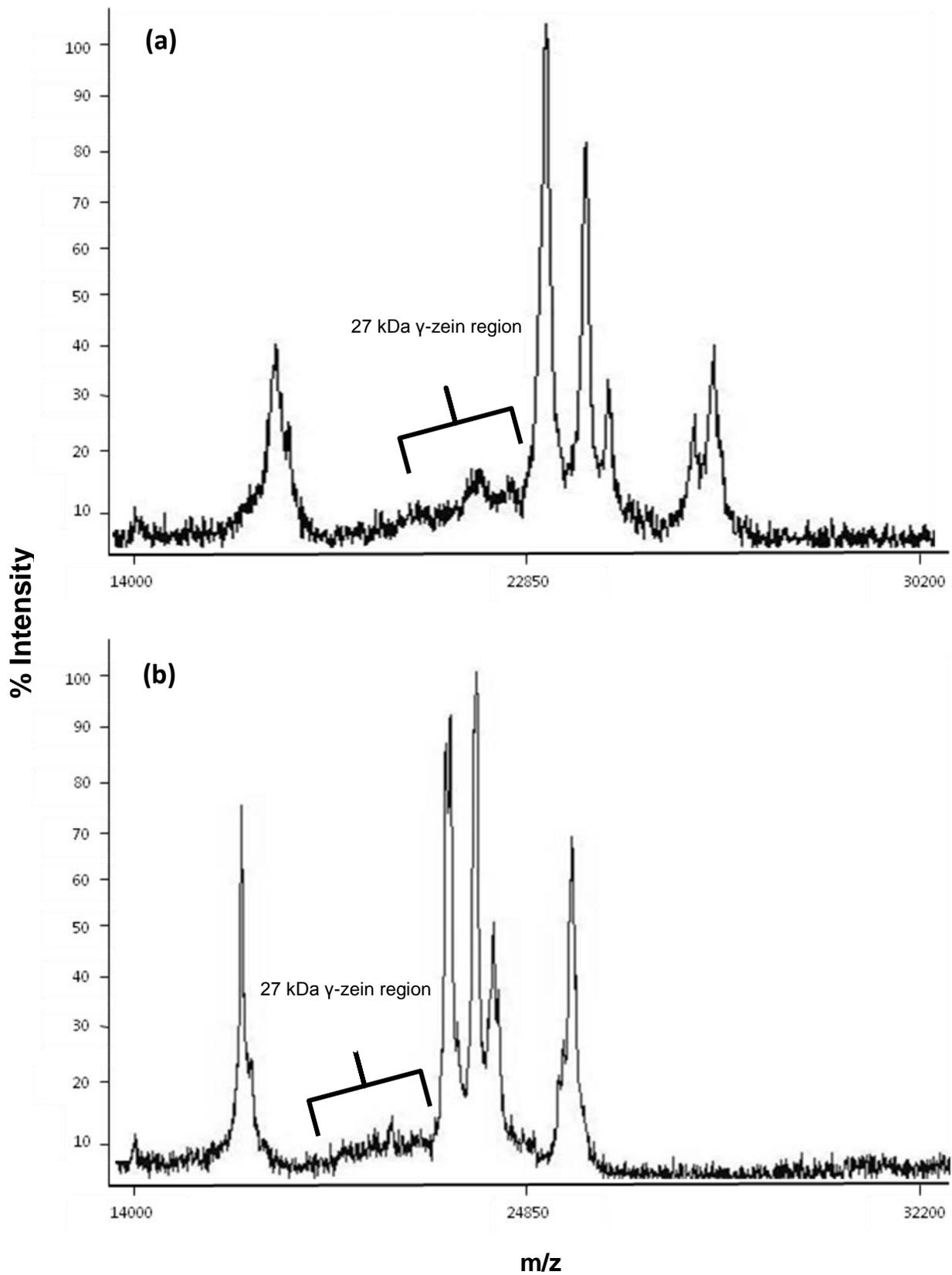


Figure 3.6 MALDI-TOF MS spectra of zein extracted at ambient temperature from samples (a) P7 and (b) H4C1 according to MP2; HABA in 50% ACN with 0.01% FA. The 27 kDa γ -zein region were absent in spectra.

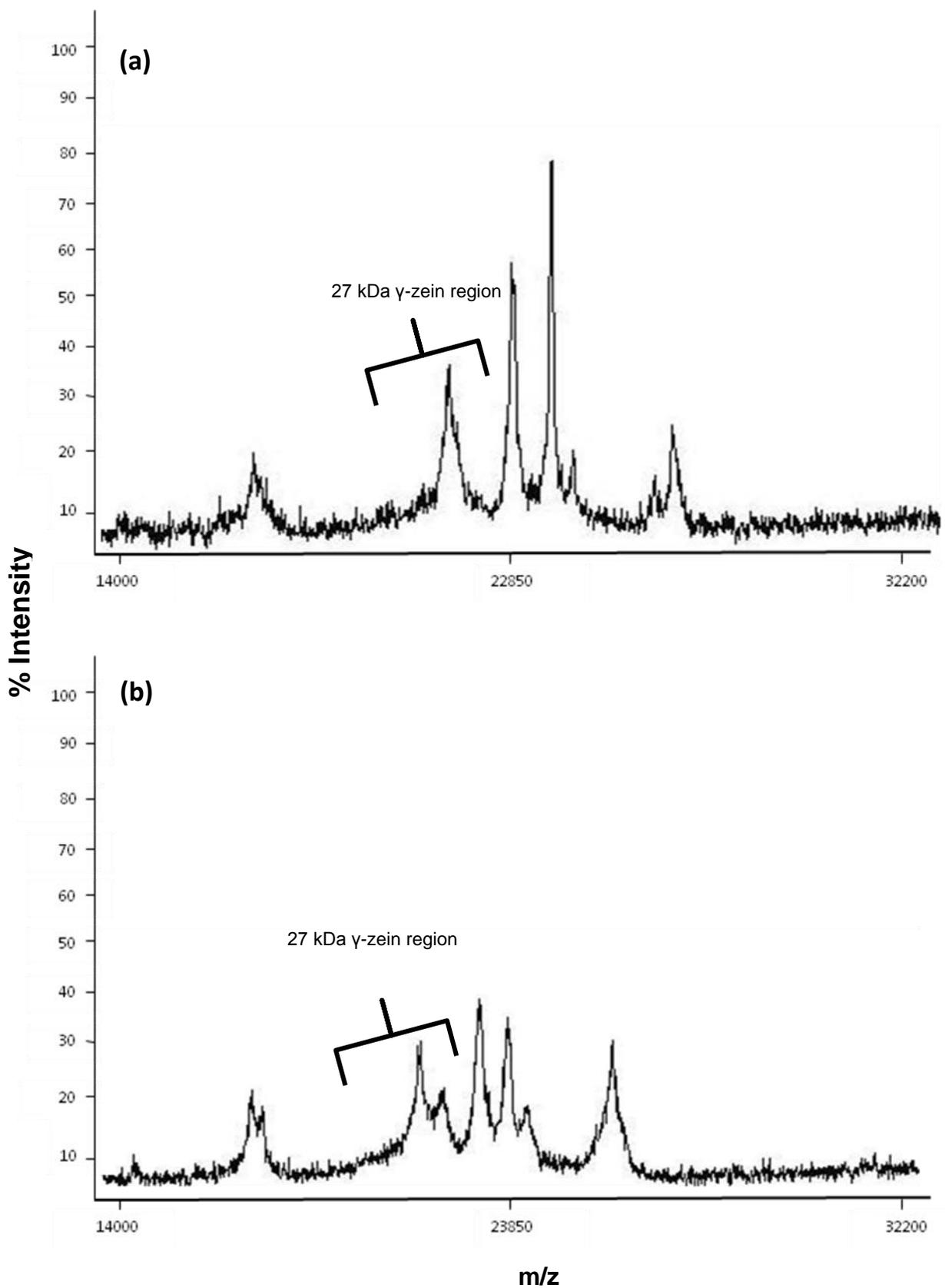


Figure 3.7 MALDI-TOF MS spectra of zein extracted at ambient temperature from samples (a) P7 and (b) H4C1 according to MP2; HABA and CHCA in 50% ACN with 0.01% FA. Peaks corresponding to 27 kDa γ -zein region were present.

Matrix preparation procedure 3 (MP3)

When only HABA was dissolved in a 70 % ACN solution containing 0.01% FA, similar spectra zein were obtained as in MP2 (Figs. 3.8a&3.9a); intense signals were observed for all zein classes except for the 27 kDa γ -zein which was absent or weak. This indicated it was not the 50% ACN (MP2) influencing the presence of the 27 kDa γ -zein but rather the pH obtained using 0.01% FA. It was initially considered the absence of the 27 kDa γ -zein was due the lower solvent (ACN) concentration used in MP2. This resulted in a longer drying period for the matrix and analyte mixture on the sample plate. The 27 kDa γ -zein perhaps stayed dissolved longer as the solvent (ACN) evaporated and therefore did not co-precipitate adequately with the HABA matrix.

TFA is a stronger acid than FA. The pH of the matrix solutions containing 0.03% TFA (MP1) and 0.01% FA were <1.5 (Adams *et al.*, 2004) and 2.9 respectively. It was possible the very low pH conditions obtained using 0.03% TFA, denatured the other zein protein classes, resulting in weaker signals than using 0.01% FA. In addition, the pH conditions obtained with 0.01% FA were not adequate for 27 kDa γ -zein to be ionised when using only the HABA matrix. By increasing the pH from <1.5 to 1.7, an improved signal for this class was observed in spectra for zein extracted from NDF maize meal at ambient temperature when only using the HABA matrix (data not shown). Relatively good signals were also obtained for the other zein classes.

As seen with MP2, the addition of CHCA to the matrix solution (pH 2.9) included the 27 kDa γ -zein peak in spectra with a reduction in intensities of the other classes (Fig. 3.8b&3.9b). This reduction can be aided, as stated earlier, due to the matrix that co-precipitated better with the 27 kDa γ -zein, compared to the other classes. Overall, HABA and CHCA at pH 2.9 gave the best S/N ratio. The signals of the internal standard added to matrix solutions of MP2 and 3 (with the addition of CHCA) were very weak. This indicated the CHCA matrix was not compatible with the standard and another standard needs to be sourced.

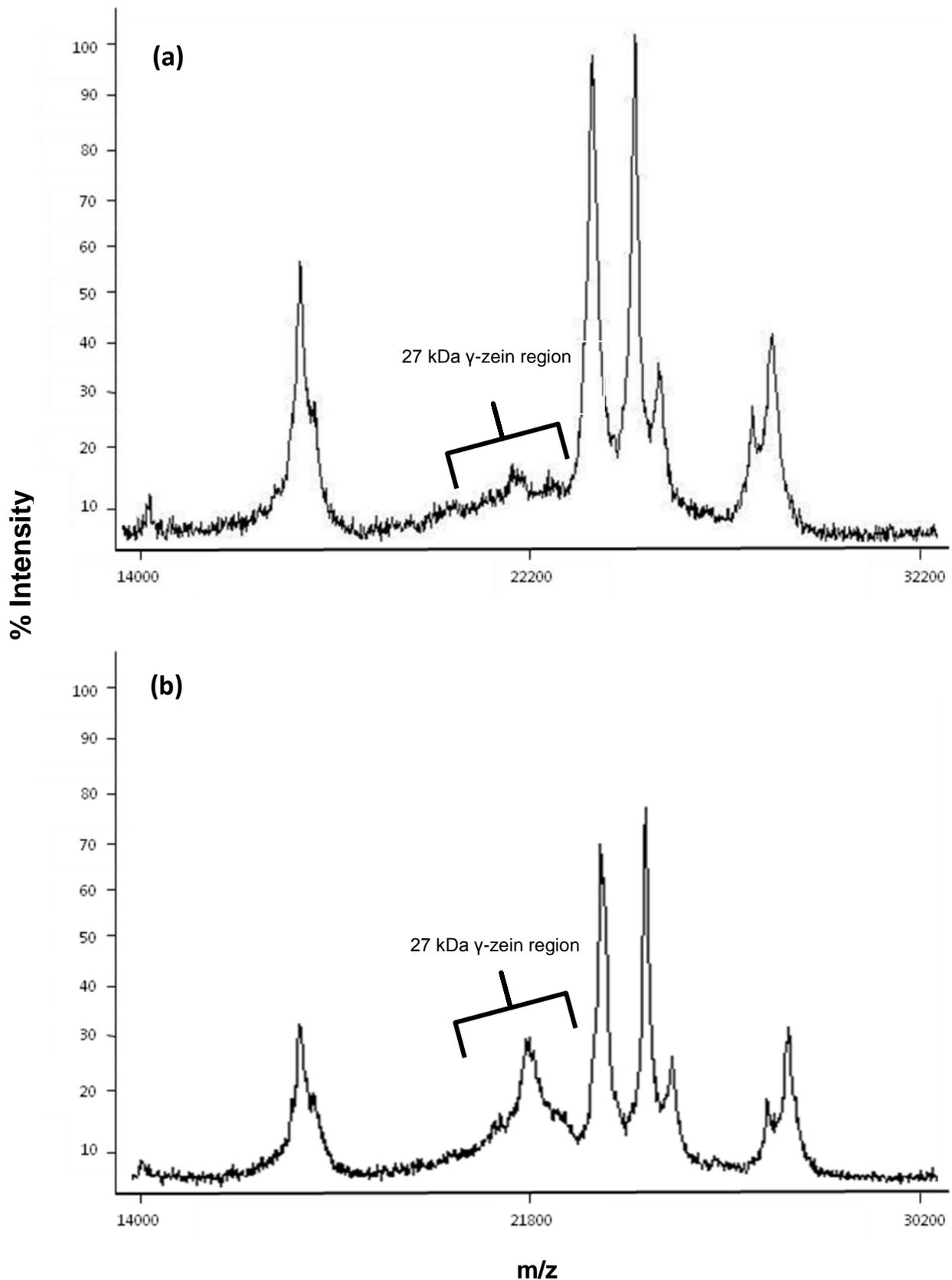


Figure 3.8 MALDI-TOF MS spectra of zein extracted at ambient temperature from a NDF maize meal of patent line P7 according to MP3; (a) HABA in 70% ACN containing 0.01% FA; 27 kDa γ -zein absent (b) HABA and CHCA in 70% ACN containing 0.01% FA; 27 kDa γ -zein present.

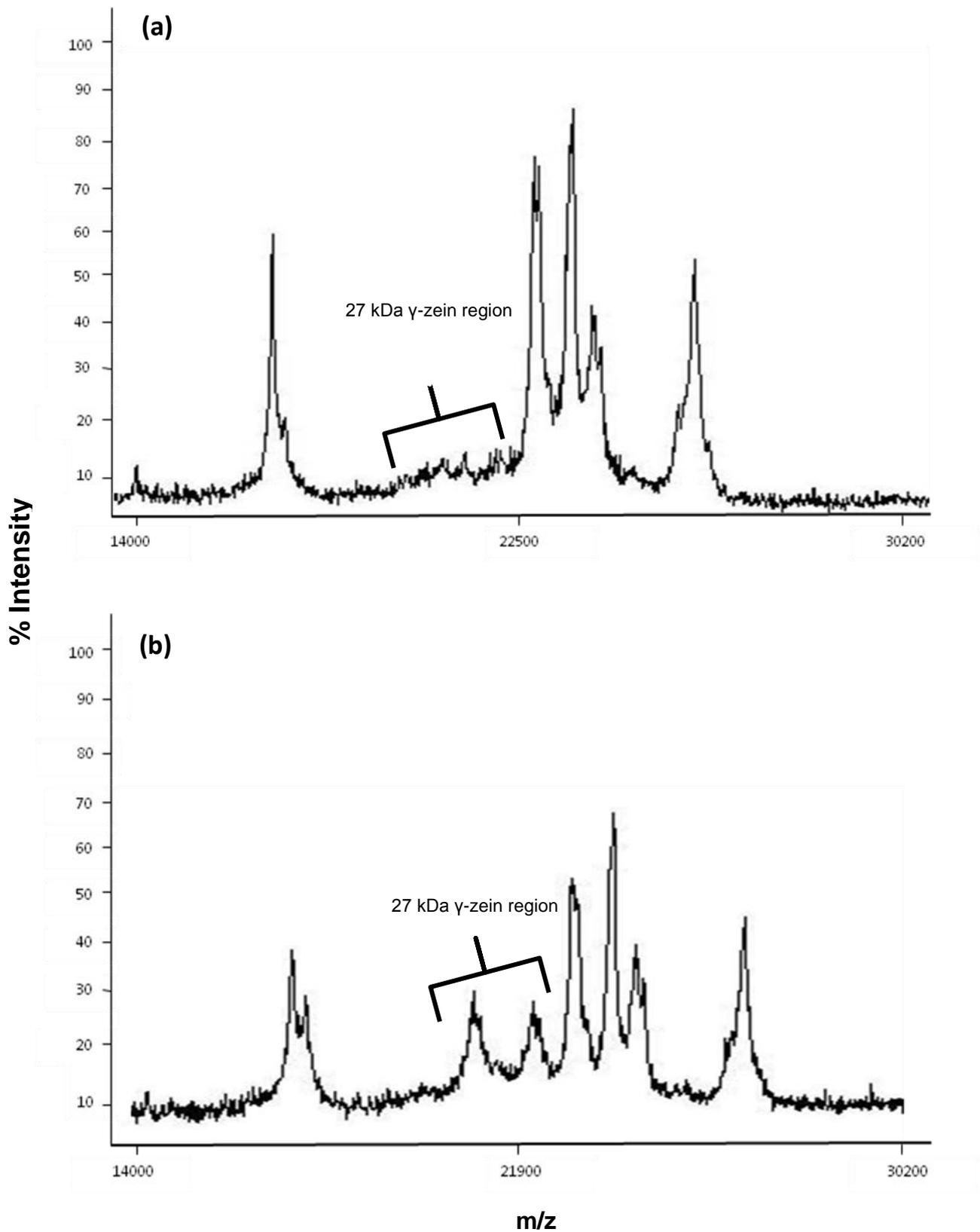


Figure 3.9 MALDI-TOF MS spectra of zein extracted at ambient temperature from a NDF maize meal sample H9B2 according to MP3 (a) 70% ACN with HABA and 0.01% FA; peaks in 27 kDa γ -zein region absent and (b) 70% ACN with CHCA and HABA and 0.01% FA; peaks in 27 kDa γ -zein region present.

Overall reproducibility of zein extraction and matrix preparation procedure

Duplicate extractions and sample plate applications of zein were done to ensure zein profiles did not differ and the extraction protocol and sample/matrix preparation were repeatable. If poor signal was obtained when analysing one spot but good signal was obtained from another spot, this would indicate matrix and analyte conditions (drying and co-precipitating of analyte with matrix) were sub-optimal, and the extraction procedure and sample preparation were not the source of the reproducibility error. All matrix preparations produced similar signal reproducibility, as determined from duplicate extracts and duplicate sample plate spots. Results for matrix preparation procedures 1, 2 and 3 are summarised in Table 3.2.

Comparison of zein extracted from maize meal of different particle size

Peaks of zein classes from maize meal of coarser and fine particle sizes, obtained using a 1 mm and 0.5 mm sieves, provided a good S/N ratio in spectra. No differences were observed between profiles and equal spot to spot reproducibility was obtained. In MALDI-TOF MS, it is important to obtain the optimum range of analyte-to-matrix ratio. A too high or too low ratio would give a poor S/N ratio. The amount of zein extracted from all three maize meal particle sizes were within the range of optimum analyte-to-matrix ratio.

Table 3.2 Comparison of matrix preparation procedures 1, 2 and 3

Matrix preparation procedure	NDF at ambient temperature	NDF at 60°C	DF at ambient temperature	DF at 60°C
1 (0.03% TFA)	S/N for 27 kDa γ -zein poor or absent. Good S/N for other zein classes	S/N for 27 kDa γ -zein poor or absent. Poor S/N for other zein classes	S/N for 27 kDa γ -zein poor or absent. Good S/N for other zein classes	Good S/N for all zein classes
2 and 3 (0.01% FA)				
Without CHCA matrix	27 kDa γ -zein poor or absent. Very good S/N for other zein classes	S/N for 27 kDa γ -zein poor or absent. Poor S/N for other zein classes	S/N for 27 kDa γ -zein poor or absent. Very good S/N for other zein classes	S/N for 27 kDa γ -zein poor or absent. Very good S/N for other zein classes.
With CHCA matrix	Good S/N for all zein classes.	All classes present. Poorer S/N of zein classes	Good S/N for all zein classes.	Good S/N for all zein classes.
3 (0.01% TFA)				
Without CHCA matrix	Good S/N for all zein classes	All classes present. Poorer S/N of zein classes	Good S/N for all zein classes	Good S/N for all zein classes

Zein profiles of remaining hybrids and inbred parent lines

Zein profiles of remaining hybrids and inbred parent lines were analysed using MP3; HABA and CHCA, pH 2.9. Spectra of the three field replicates per hybrids were averaged. Similarities between parent lines and hybrids were observed with respect to the minor zein classes; the 27 kDa γ , 10 kDa δ and 15 kDa β -zein regions (Fig. 3.10-19). A summary of these similarities are given in Table 3.3.

A peak with a molecular weight of ~17150 Da was observed for H1, H2, H7 and H10. Spectral profiles of the parents, P6 and P3, also contained this peak. This peak was also observed by Adams *et al.* (2004) and was identified by IAA derivitisation as the 15 kDa β -zein. In the current study, a peak with a mass (~17450 Da) closer to the calculated mass (17458 Da) was observed and assigned as the 15 kDa β -zein. The 15 kDa β -zein was absent in P3 and P6. Analysis of zeins with RP-HPLC indicated two retention times for β -zein from different inbred lines and it was suggested these two β -zein behave as if their genes were allelic at the same locus (Wilson, 1991). β -Zeins eluted as doublets in chromatograms, which indicated both genes existed for the β -zein in some cultivars (Mestres & Matencio, 1996). The 17150 Da peak was observed in conjunction with the 15 kDa β -zein in these four hybrids (H1, H2, H7 and H10). This also indicates both these proteins were expressed in these hybrids

Spectra of hybrids H4-10 (Fig. 3.14-19) each contained two peaks in the 27 kDa γ -zein region except for hybrid H4B which contained three peaks. Profiles in the 27 kDa γ -zein region of hybrids H5-10 were combinations of their parents. It is possible allelic variation, as suggested for 15 kDa β -zein (Wilson, 1990), also occurred for γ -zein. More detailed genetic studies, which is outside the scope of this study, is needed to confirm this observation.

Hybrid 2 had an additional peak (labeled as B) with a molecular weight of ~15150 Da close to the 10 kDa δ -zein. Spectra of zein from the maternal parent line, P3, had a peak with the same molecular weight. However, the 10 kDa δ -zein was absent. The paternal parent, P2, had the 10 kDa δ -zein. Both proteins were expressed in H2, possibly as a result of allelic variation but this needs to be confirmed.

The 22 kDa α -zeins region was not as easily interpreted when comparing hybrids and parents. This was due to the low signal associated with these less abundant zeins. More peaks (up to three) were observed than from hybrids used for method optimisation. Overall, the zein profiles appeared to be a combination of the parent lines.

MALDI-TOF MS was a useful technique to compare zein profiles from inbred parent lines and their associated hybrids to establish genetic heritability of classes. It was suggested to use this technique for investigation of the molecular genetics of zein protein expression, which is usually difficult to study due to the intricacy of their multigene families (Adams *et al.*, 2004).

Table 3.3 Similarities between zein profiles of inbred parent lines and their associated hybrids.

Hybrid and respective parents lines	27 kDa γ-zein region	15 kDa β- and 10 kDa δ-zein region
H1	X	A
M (P6)	X	A
P (P7)	X	-
H2	X	AB
M (P2)	X	B
P (P3)	X	A
H3	X	-
M (P7)	X	-
P (P13)	X	-
H4A	XZ	-
H4B	XYZ	-
H4C	YZ	-
M(P9)	Y	-
P (P8)	X	-
H5	XZ	-
M (P5)	X	-
P (P10)	Z	-
H6	XZ	-
M (P7)	X	-
P (P10)	Z	-
H7	XZ	A
M (P6)	X	A
P (P10)	Z	-
H8	XZ	-
M (P12)	X	-
P (P10)	Z	-
H9	YZ	-
M (P4)	Z	-
P (P1)	Y	-
H10	XZ	A
M (P11)	Z	-
P (P6)	X	A

M=maternal parent; P=paternal parent; A~17150 Da, B~15150 Da, X=27 kDa γ -zein, Y~21200 Da and Z~22400 Da.

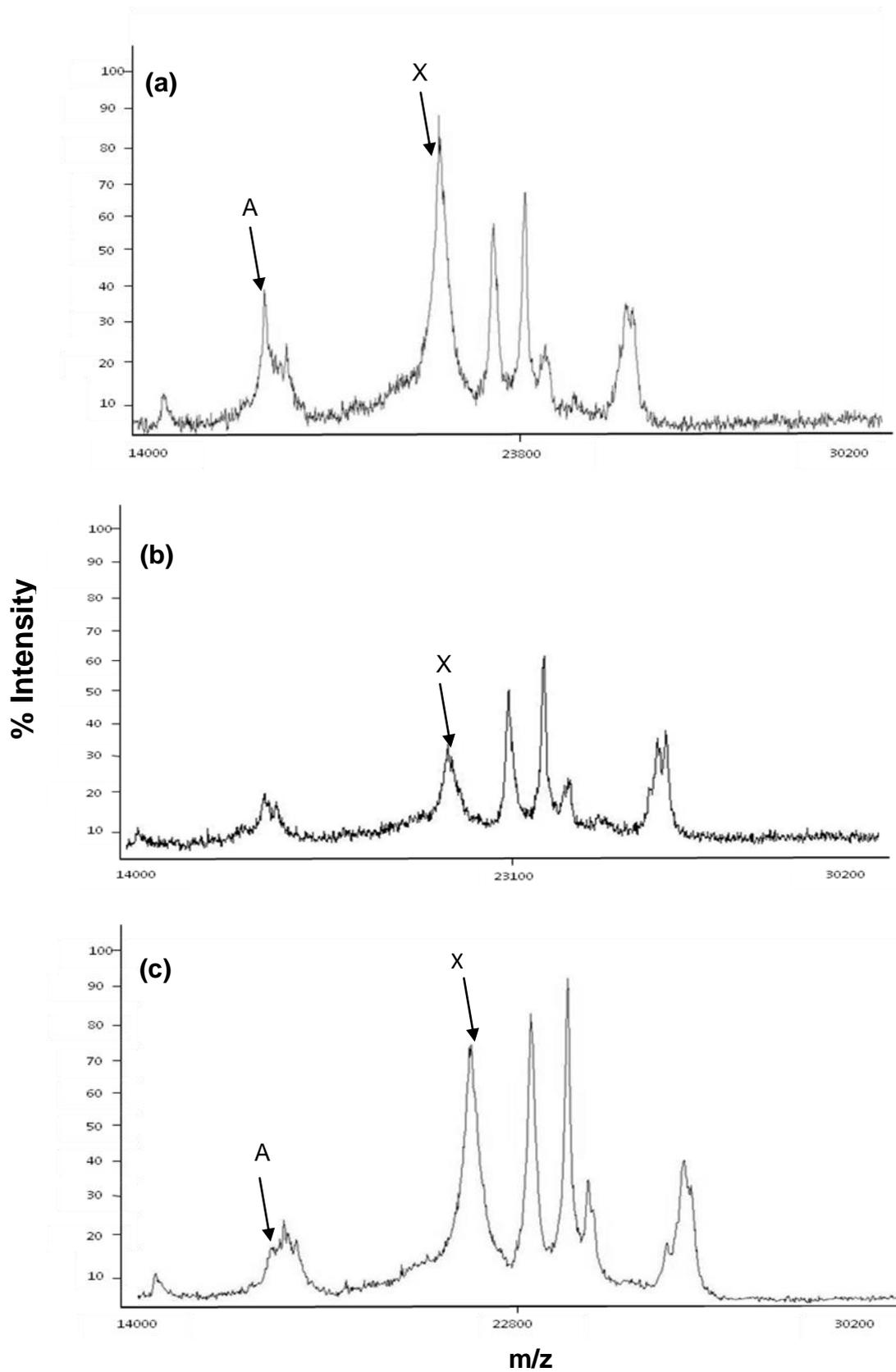


Figure 3.10 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P6, (b) paternal parent line P7 and (c) the associated hybrid H1. (A~17150 Da and X=27 kDa γ -zein)

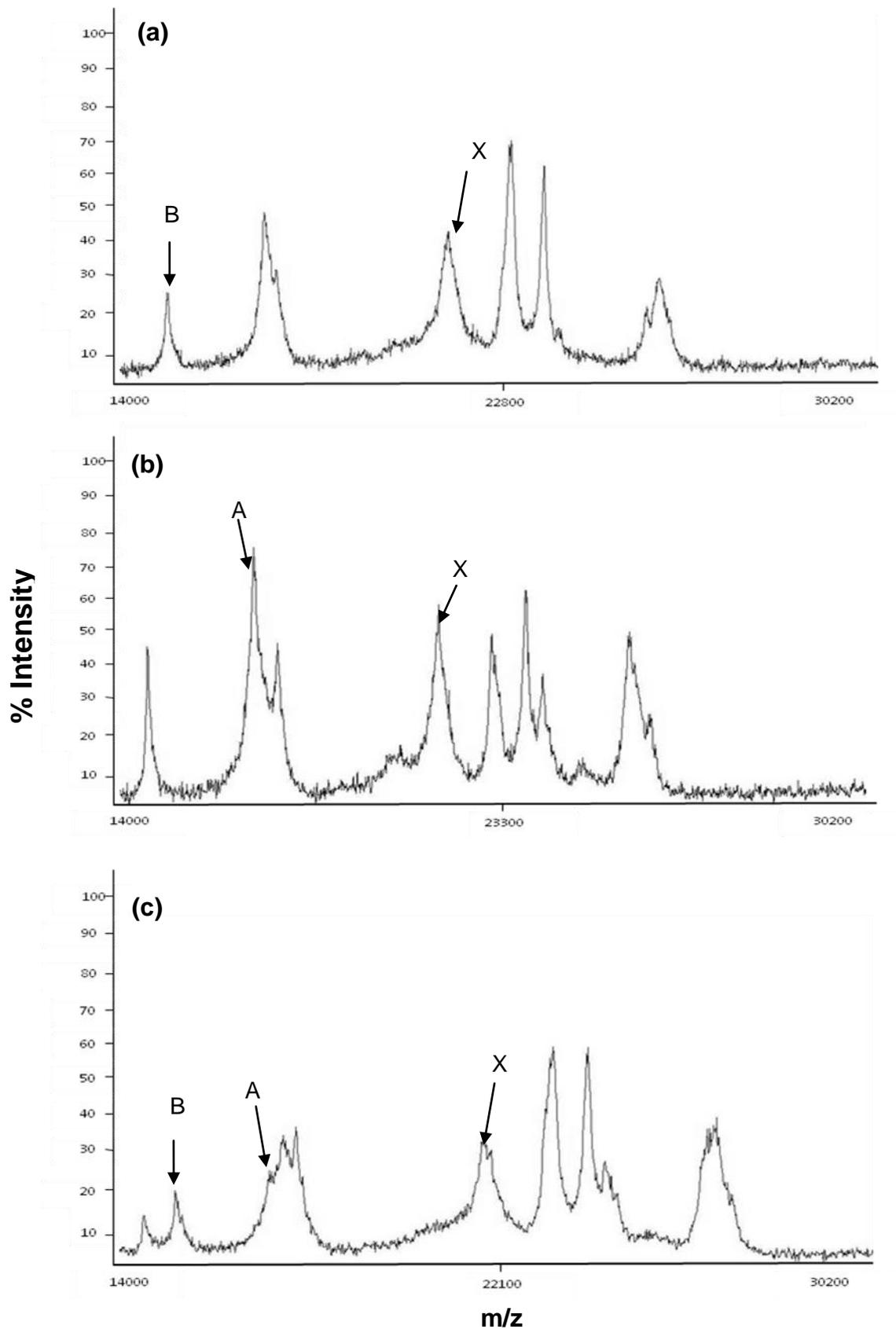


Figure 3.11 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P2, (b) paternal parent line P3 and (c) the associated hybrid H2. (A~17150 Da, B~15150 Da, X=27 kDa γ -zein)

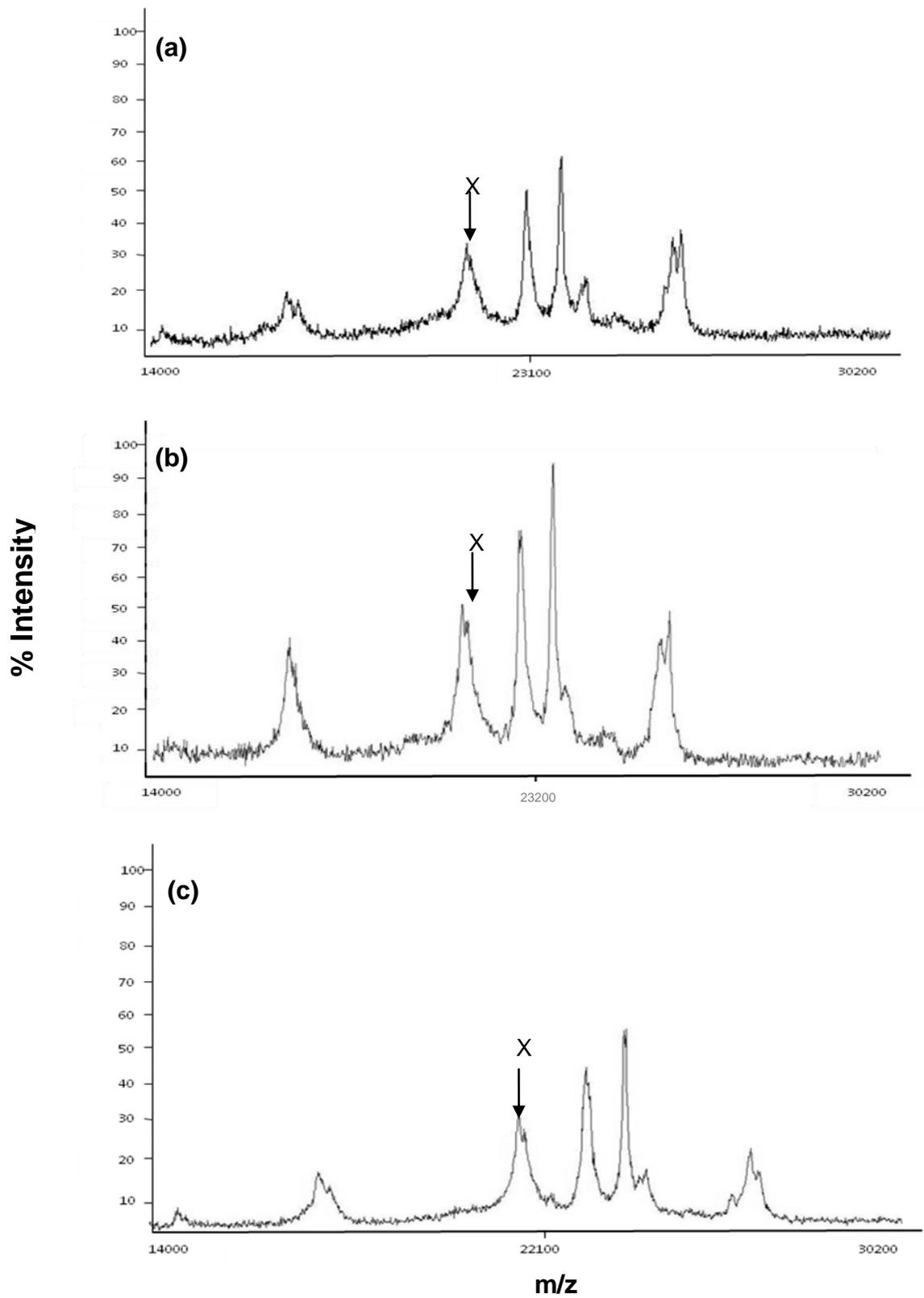


Figure 3.12 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P7, (b) paternal parent line P13 and (c) the associated hybrid H3. (X=27 kDa γ -zein)

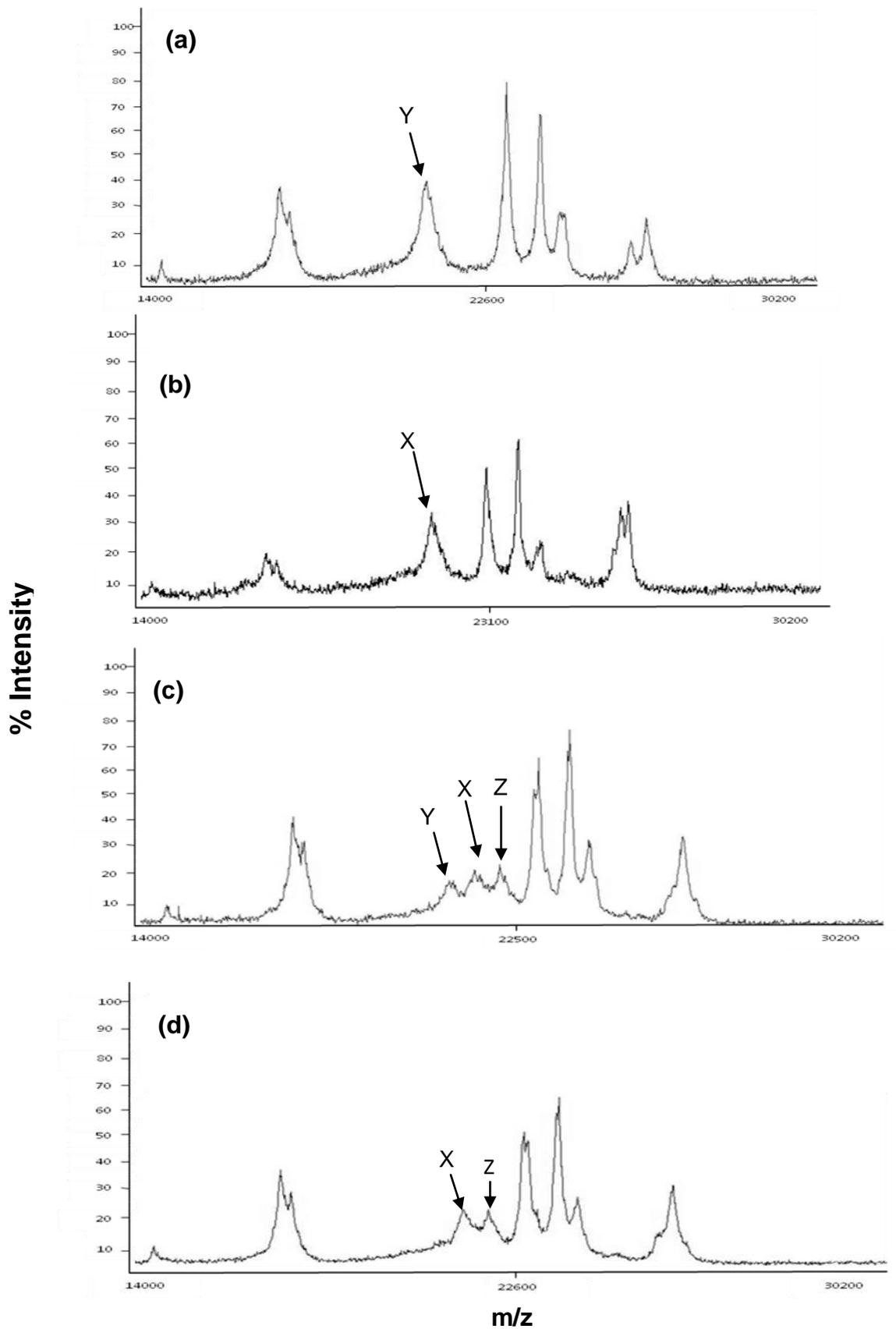


Figure 3.13 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P9, (b) paternal parent line P8 and (c) the associated hybrid H4B and (d) H4A. (X=27 kDa γ -zein, Y~21200 Da and Z~22400 Da)

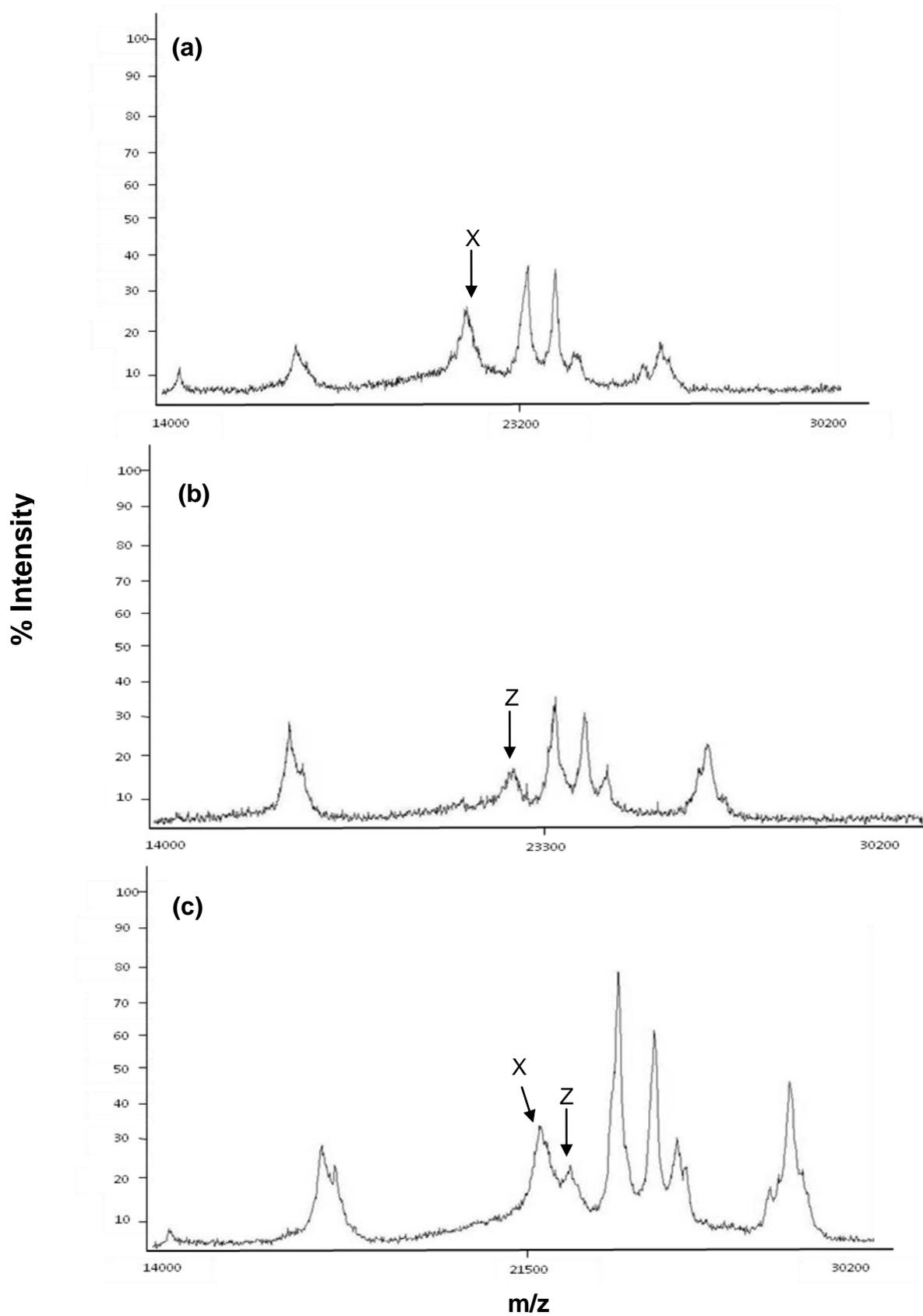


Figure 3.14 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P5, (b) paternal parent line P10 and (c) the associated hybrid H5. (X=27 kDa γ -zein and Z~22400 Da)

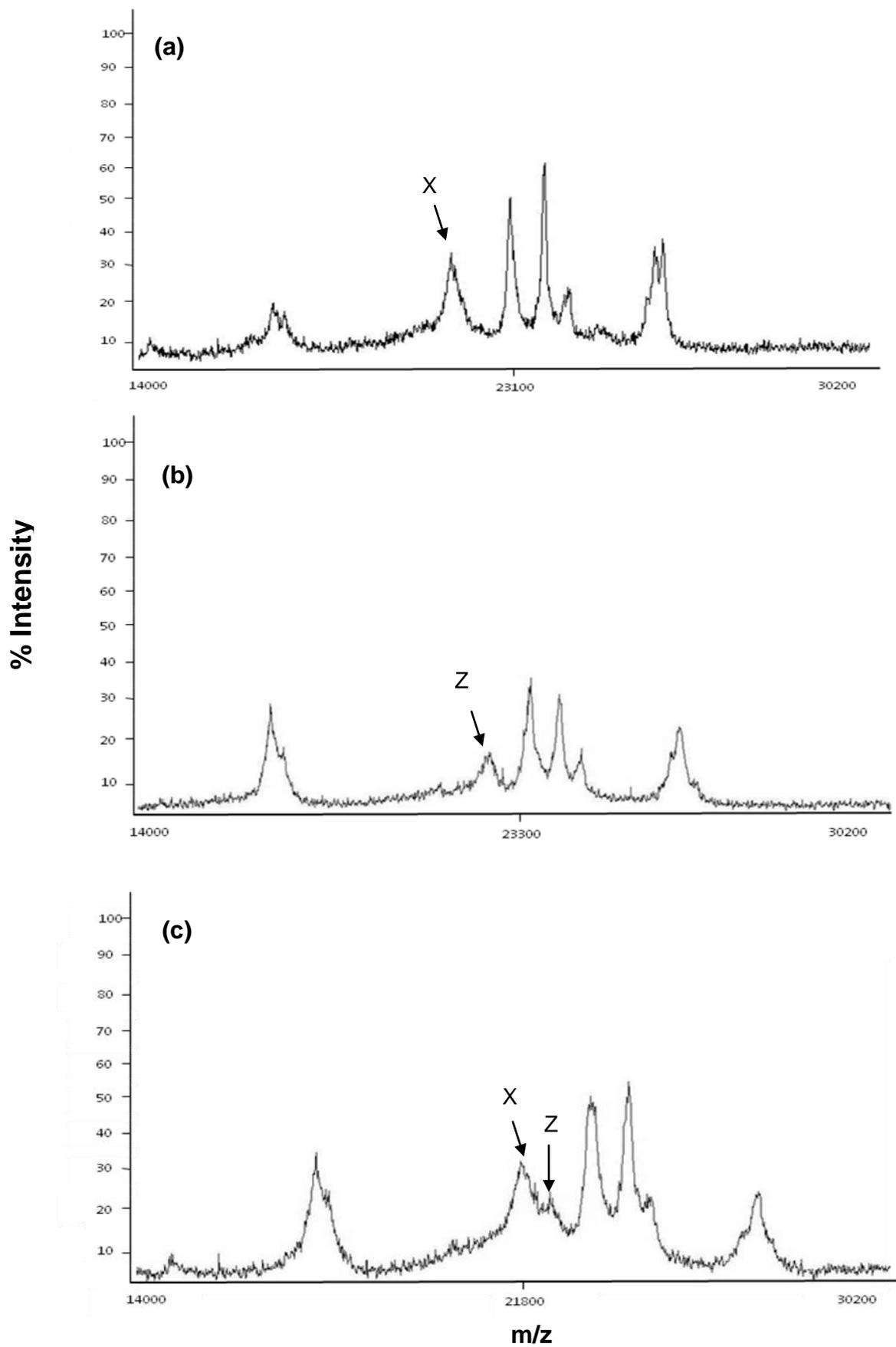


Figure 3.15 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P7, (b) paternal parent line P10 and (c) the associated hybrid H6. (X=27 kDa γ -zein and Z~22400 Da)

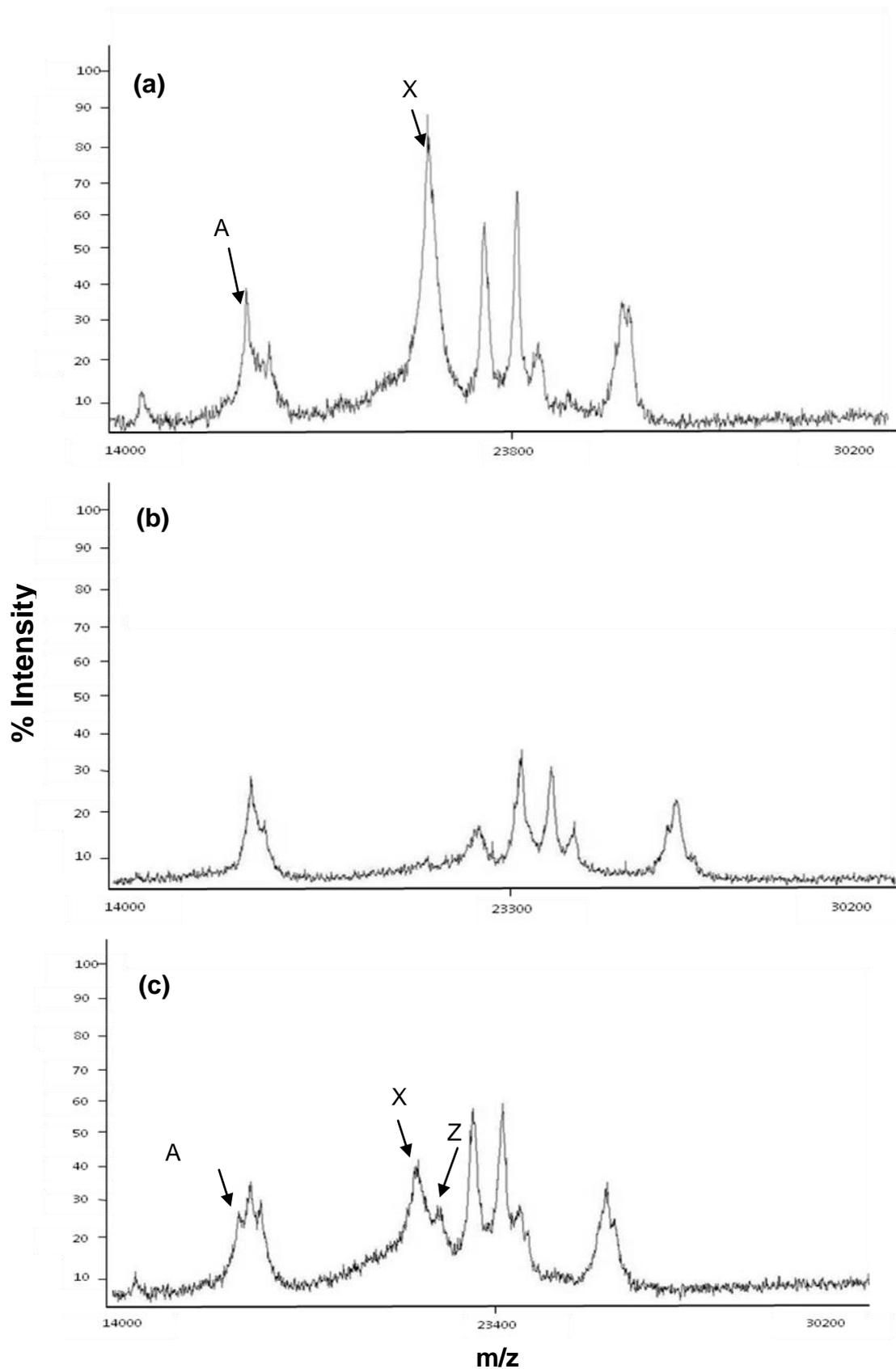


Figure 3.16 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P6, (b) paternal parent line P10 and (c) the associated hybrid H7. (A~15150, X=27 kDa γ -zein and Z~17150 Da)

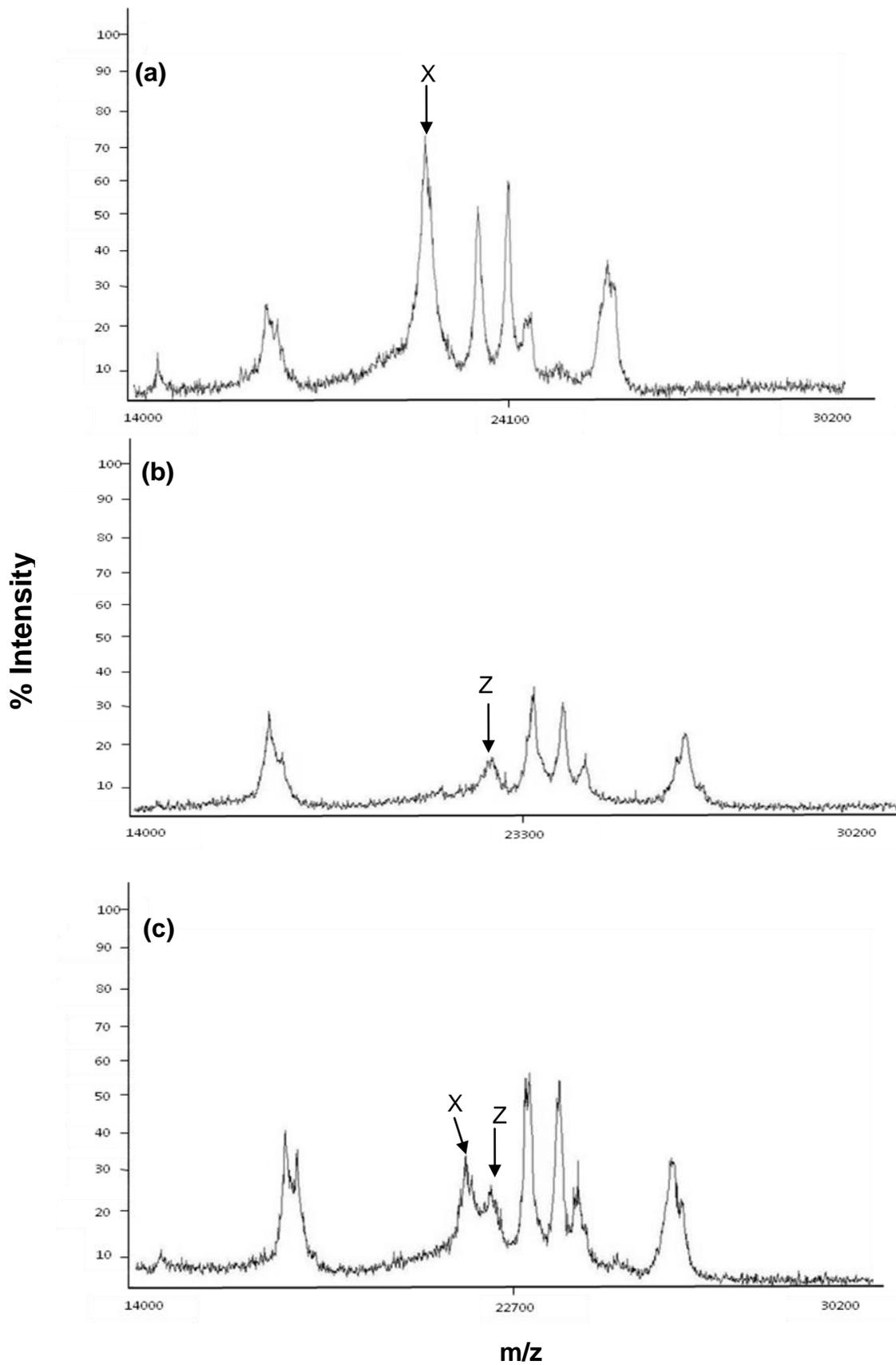


Figure 3.17 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P12, (b) paternal parent line P10 and (c) the associated hybrid H8. (X=27 kDa γ -zein and Z~22400 Da)

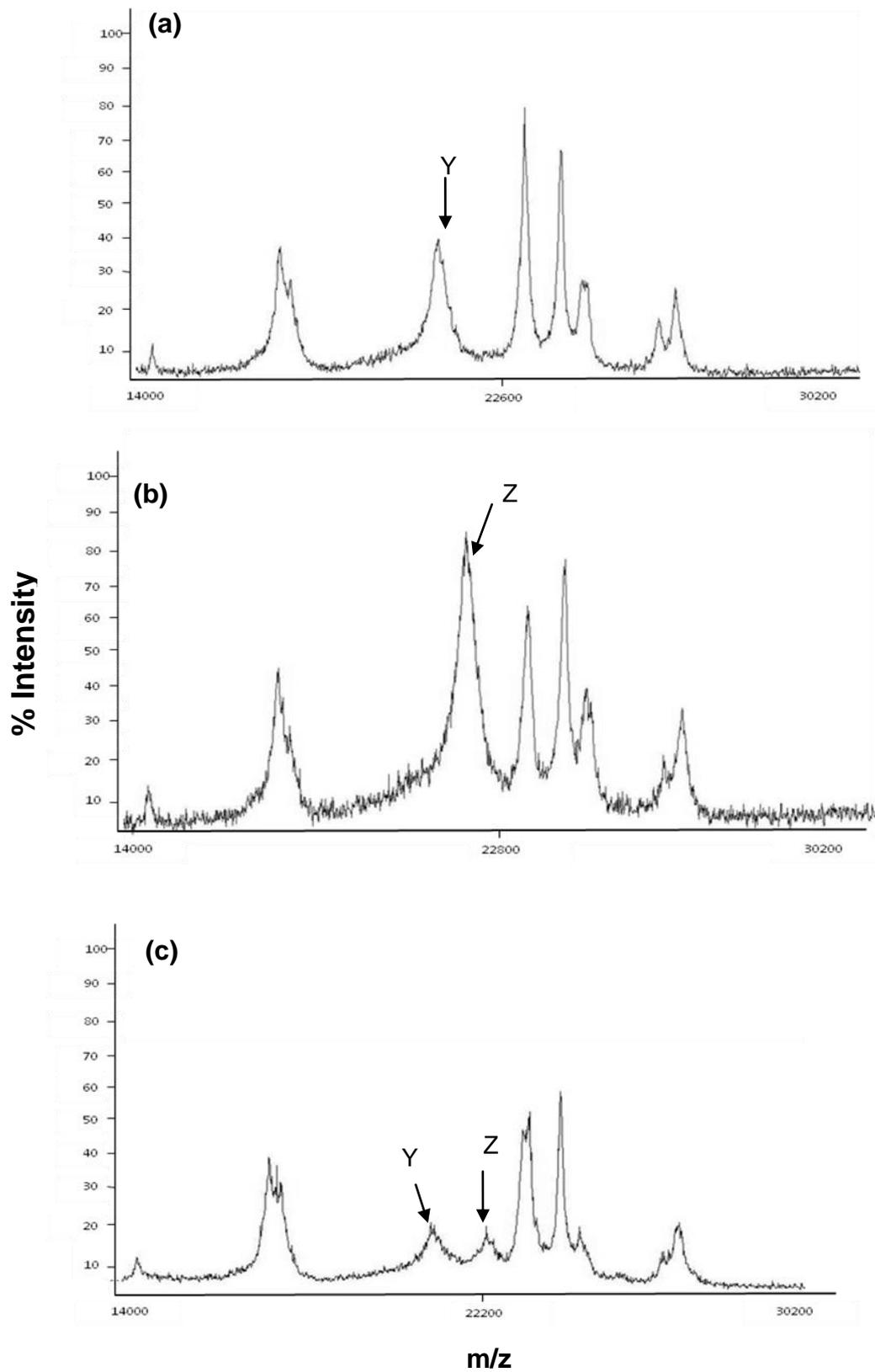


Figure 3.18 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P4, (b) paternal parent line P1 and (c) the associated hybrid H9. (X=27 kDa γ -zein and Z~22400 Da)

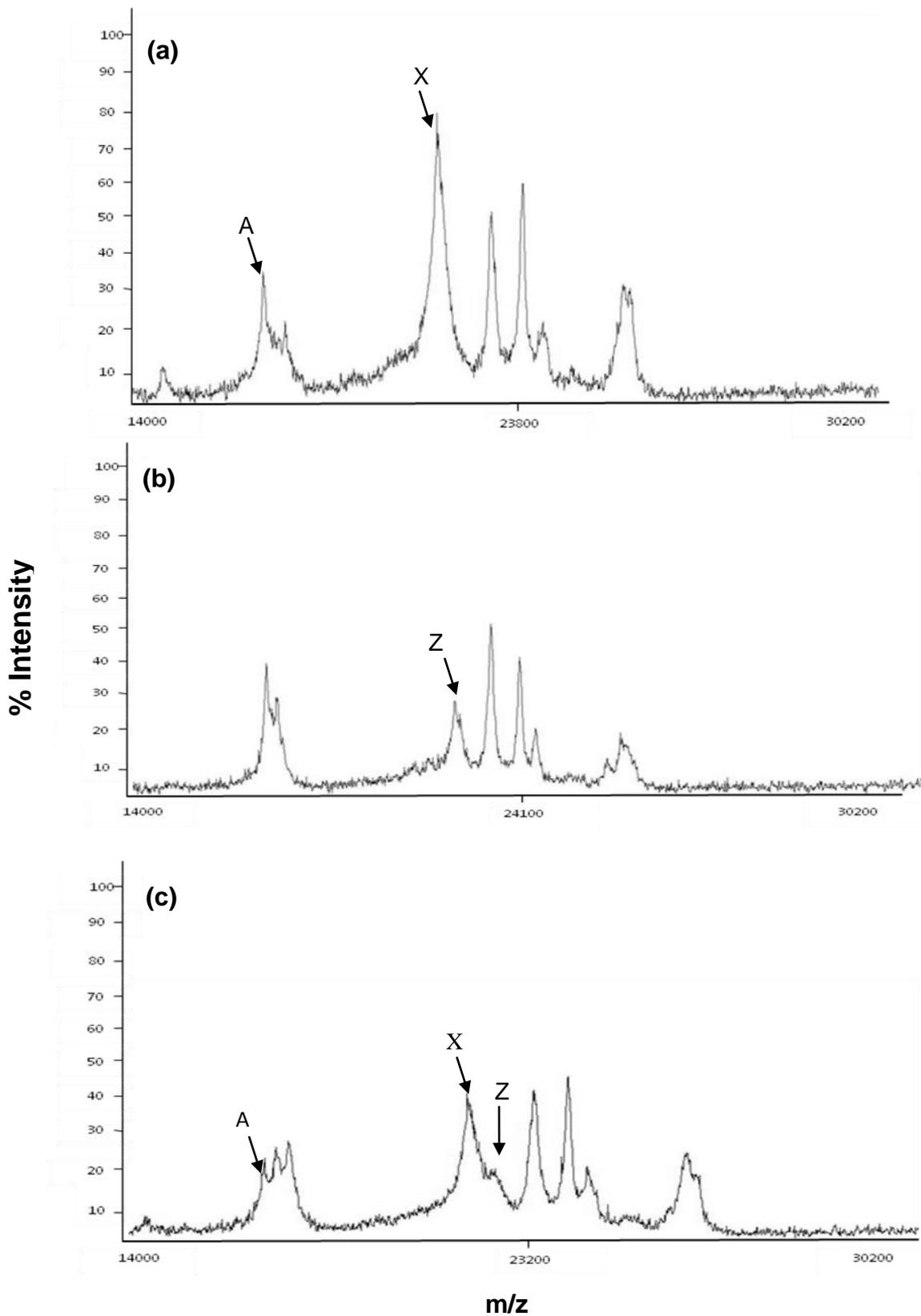


Figure 3.19 MALDI-TOF MS spectra of zein profiles of (a) maternal parent line P6, (b) paternal parent line P11 and (c) the associated hybrid H10. (A~17150, X=27 kDa γ -zein and Z~22400 Da)

Prolamin profiles of sorghum using optimised procedure

Similar to zeins, kafirins (prolamin of sorghum) consist of four main classes; α -, β -, γ - and δ – kafirin. Only α -, β - and γ -kafirin have been observed on a protein level using SDS-PAGE while δ -kafirin has only been observed on a genetic level (Belton *et al.*, 2006). The α -kafirins are divided into two sub-classes; 19 kDa and 22 kDa α -kafirin with true molecular weights ranging from 26800 – 27100 Da (Belton *et al.*, 2006). Three proteins with molecular weights of 15000, 17000 and 20000 Da, based on SDS-PAGE results, have been identified as β -kafirin (Shull *et al.*, 1991a). Kafirins were tested for immunogenic reactivity with zeins and only the 20000 Da protein of the β -kafirin class reacted with the β -zein antiserum. This indicated 15000 Da and 17000 Da proteins were perhaps not β -kafirins but proteins with similar solubility behaviour to β -kafirin (Shull *et al.*, 1991a). Analysis of kafirin genes indicated only one gene for β -kafirin is present (Chamba *et al.*, 2005). The γ -kafirin comprises a single protein, 28 kDa γ -kafirin, which shares sequence homology with the 27 kDa γ -zein. Due to similarities between the kafirins and zeins it was decided to also apply the optimised procedure to kafirins.

Spectra of kafirins (extracted from NDF flour at ambient temperature) are depicted in Fig. 3.20. Kafirin profiles were similar for both sorghum hybrids. Two peaks with molecular weights of ~26800 Da and ~27040 Da corresponded to true molecular weights of the 22 kDa α -kafirins were observed. The 22 kDa α -kafirins comprise a large group of proteins with an even more narrow mass range than α -zeins. Therefore, it was not possible to distinguish between these proteins using MALDI-TOF MS. A low abundance peak (~25400 Da) was thought to be the 19 kDa α -kafirin (Fig. 3.20b). The difference, however, between the observed and true molecular weight of 19 kDa α -kafirin was large, ~1000 Da (Belton *et al.*, 2006). An unidentified peak at ~29550 Da (H), was also observed from both sorghum samples. The identities of these peaks were not conclusively assigned.

A peak at ~18850 Da, corresponding to β -kafirin (encoded by the single gene), was present in both spectra. Two other peaks, ~22860 Da (E) and ~23600 Da (F), were also present. These peaks did not correspond to gene sequence data available for kafirins (Belton *et al.*, 2006) and are perhaps the other two proteins suggested to have similar solubility behavior as β -kafirin (Shull *et al.*, 1991a).

The peak corresponding to γ -kafirin (~20210 Da) behaved in a similar manner as γ -zein. Signal for this class appeared only when NH_4OH was included in extraction solutions. Better signals were obtained with 0.01% TFA concentration than 0.01% FA (Figs. 3.20a&b). When using FA, the γ -kafirin peak was rarely observed. The addition of CHCA together with HABA did not improve the signal much compared to improvement seen with 27 kDa γ -zein.

A low abundance peak, ~12900 Da (I), was present in both spectra (Fig. 3.20). It corresponded to the mass of the δ -kafirin (true molecular weight 12 961 Da).

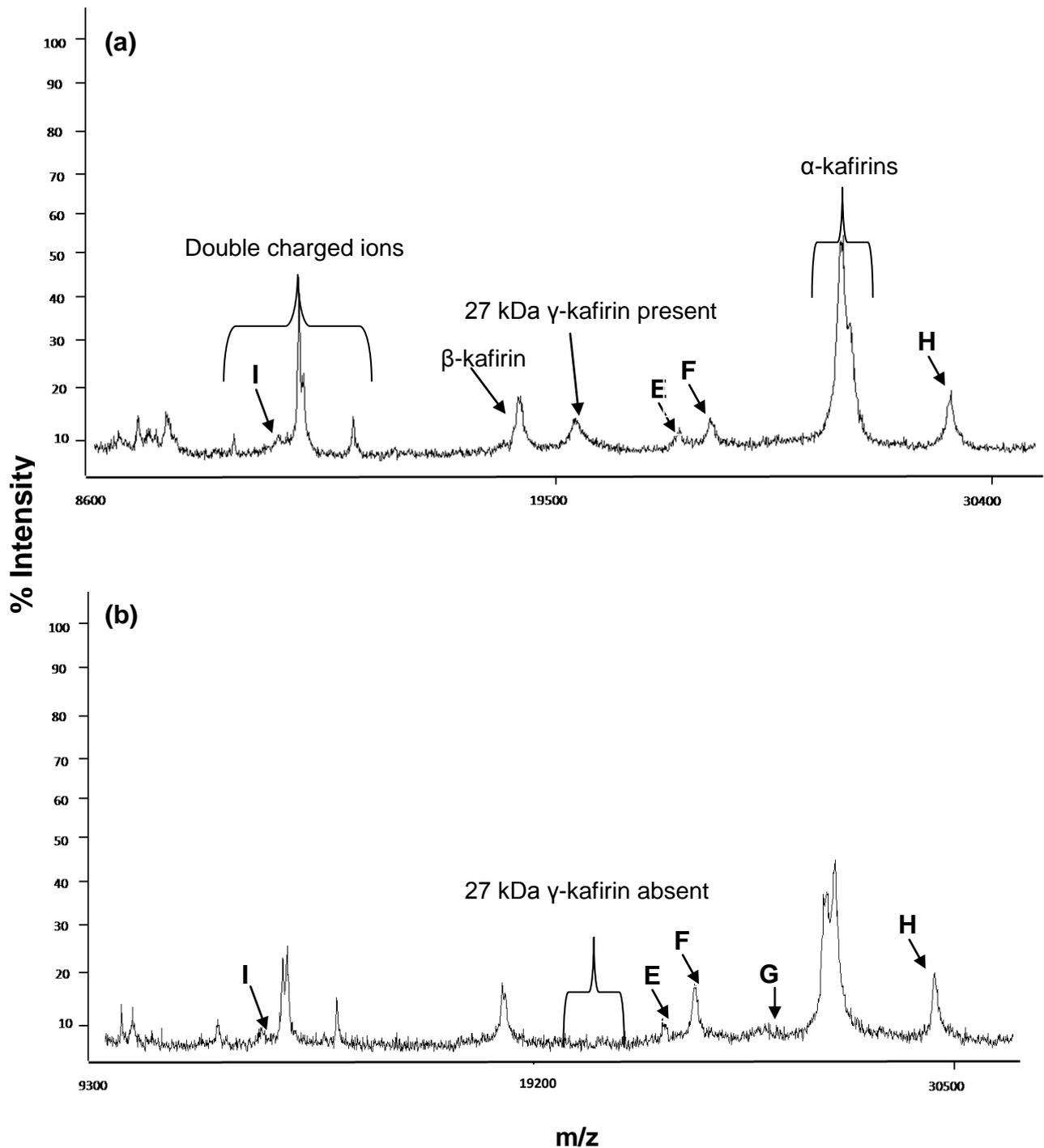


Figure 3.20 MALDI-TOF MS spectra of kafrin profiles; (a) with 0.01% TFA, where γ -kafrin is present and (b) 0.01% FA where γ -kafrin is absent. (E~22840, F~23600, G~25900, H~29550 and I~12900)

It also corresponded to the mass of the double charged ion of the low abundant class, ~25 400 Da (Fig. 3.20b). This peak, ~25400 Da, appeared to be absent in Fig. 3.20a. Further investigation is necessary to confirm if the ~12900 Da peak is δ -kafirin.

Although poorer S/N for kafirins was obtained compared to zein, this procedure does show potential for kafirin characterisation. Iodoacetamide (IAA) derivatisation can be used in future to positively identify the classes. The extraction and matrix preparation procedure should be optimised to obtain a better S/N ratio.

Conclusion

MALDI-TOF MS allowed for good detection of zein sub-classes. This was especially true for the α -zeins. Profiles of zein from hybrids indicated their proteins are expressed as a combination of those present in the parent lines. Signals of zein in spectra, using only the HABA matrix for analyses, from NDF maize meal extracted at 60°C had a lower S/N ratio, compared to zein extracted from DF maize meal. The peak corresponding to 27 kDa γ -zein was absent or had very poor signal from zein extracted from NDF and DF maize meal at ambient temperature. The addition of a more water soluble matrix (CHCA) at pH 2.9 or increasing the pH from <1.5 to 1.7 (only including HABA) allowed for the more water soluble 27 kDa γ -zein to appear when zein was extracted at ambient temperature. This was true when extracting from NDF and DF maize meal at ambient temperature. Increasing the pH of the matrix solution to 2.9 resulted in improved signals in spectra. Similar spectra were obtained when zein was extracted from either coarser or fine maize meal samples. Kafirins were also analysed using the optimised extraction and matrix preparation procedures. This indicated these optimised procedures can be applied for analysis of prolamins from other related cereals.

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Chapter 4

Evaluation of total protein and zein contents of
maize hybrids differing in kernel texture

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Abstract

The total protein and zein contents (assessed using reverse phase high performance liquid chromatography (RP-HPLC)) and degree of hardness of 10 hybrids grown at three localities were determined to establish a relationship between these variables. Particle size index (PSI) was used to evaluate the kernel texture (degree of hardness) of the hybrids. Protein content correlated positively with a harder kernel texture. Hybrids which differed significantly ($P < 0.01$) in protein content, did not all differ significantly ($P < 0.01$) in degree of hardness. Internal morphological structure (evaluated using scanning electron microscopy) of floury endosperms of hard and soft hybrids differed. The starch granules of the harder hybrid appeared to be more tightly packed. Total zein, expressed as total area of RP-HPLC peaks, correlated positively with protein and a harder kernel texture. Correlations between zein peaks expressed as percentage peak areas of total area differed than when expressed as absolute area given as arbitrary units (AU). Most zein-2 peaks (β - and γ -zein), expressed as percentage peak areas correlated negatively with a harder kernel texture whereas opposite trends were observed when, expressed as area (AU). The majority of the individual α -zein peaks correlated positively with protein content and a harder kernel texture. PCA loadings plots of the first two principal components indicated similar trends to correlations and differentiated between hardest and softest hybrids. Trends indicated total protein content and particular zein proteins have an impact on kernel texture.

Introduction

The relation of the endosperm chemical composition to maize kernel texture has been extensively studied, but some aspects remain unexplained. The endosperm constitutes the largest portion of the total kernel (Sofi *et al.*, 2009). It comprises starch granules which are surrounded by protein bodies (PBs) (Lending & Larkins, 1989). Endosperm varies in texture containing both hard and soft endosperm types. The hard endosperm is referred to as horny, translucent, glassy or vitreous whereas the soft endosperm is referred to as floury or opaque (Dombrink-Kurtzman & Beitz, 1993). Vitreous endosperm is considered to be more developed than floury endosperm due to PBs being larger, more abundant and mature (Lending & Larkins, 1989). The ratios in which these endosperm types are present will determine the overall texture of the maize kernel. In South Africa, a dry milling method is predominantly used and breeders aim to reduce the percentage floury endosperm due to this endosperm type “getting lost” during the milling process (Evan Brauteseth, PANNAR, Greytown, South Africa, personal communication, 2010). The floury endosperm breaks down into fine particles while vitreous

endosperm remains as larger grits. Maize grits are of high value and are used in various food products (Rodriguez-Nogales *et al.*, 2006). Thus, a higher percentage of vitreous endosperm is needed to obtain a high yield and quality of these grits. Wet milling, on the other hand, requires softer kernels (Dombrink-Kurtzman & Knutson, 1997; Chandrashekar & Mazhar, 1999). Kernel shape should also be considered when assessing kernel texture. Flint (long and flat kernels, with an oval, distal end) and dent (short and flat kernel with a dented distal end) (Fox & Manley, 2009) are the more common types grown, with flint having a harder texture than dent.

Both cultivar and environment has been shown to influence kernel texture (Fox & Manley, 2009). Biochemical constituents including **starch** (amylopectin and amylose) (Dombrink-Kurtzman, 1994; Dombrink-Kurtzman & Knutson, 1997; Gibbon *et al.*, 2003), **zein**, the major storage protein of maize, (Paiva *et al.*, 1991; Dombrink-Kurtzman & Beitz, 1993; Eyherabide *et al.*, 1996; Robutti *et al.*, 1997; Robutti *et al.*, 2000; Gibbon & Larkins, 2005; Holding & Larkins, 2006; Lee *et al.*, 2006) and total **protein** content have been shown to correlate with kernel texture.

Total protein content has been positively associated to kernel texture when correlated with degree of hardness determined using different hardness measurements (Mestres & Matencio, 1996; Blandino *et al.*, 2010). The protein content can range from 6-14% and zein can contribute up to 70% of the total protein (Prasanna *et al.*, 2001). Zein consists of four main classes, namely α -, β -, γ , and δ -zein, and are located within the protein bodies in starchy endosperm (Lending & Larkins, 1989). Reverse-phase high performance liquid chromatography (RP-HPLC) has been used to establish the relationship between zein classes and kernel texture (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994; Eyherabide *et al.*, 1996; Mestres & Matencio, 1996; Robutti *et al.*, 1997). The α -zeins have been positively correlated with hardness when comparing the contents of this class from vitreous and floury endosperm respectively or when correlated with the degree of hardness obtained using various hardness measurements (e.g. density, virtuousness, grinding time and energy). Both 15 kDa β -zein and 27 kDa γ -zein have been positively (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006) and negatively (Dombrink-Kurtzman & Beitz, 1993; Pratt *et al.*, 1995) linked to a harder kernel texture. Thus, uncertainty exists for the relationship between the γ - and β -zeins content and kernel texture evaluated using various hardness measurements.

Many indirect techniques have been used over the past few decades to assess hardness. These include determinations of density, grinding resistance, grinding (milling) followed by sieving and determining amount of throughs, starch gelatinization properties, test weight or determinations using near infrared spectroscopy, both reflectance and transmission (Pratt *et al.*, 1995; Fox & Manley, 2009). A more common method to assess hardness has been to mill maize, followed by fractionating the meal into course and fine material, using a series of sieves. This method has been referred to as Particle Size Index (PSI). Little consistency has been reported for this method where variation exists in sieve sizes and mills used (Abdelrahman &

Hoseney, 1984; Wu, 1992). The sieve size used is dependent on the milling process and size of the holes of the sieve (Fox & Manley., 2009). Useful information, in terms of variation in hardness and calculating average particle sizes of samples, can be gained using this method. The ratio of course to fine (C/F) material can also be calculated to obtain a single value for hardness, with higher value indicating a higher degree of hardness. C/F ratio of commercial hybrids has recently been reported to be the best indicator of dry-milling ability (Blandino *et al.*, 2010).

Univariate statistical analyses have been used to establish a relationship between RP-HPLC peaks containing the various zein classes and kernel texture. A multivariate approach has not been reported to simultaneously take into account all zein classes analysed by RP-HPLC and results from hardness measurements. Principal component analysis (PCA) has been applied to zein RP-HPLC data to identify and group maize cultivars but not to establish a relationship with hardness (Robutti *et al.*, 2000). PCA has also been applied to RP-HPLC zein data and maize characteristics (fat, ash, protein, starch, seed weight) (Flint-Garcia *et al.*, 2009). Multivariate data analysis techniques have been applied to generate new sets of variables to characterize maize hardness (Lee *et al.*, 2004). However, zein and individual zein classes were not one of the variables included in this study. It would be beneficial to know how the individual classes associate with kernel texture and to one another using multivariate techniques. To date, the α -zeins peak areas have only collectively been used, to establish a relationship to kernel texture. Therefore, it is not known if a specific α -zein sub-class would perhaps have a stronger relationship to kernel texture than another sub-class.

In this study, the relationship between zein proteins (assessed with RP-HPLC), total protein content and kernel texture using 10 hybrids grown at three localities, was established by means of univariate and multivariate approaches.

Materials and Methods

Plant materials and material preparation

The sample set consisted of two field replicates of 10 white maize hybrids (H1-10) grown at three localities (A=Greytown, B=Klerksdorp and C=Delmas). Sample numbers were assigned by referring to the hybrid, locality and replicate, e.g. H4C1 would be hybrid 4, locality C, replicate 1. Equal portions (50g), of maize hybrid samples were milled using a hammer mill (Scientific, Cape Town, South Africa) (fitted with 1 mm sieve) and stored in sealed jars for a period of six months.

Moisture determination

Moisture content was determined according to AACC Approved Method 44-15A. The moisture dish and lid was weighed to the nearest 0.001 g. Thereafter, 5 ± 0.001 g of maize meal was weighed into the moisture dish and mass recorded. Moisture dishes were placed uncovered in a Heraeus vacuum oven (Thermo Scientific, Johannesburg, South Africa) at 130°C for 2 hours. After drying, the lids were placed back on the moisture dishes. Moisture dishes were then removed from the oven and placed in a desiccator for 40 minutes to cool. After cooling, the mass of the covered dishes was recorded to the nearest 0.001g and moisture content determined.

Protein content

The protein content of the samples was determined according to the AACC Approved Method 46-30 (AACC, 2008) using a Dumas combustion analyser (Model Truspec[®] N elemental Determinator, Leco Africa, Kempton Park, South Africa). A number of blank samples followed by a number of ethylenediaminetetraacetic acid (EDTA) samples were analysed prior to protein determination to ensure the instrument was performing within specifications. EDTA (a chemical standard with known nitrogen content; 9.57%) was used to calibrate the instrument. The EDTA standard (0.1 ± 0.001 g) was weighed into a tin foil sample cup (Leco Africa, Kempton Park, South Africa), twisted and rolled into an egg shape and placed on the carousel loading head of the instrument. After calibration of the instrument, samples of maize meal (0.35 ± 0.001 g) were weighed into a tin foil cup, folded and loaded in the instrument and nitrogen content measured. To convert nitrogen to protein, a conversion factor of 6.25 was used. The protein content was then expressed on a 12% moisture basis (mb).

Particle size index measurement

The Particle Size Index (PSI) was determined using a two sieve method. A 150 micron sieve was placed on a 75 micron sieve, fitted with a receiving pan. The sieves and receiving pan were each weighed to the nearest 0.001 g (W1). Thereafter, 10 ± 0.01 g of ground maize was weighed in the 150 micron sieve (W2) together with 10 ± 0.01 g whole wheat (W3). Another 10 ± 0.01 g whole wheat was weighed in the 75 micron sieve. Six sieves (2 replications) were sieved at a time in the Retsch AS200Tap (Haan, Germany) sieving apparatus for 10 min. The fine maize meal adhering to bottom of each sieve was gently brushed off into respective receiving sieve or pan. The sieves and receiving pan were weighed to the nearest 0.001 g. The mass (W4) in each of the 150 sieve, 75 sieve, and in the bottom pan were recorded and three percentages, PSI-1, PSI-2 and PSI-3, were calculated respectively according to the following equation.

$$\text{PSI} = (W4 - (W2 + W3)) / W1$$

Where W1 = mass of maize meal

W2 = mass of sieve or receiving pan

W3 = mass of wheat kernels*

W4 = mass of sieve or pan with maize meal and kernels*

*For receiving pan mass of wheat kernels = 0 g

A higher PSI-1 value will indicate a harder kernel texture and higher PSI-2 and -3 values will indicate the opposite. A coarse-to-fine (C/F) ratio was calculated as follow; $C/F = PSI-1/(PSI-2 + PSI-3)$ where a higher ratio will indicate a higher degree of hardness.

Visual evaluation of whole maize kernels

Light transmission photographs were taken of a random selection of kernels per hybrid. A light box consisting of a wooden box fitted with UV fluorescent lights, covered with a one centimetre thick white Perspex layer, was used. Kernels were placed germ side facing down on the perspex layer together with a 10 cm ruler. Photographs were acquired with a Nikon D90 (Nikon, Tokyo, Japan) camera, fitted with a 18-55 mm VR lens, using a focal length of 20 and shutter speed of 1/8 of a second. Adobe® Photoshop® CS3 2007 (Adobe Systems Incorporated, USA) was used to transform the white background to black and create greyscale images of kernels.

Scanning electron microscopy (SEM)

Imaging of the samples was accomplished using a Leo® 1430VP Scanning Electron Microscope (Zeiss, Germany). Prior to imaging or analysis the samples were sputter-coated with gold. Samples were identified with secondary electron images. Beam conditions during imaging were 7 KV and approximately 1.5 nA, with a working distance of 13 mm and a spot size of 150.

RP-HPLC analysis of zein

Zein extraction

Zein extraction was adapted from the extraction method as described by Dombrink-Kurtzman (1994). Zein fractions were extracted with 70% (v/v) ethanol, 5% (v/v) 2-mercaptoethanol (2ME) and 0.5% (w/v) sodium acetate in 2-mL eppendorf tubes by continuous agitation on a vortex apparatus for two and a half hours at ambient temperature. A solvent:endosperm ratio of 5:1 (v/w) was used for quantitative extraction of zeins. Suspensions were centrifuged twice for 15 minutes at 6000 g. The supernatants obtained the first time were transferred to a clean 2-ml eppendorf tube and centrifuged again (to ensure removal of solid particles), where after supernatants were kept for RP-HPLC analysis.

RP-HPLC analysis

Zein proteins were separated with a Phenomenex Luna C18 column (250 mm x 4.6 mm, 5- μ m pore size) preceded by a Security Guard cartridge column (Phenomenex, Johannesburg, South Africa). The RP-HPLC separation procedure was adapted from (Eyherabide *et al.*, 1996; Robutti *et al.*, 2000). Acetonitrile (ACN) (B) and distilled water (A) both containing 0.1% trifluoroacetic acid were used as solvents. Prior to analyses solvents were filtered through a Durapore Membrane (PVDF, hydrophilic, 0.45 μ m). Samples (20 μ L) were eluted at 0.9 mL/min with a 50 min linear 28-60.5% solvent B gradient followed by a 10 min isocratic elution at 60.5% B. The eluate was monitored by UV absorbance (210 nm). RP-HPLC instrumentation was supplied by Waters Corporation (Milford, USA). Peaks were integrated using Empower Pro 2, (2006) software (Waters Corporation Milford, USA) and percentage peak areas were calculated. A few of the respective inbred parent lines were used to compare temperature conditions (ambient temperature vs. 60°C) for the column.

Data analysis

Univariate statistical analysis

Statistical analysis was performed using STATISTICA version 9.0 (StatSoft, Inc., Tulsa, OK, USA). Two way analysis of variance (ANOVA) was performed to compare protein content and PSI values between localities. Error bars were used to indicate 0.95% confidence intervals and least significant difference (LSD) post hoc testing was used. Spearman correlations coefficients (r) between protein content, RP-HPLC peaks and PSI index values, PSI-1, -2 and -3 were determined.

Multivariate data analysis

Principal component analysis was conducted using LatentIX v.2.0 (2008) (Laten5, Copenhagen, Denmark) software. Prior to analysis, all data was mean centred and scaled. Results were given as separate loadings and score plots.

Interpretation of PCA loadings and scores plots

A PCA model consists of principal components each explaining a certain percentage of the variation. The first principal component (PC1) will explain the largest percentage followed by PC2, PC3 and so forth. PCA models consist of two plots, i.e. a principal component (PC) score plot, indicating how samples associate with one another, and a corresponding a PC loadings plot, indicating why the samples associate. The loadings plot thus refers to the variables. A simple example of a PCA loadings and scores plot (bi-plot) of PC1 vs. PC2 is given in Fig. 4.1. PC1 is in the direction of the horizontal axis and PC2 in the direction of the vertical axis. Scores (a-c) are indicated in red and loadings in black (u-z). Variables u and v have high positive loadings on PC1 and are positively associated. Variable w has a high positive loading on PC2

and is also positively associated to v, u due to the angle being smaller than 90°. The sharper the angle the stronger the association will be between variables. Sample b has a high amount of those variables and this can be seen from its position; it is closely associated to these variables. Variable x has a high positive loading on PC1 and is negatively associated to w, u and v due to angled being larger than 90°C. Samples a and c are positively and negatively associated with sample b. Sample has a high amount of variable w and sample c a higher amount of variable x. If the angel is 90°C there is no association (Everitt, 1978). Variables y and z are close to the origin indicating they are not well explained by the principal components. Therefore, not much can be concluded regarding these variables (Kjeldahl & Bro, 2010).

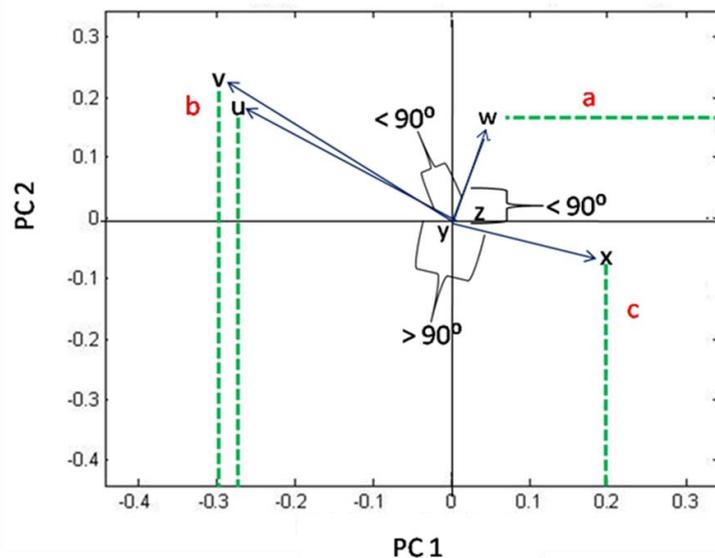


Figure 4.1 Example of a PCA scores and loadings plot (bi-plot). Scores indicated in red and variables in black. Green lines indicating loadings on relevant principal components. Black brackets indicate angles.

Results and Discussion

Evaluation of protein content and PSI-1 values

(Detailed results in Appendix 1)

Hybrid H1 was not grown in Delmas (C) and only hybrids 2-10 were subjected to two way ANOVA for protein content and PSI values. Significant interactions ($P < 0.01$) were observed between the protein content of hybrids and the three localities (Fig. 4.2a). The protein content did not differ significantly ($P > 0.05$) for hybrids H3, H4, H6 and H7 when grown at Greytown (A) and Klerksdorp (B). Protein content for most hybrids grown at Delmas was significantly ($P < 0.01$) lower than Klerksdorp (B) or Greytown (A). Hybrids H4 and H10 were the only hybrids, when grown at Delmas, with a significant ($P < 0.01$) higher protein content than when grown at the other localities. Field replicates had similar protein contents. The average protein content of hybrids grown at each locality differed significantly ($P < 0.01$) (Fig. 4.2b). This indicated

environmental factors and/or genotype contributed to differences in protein content. Various environmental factors including soil nutrient profile, soil acidity, available moisture and temperature as well as maturity influence the development of the maize and subsequently the constituents of maize.

Significant ($P<0.01$) locality and hybrid interactions were observed for PSI-1 values (Fig. 4.3a). A range of PSI-1 values from 58 to 74% were obtained. PSI-1 values for hybrids from Greytown ranged from 65 to 74%. For Klerksdorp, a similar range of 65 to 73% was observed and Delmas exhibited a range of 58 to 74%. Overall, hybrids grown at Delmas had lower PSI-1 values. Significant ($P<0.01$) differences were observed between Greytown and Delmas for PSI-1 values for hybrids H2, H3, H5, H6, H9. The average of PSI-1 values obtained for Greytown and Delmas differed significantly ($P<0.01$) (Fig. 4.3b). Due to the differences in protein content between Greytown and Delmas, this trend was expected. It was expected Klerksdorp would also differ significantly in higher degree of hardness from Delmas due a significant ($P<0.01$) difference in protein content. This was, however, not the case.

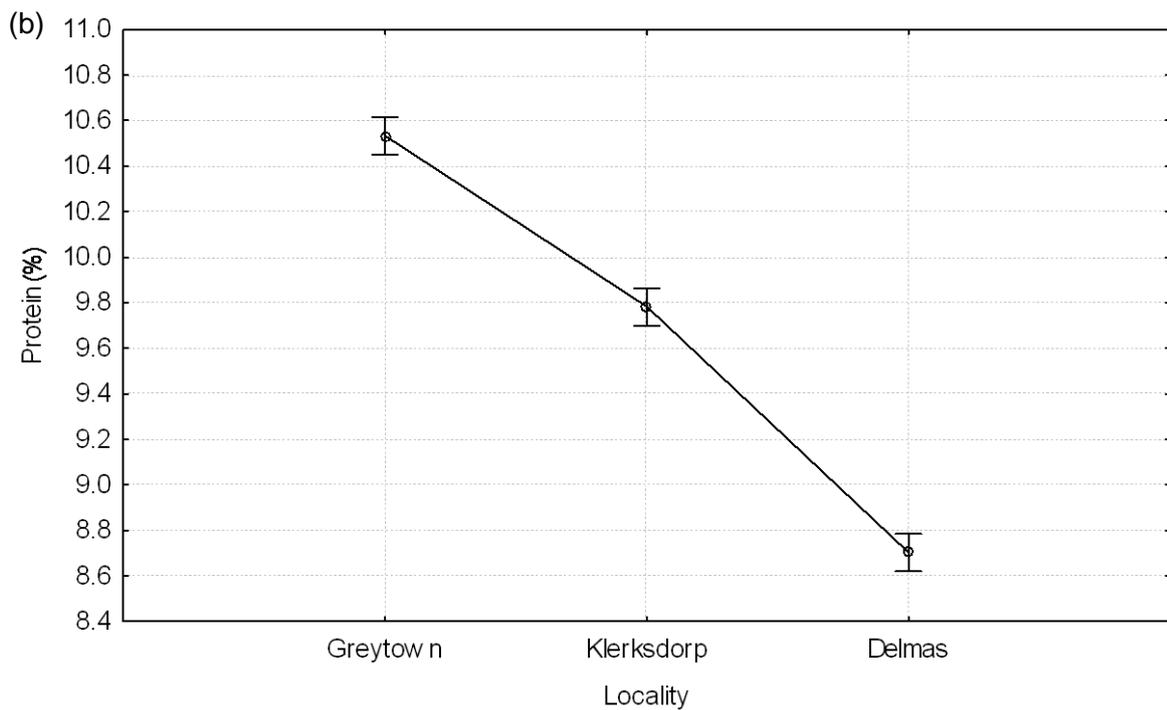
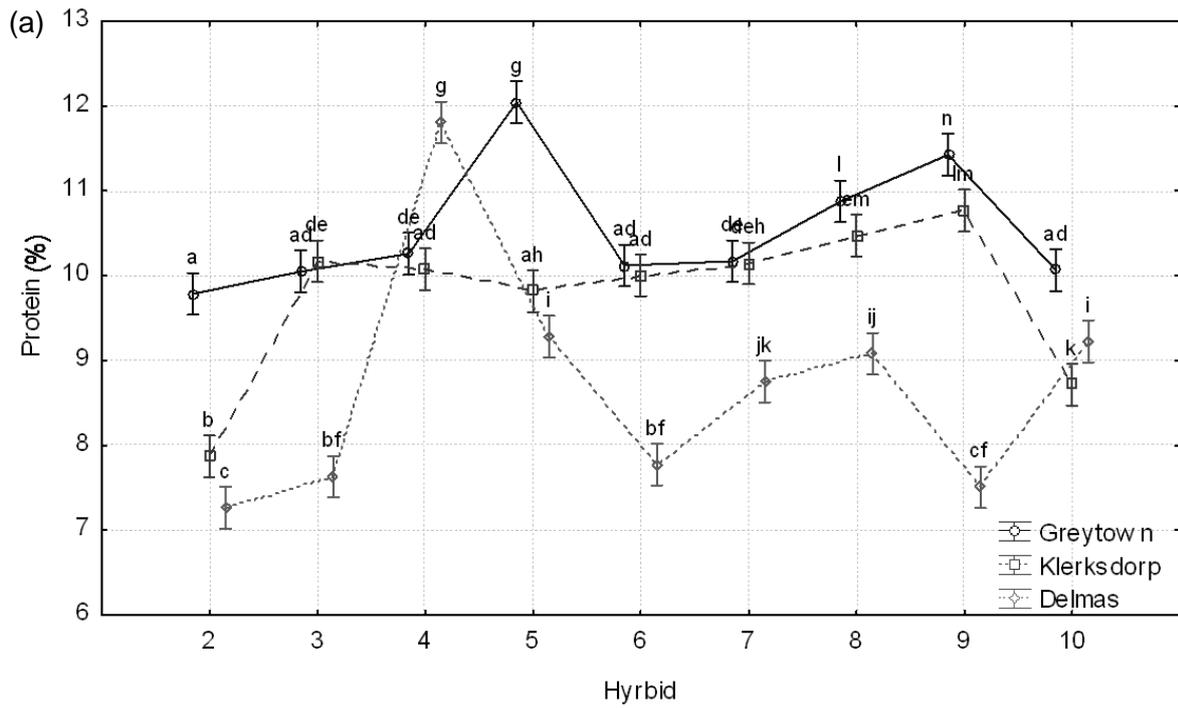


Figure 4.2 Results (a) for locality by hybrid interaction for protein content and (b) differences between average protein content obtained for localities as determined by ANOVA ($P < 0.01$). Error bars denote 0.95 confidence intervals.

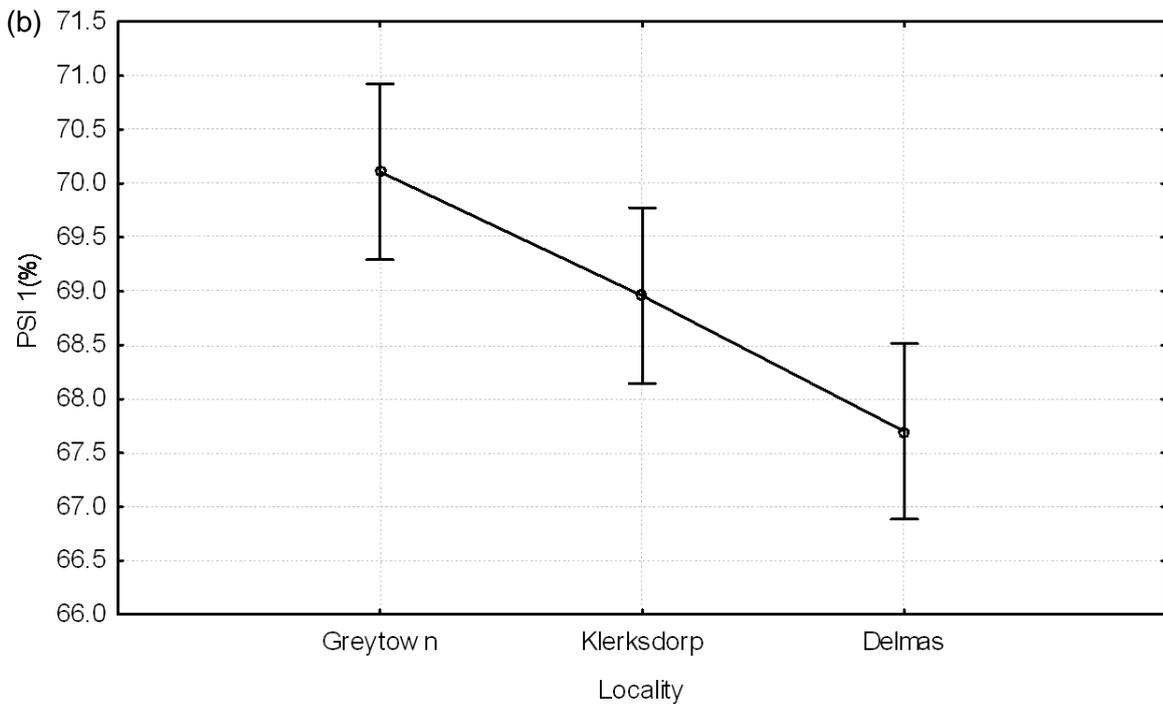
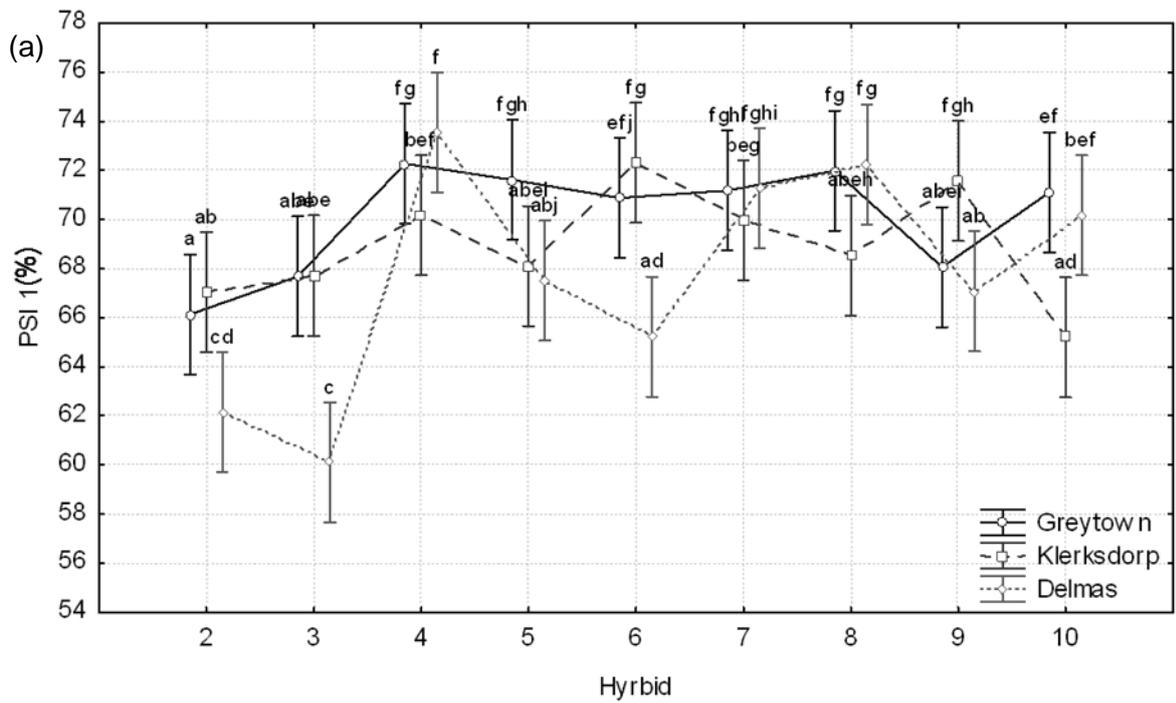


Figure 4.3 Results (a) for locality by hybrid interaction for PSI-1 values and (b) differences between average PSI-1 values obtained for localities as determined by ANOVA ($P < 0.01$). Error bars denote 0.95 confidence intervals.

Visual evaluation of overall texture

Light transmission photographs of hybrids with highest and lowest PSI-1 values, similar protein content or PSI-1 values are depicted in Fig.4.4 PSI-1 measurement indicated hybrid H4C2, with a relatively high protein content of 11.90%, as the hardest sample (PSI-1=74.3%) and hybrid H3C2, having a relatively low protein content of 7.45%, as the softest (PSI-1=58.4%). Comparisons of light transmission photographs of these two hybrids indicated obvious differences in kernel texture. Kernels from hybrid H4C1 were large, uniformly shaped with little evidence of floury regions (indicated by dark regions in the kernels). Hybrid H3C2 had irregular shaped kernels as well as floury endosperm present in front regions of the kernels. These visual differences together with protein content coincided well with the PSI measurements theory. However, discrepancies existed when comparing hybrids with either similar protein content or PSI-1 values. When comparing samples with a low protein content range of 7.19 - 7.95%, their PSI-1 values did not increase in the same order of protein increase. Hybrid H2C2 had the lowest protein content in the sample set and a higher PSI-1 value than hybrid H3C2 and H3C1. Comparisons of visual inspection of kernels indicated hybrid H2C2 had more kernels with less floury regions than H3C1 and 2. Both field replicates of hybrid H9C had a similar PSI-1 value, a slight difference in protein content and mixture of floury and vitreous kernels. Similar observations were made with two field replicates of H6C except for the PSI-1 value differing to a greater extent. Hybrids H9B2 and H10C2 both had a similar PSI-1 value but a large difference in protein content existed. These observations suggest this PSI method did not distinguish as well between hardness levels of the hybrids, and could be optimised.

Correlations between protein content and PSI values

Protein correlated ($P<0.01$) positively with PSI-1 and negatively with PSI-2 and PSI-3 (Table 4.1). A slightly weaker correlation was obtained when correlated with C/F ratio. The correlation between PSI-1 and protein content was not very strong but it did show a trend. A lower correlation ($r=0.55$; $P<0.05$) has been reported between total nitrogen and hardness measurement method similar to PSI, where regular coarse grits was separated from the fine material obtained from milling (Mestres & Matencio, 1996). Higher negative correlations between total nitrogen and friability ($r=-0.69$, $P<0.01$) and fine material obtained after milling ($r=-0.72$, $P<0.01$) were reported in the same study. A higher significant correlation ($r=0.76$; $P<0.01$), was obtained using a PSI method with different sieve sizes to obtain maize meal fractions preferred in the industry (Blandino *et al.*, 2010). Maize meal was also milled to a coarser texture using a 2 mm sieve. This indicated particle size of maize meal and sieve size should perhaps be more representative to that used in the industry.

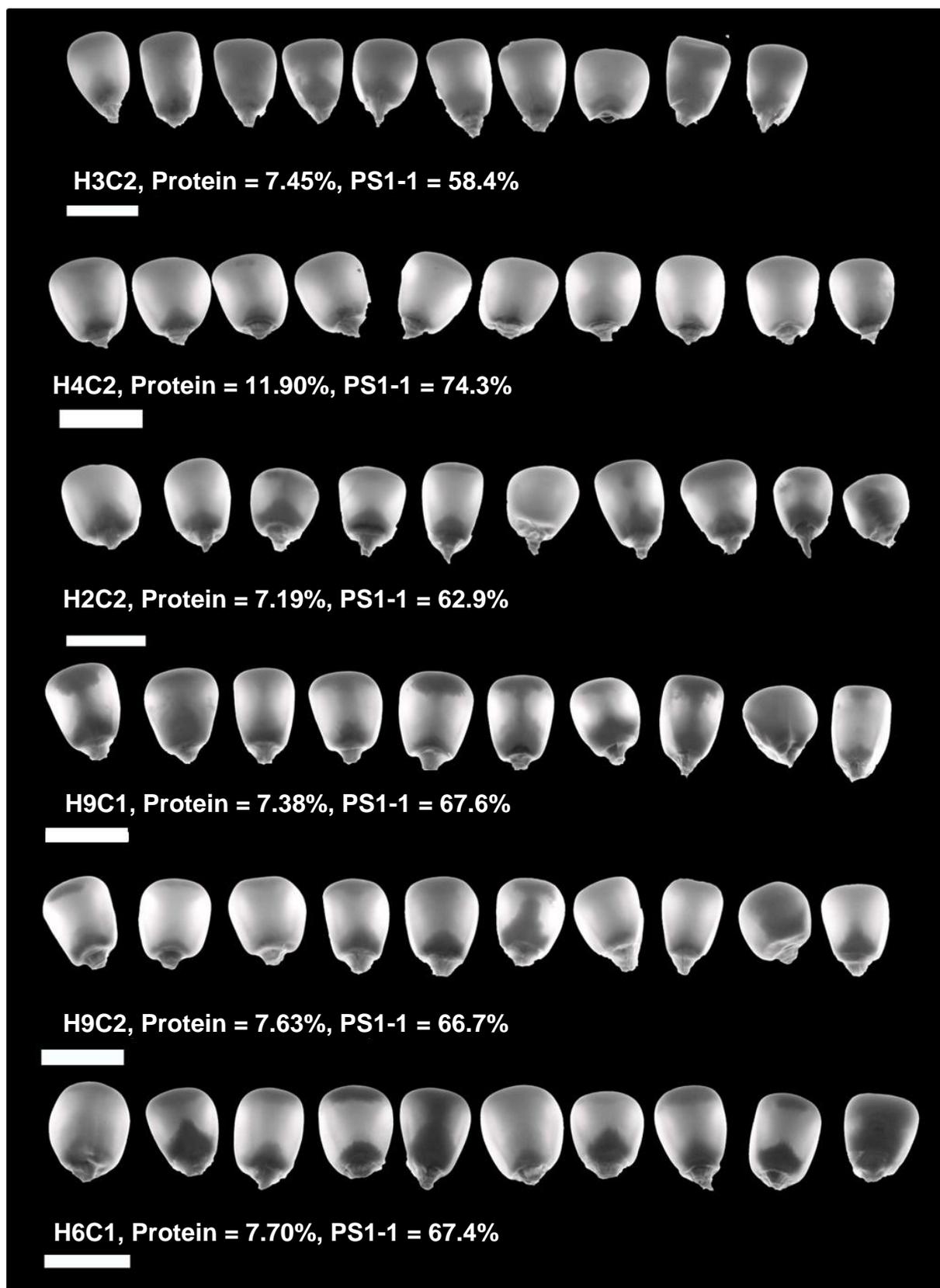


Figure 4.4 Light transmission photographs of a selection of hybrids where, identity, protein content and PSI-1 values are indicated below samples. The white bar at the bottom of each sample denotes one centimetre.

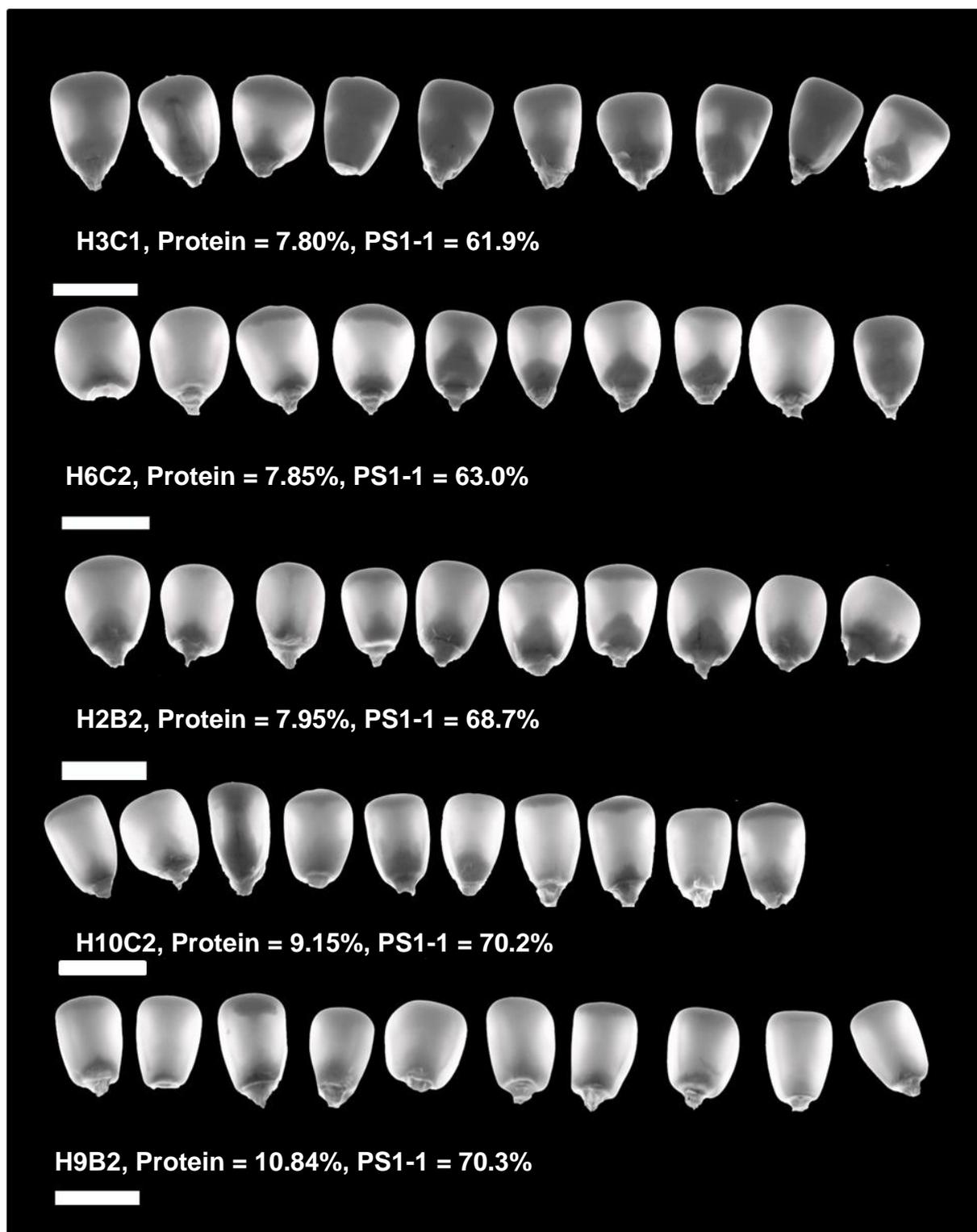


Figure 4.4 continued.

Table 4.1. Spearman correlation coefficients between RP-HPLC zein peaks, protein content and PSI values

	Protein content (%)	PSI-1	PSI-2	PSI-3	C/F (PSI-1/(PSI-2+ PSI-3))	Total area (zein)	Zein-1 (α -zeins)	Zein-2 (β -+ γ -zein)
Protein	-	0.60**	-0.58**	-0.48**	0.57**	0.68**	-0.53**	0.56**
Total area (zein)	0.68	0.30*	-0.34*	-0.26	0.33*	-	-	-
Percentage peak areas								
Zein 1	0.56**	0.19	-0.22	-0.15	0.21	-	-	-
Zein 2	-0.53**	-0.16	0.21	0.11	-0.18	-	-	-
Peak 5	-0.38*	-0.22	0.23	0.28	-0.28	-0.39*	-0.54**	0.53**
Peak 6	-0.05	0.19	-0.08	-0.36*	0.23	-0.36*	-0.35*	0.38*
Peaks 5+6	-0.43**	-0.10	0.20	0.04	-0.06	-0.69**	-0.85**	0.85**
Peak 10	-0.46**	-0.08	0.07	-0.07	-0.00	-0.57**	-0.73**	0.73**
Peak 19	0.36*	0.07	-0.16	-0.01	0.08	0.60**	0.40**	-0.41**
Peak 21	0.20	0.46**	-0.40**	-0.30*	0.46**	0.07	-0.09	0.12
Peak 22	-0.22	-0.31**	0.26	0.13	-0.29	-0.13	0.01	-0.03
Peak 23	0.34*	0.52**	-0.50**	-0.30*	0.51**	0.28	0.10	-0.07
Peak 24	-0.33*	-0.33*	0.32*	0.12	-0.27	-0.45**	-0.18	0.16
Peak areas given as AU units								
Zein 1	0.69**	0.29	-0.34*	-0.21	0.20	-	-	-
Zein 2	0.19	0.14	-0.13	-0.10	0.15	-	-	-
Peak 5	0.36*	0.12	-0.20	0.05	0.05	0.60**	0.58**	0.48**
Peak 6	0.03	0.19	-0.07	-0.40**	0.22	-0.29	-0.30	-0.05
Peak 5+6	0.50**	0.34*	-0.33*	-0.25	0.27	0.56**	0.53**	0.65**
Peak 10	-0.05	0.09	-0.07	-0.16	0.13	0.05	-0.03	0.60**
Peak 16	0.62**	0.19	-0.17	-0.19	0.09	0.72**	0.75**	0.02
Peak 19	0.51**	0.15	-0.29	-0.01	0.12	0.84**	0.83**	0.34*
Peak 21	0.51**	0.49**	-0.43**	-0.37*	0.44**	0.51**	0.48**	0.38*
Peak 23	0.47**	0.47**	-0.45**	-0.33*	0.45**	0.56**	0.54**	0.40**
Peak 24	0.41**	0.09	-0.14	-0.16	0.05	0.56**	0.59**	-0.03

*Significant at $P < 0.05$; **Significant at $P < 0.01$

Significant correlations ($P < 0.01$) also existed between protein content and results from other hardness measurements; total grit yield ($r = 0.70$), test weight ($r = 0.69$), hard/soft endosperm ratio ($r = 0.68$), milling time ($r = 0.73$) and total milling energy ($r = 0.71$). This suggests other hardness measurements should also be considered to assess hardness.

Evaluation of internal structure

Hybrids H4C2 (PSI-1=74.3%) and H3C2 (PSI-1=58.4%) were subjected to SEM. Noticeable differences between the vitreous and flourey regions of hybrid H4C2 were observed. The flourey endosperm was irregular and no distinct exposed starch granules were present. This irregular structure was observed adjacent to the germ through to the vitreous endosperm (Fig. 4.5). A network of protein bodies was observed which created a granular surface. The area where the two endosperms types met, was referred to as the junction (Dombrink-Kurtzman, 1994). Between vitreous and flourey endosperm, a different type of endosperm seemed to occur (Fig. 4.6a) and was not as abrupt as previously reported (Dombrink-Kurtzman, 1994). This was referred to as the transition phase. The starch granules became smaller, more compact and exposed. At a higher magnification, a network of protein bodies was clearly visible. At the start of the vitreous endosperm area, the starch granules became more densely packed. A smooth surface observed toward the edge of the kernel (Fig. 4.6b). Individual starch granules were not clearly visible.

Two kernels of hybrid H3C2 were subjected to SEM. The first kernel (similar to third kernel from left in Fig. 4.4) consisted almost entirely of flourey endosperm and the second (similar to sixth kernel from the left in Fig. 4.4) of vitreous and flourey endosperm. In most of the interior region of the flourey kernel, loosely irregular packed starch granules with little evidence of a protein network were observed (Fig. 4.7a). Near the edge, more compact starch granules (less than for the vitreous endosperm of H4C2) together with loosely packed starch granules were present (Fig. 4.7b). Near the crown of the kernel, the starch granules were almost completely spherical (Fig. 4.7c). The vitreous regions of the second kernel were smoother and more compact, similar to that of hybrid H4C2 (Fig. 4.8a). The flourey endosperm of the second kernel also varied in endosperm texture. Compacted smooth starch regions were observed adjacent to more abundant loosely and irregular packed starch granules (Fig. 4.8b). No transition phase was observed, as was the case for H4C2.

It was evident, the flourey endosperms of the two hybrids differed. The flourey endosperm of hybrid H4C2 appeared to be more vitreous due to more densely packed starch granules that were present (Fig. 4.4b). The protein bodies in the flourey region of hybrid H3C2 were less abundant than in hybrid H4C2 as well as much more loosely packed around the starch granules.

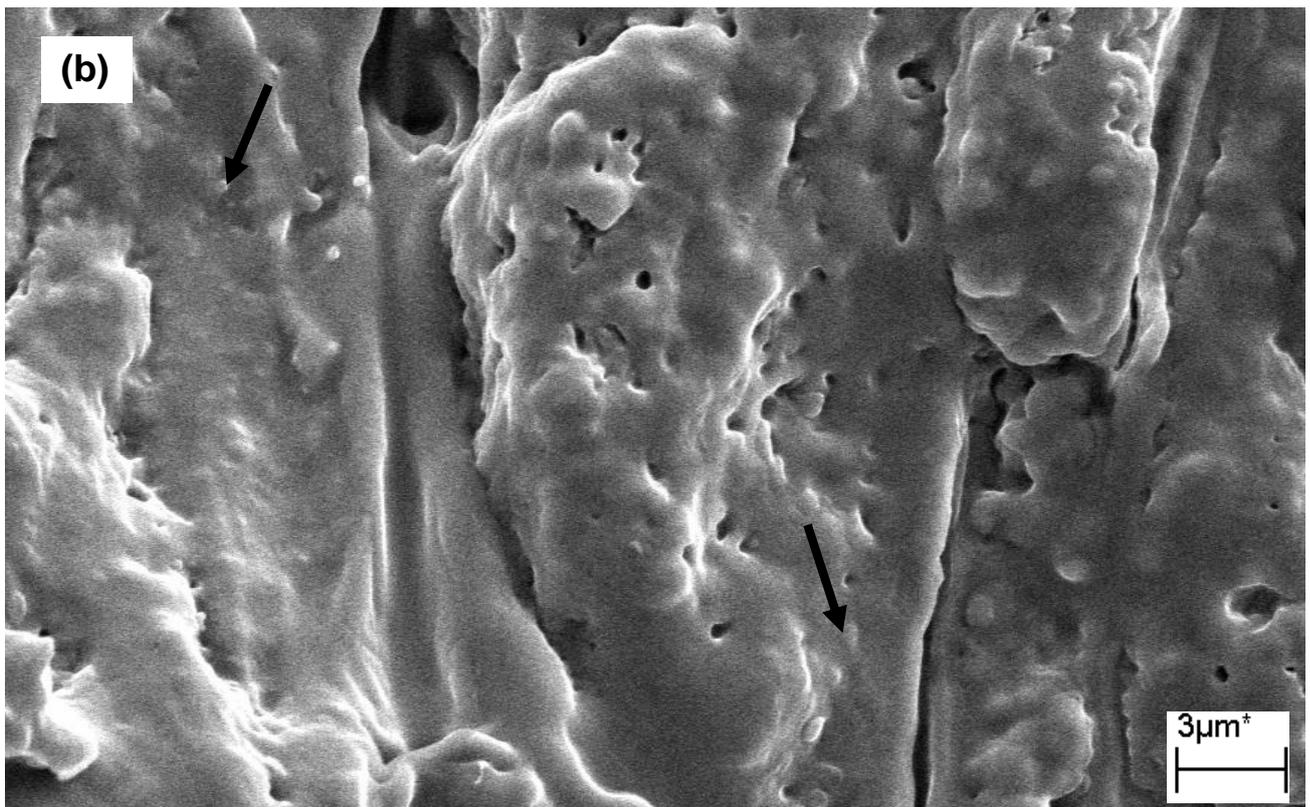
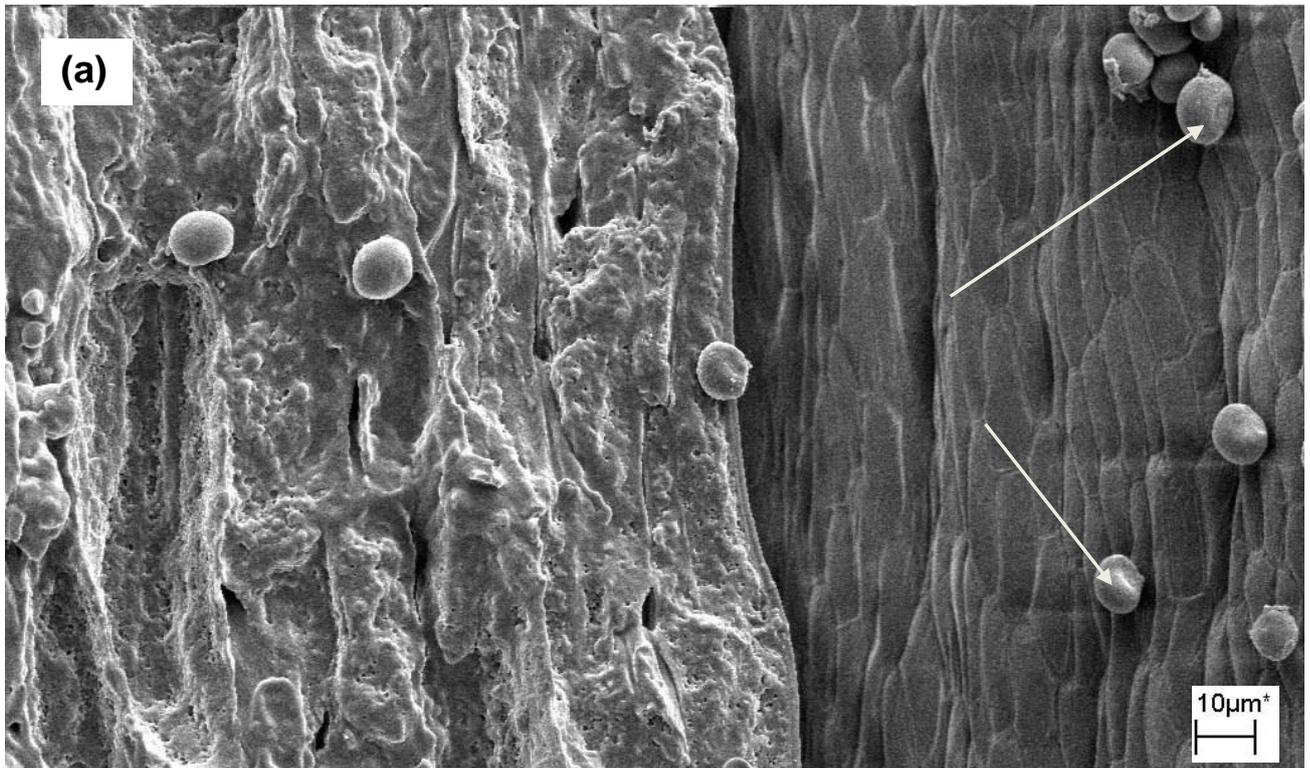


Figure 4.5 Scanning electron micrographs of floury endosperm of hybrid H4C2 (a) adjacent to germ and (b) higher magnification towards interior region of floury endosperm where an irregular structure is observed. White arrows indicate loose starch granules. Black arrows indicate protein bodies.

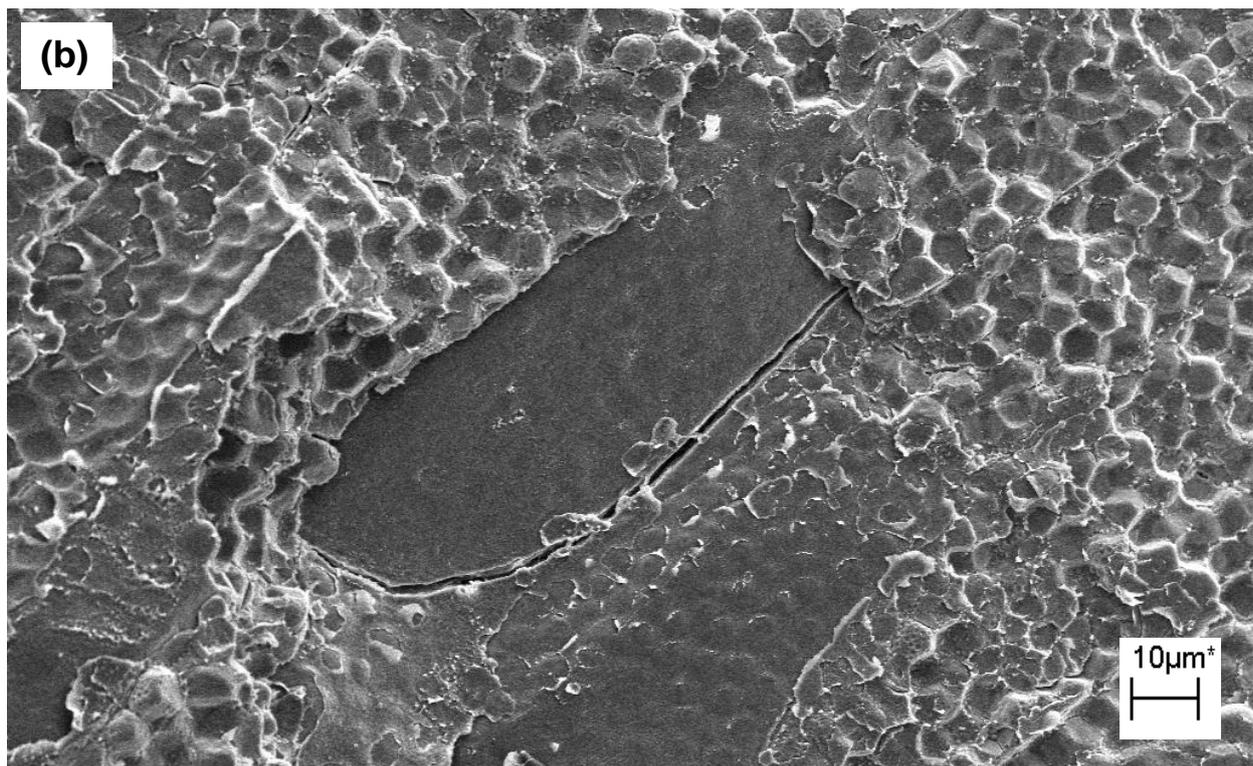
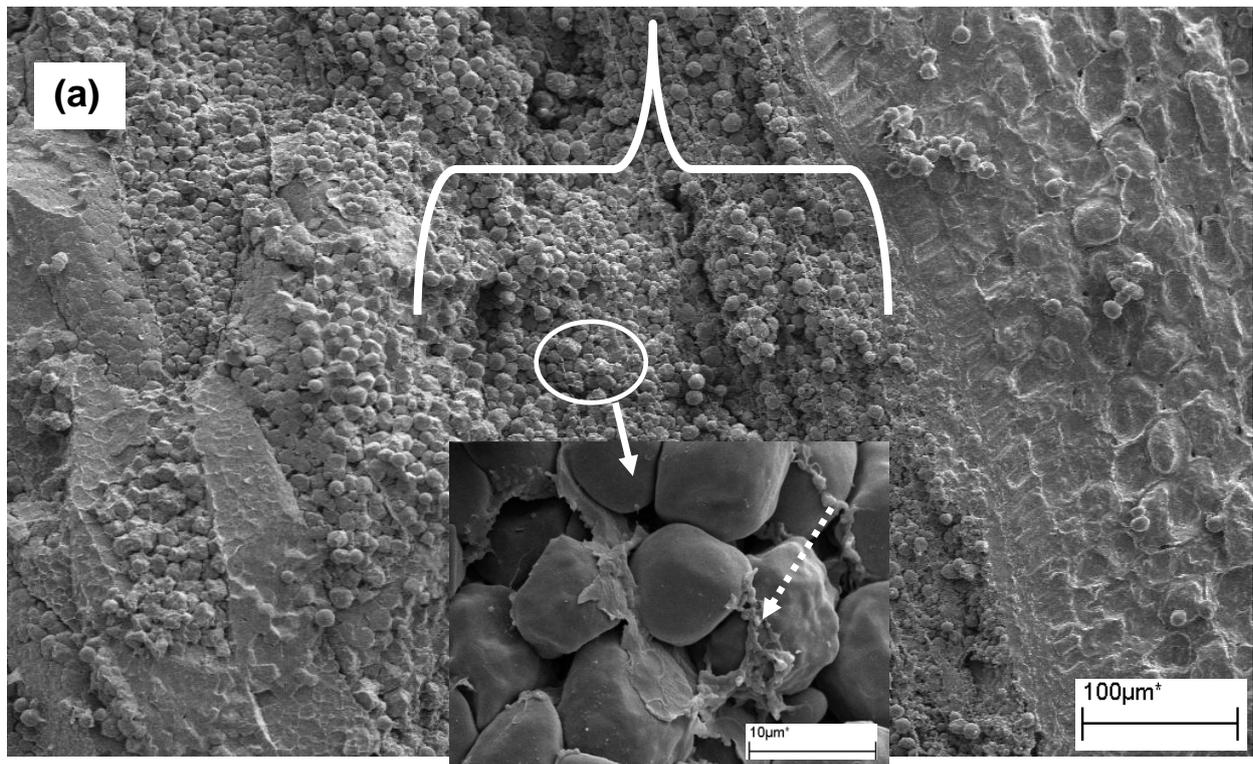


Figure 4.6 Scanning electron micrographs of hybrid H4C2. (a) Transition from floursy endosperm (right) to the vitreous endosperm (left). The transition phase is indicated by the white bracket. An area indicated by the white oval was magnified to obtain a more detailed structure. The white dashed arrow indicates the protein network. (b) Vitreous endosperm towards edge of kernel. The starch granules is densely packed forming a layer over a smooth and more densely packed region of starch.

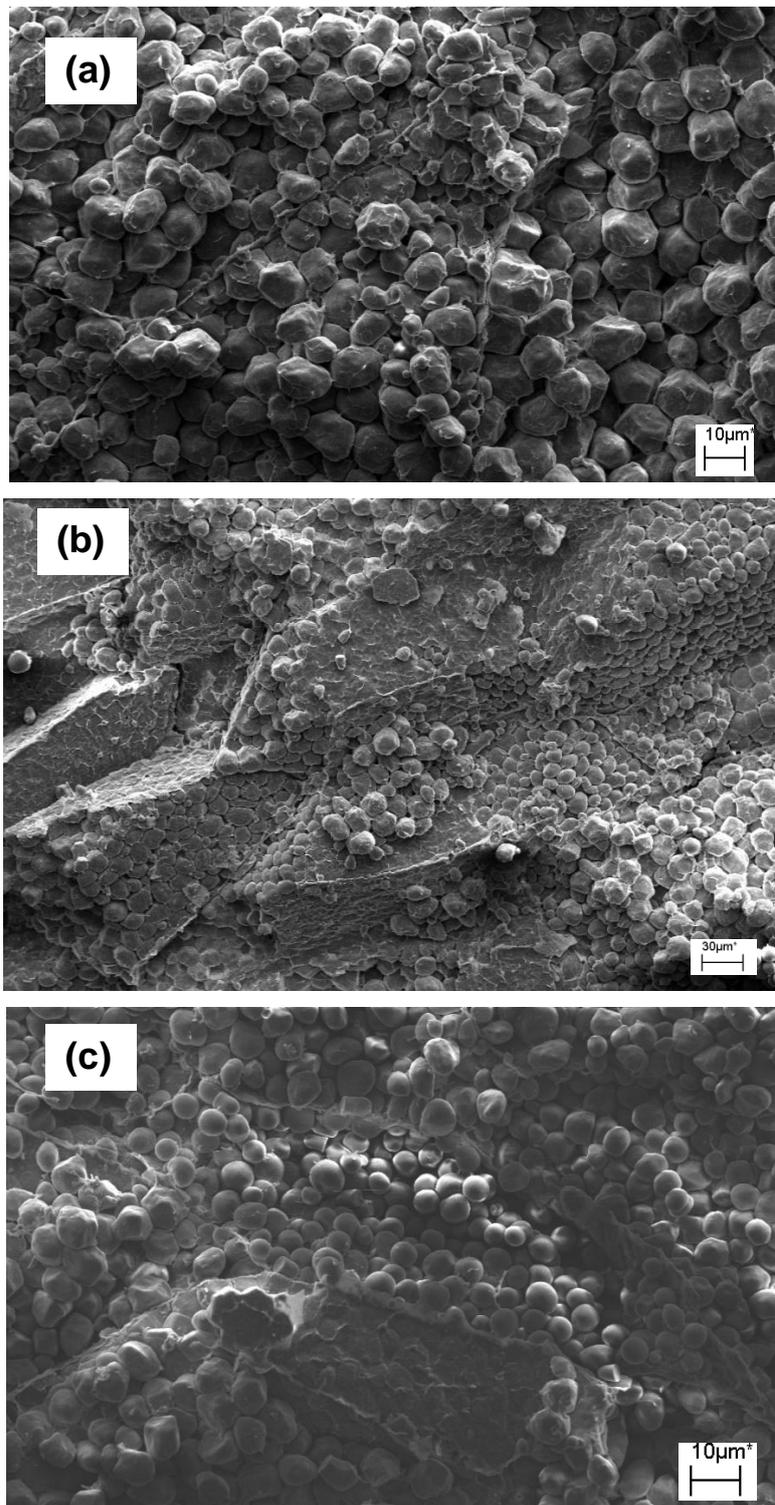


Figure 4.7 Scanning electron micrographs of a floury kernel of hybrid H3C2. The starch granules are more loosely packed and irregular in structure and surrounded by little protein network at (a) the central region of kernel, (b) the edge of the kernel where starch granules are also more densely packed and (c) the crown of the kernel.

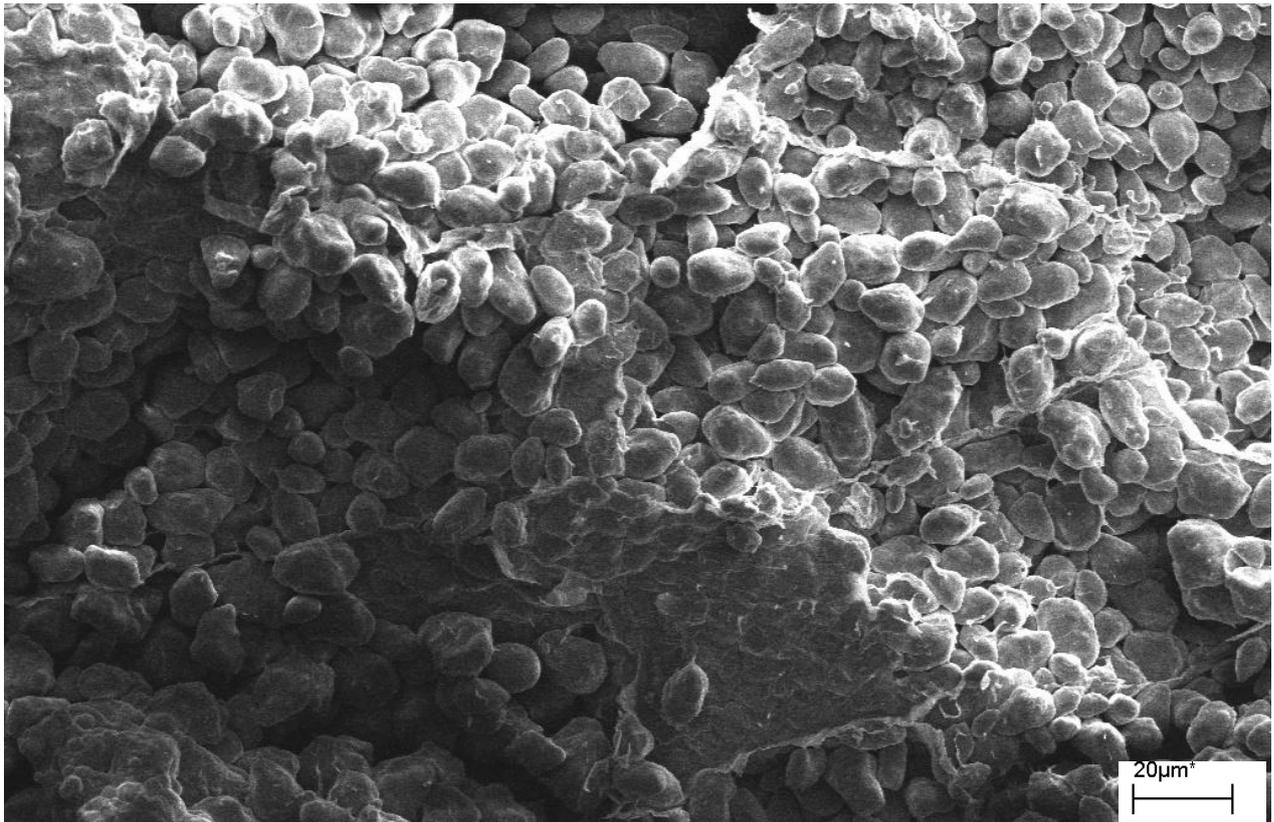
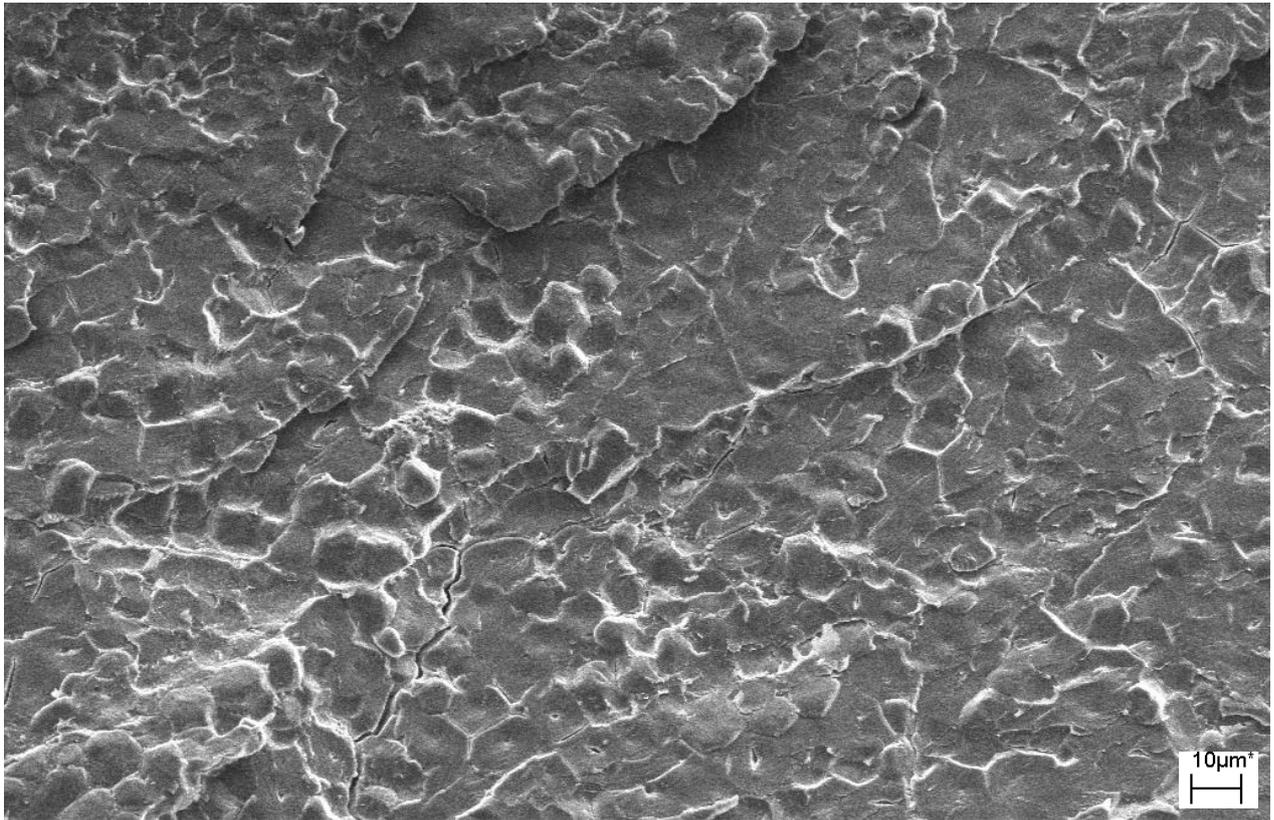


Figure 4.8 Scanning electron micrographs of kernel of hybrid H3C2 a where a floursy and vitreous region was present. (a) The edge of the kernel where starch granules are more compact and (b) the central region of the floursy endosperm where starch granules are more loosely packed.

It has been reported, apart from the size and structure of the zeins and starch granules, the bond between zein and starch also determines the degree of hardness (Chandrashekar & Mazhar, 1999). A stronger degree of bonding will result in a higher degree of hardness. The difference in morphology indicated starch analysis in conjunction with protein should be considered when assessing hardness. A negative correlation ($r=-0.93$) between damaged starch and friability has also been reported (Mestres & Matencio, 1996). By enforcing mechanical stress, kernels with more undamaged (intact) starch will be more friable. Starch granules were less damaged in harder kernels and it was suggested it acted as a passive filler in these kernels resulting in a stronger compact starch structure (Mestres & Matencio, 1996).

Amylose has been positively linked with kernel texture (Dombrink-Kurtzman & Knutson, 1997; Gibbon *et al.*, 2003; Blandino *et al.*, 2010). Amylose content (%) differed significantly ($P<0.05$) between vitreous and flourey endosperm being lower in the latter. SEM revealed the surface of starch granules were rough and wrinkly (caused by the electron beam) with pores randomly distributed on the surface, suggesting increased amylose content would enhance compressibility, resulting in compact starch granules. Starch granules in flourey endosperms were higher in amylopectin, resulting in less compressing due to its crystalline structure. Amylose content was positively correlated with degree of hardness assessed using different hardness measurements (Blandino *et al.*, 2010).

Zein profiles assessed with RP-HPLC

Evaluation of chromatograms

The areas of peaks corresponding to α -zeins were summed together to obtain the total α -zein (zein-1) content for each sample. The β - and γ -zein peaks were summed together to obtain zein-2. Retention times obtained in this study were slightly longer, especially for zein-1 peaks, to times previously reported using a similar separation gradient (Robutti *et al.*, 2000) (Fig. 4.9a&b). This can be attributed to the slower elution rate of 0.9 mL/min compared to 1 mL/min (Robutti *et al.*, 2000).

The zein-2-peaks consisted of three prominent peaks as well as low abundant (<2% of total area) peaks. The identities of these peaks will be discussed in the next section. The most hydrophobic proteins, α -zeins, eluted last. A single peak containing the 10 kDa δ -zein eluted after the peaks containing α -zeins (Fig. 4.9c) (Dombrink-Kurtzman & Beitz, 1993; Flint-Garcia *et al.*, 2009). Low abundant peaks eluting after the α -zeins, at 49.9-50.1 and 52.3 min, were only observed for certain hybrids in the current study. MALDI-TOF MS spectra of zein (Chapter 3, p. 74-83) indicated δ -zein was present for all hybrids. Peaks containing δ -zein were not indicated in previous studies using the same linear gradient (Eyherabide *et al.*, 1996; Robutti *et al.*, 2000). Therefore, the peaks in the current study were likely α -zeins, eluting later..

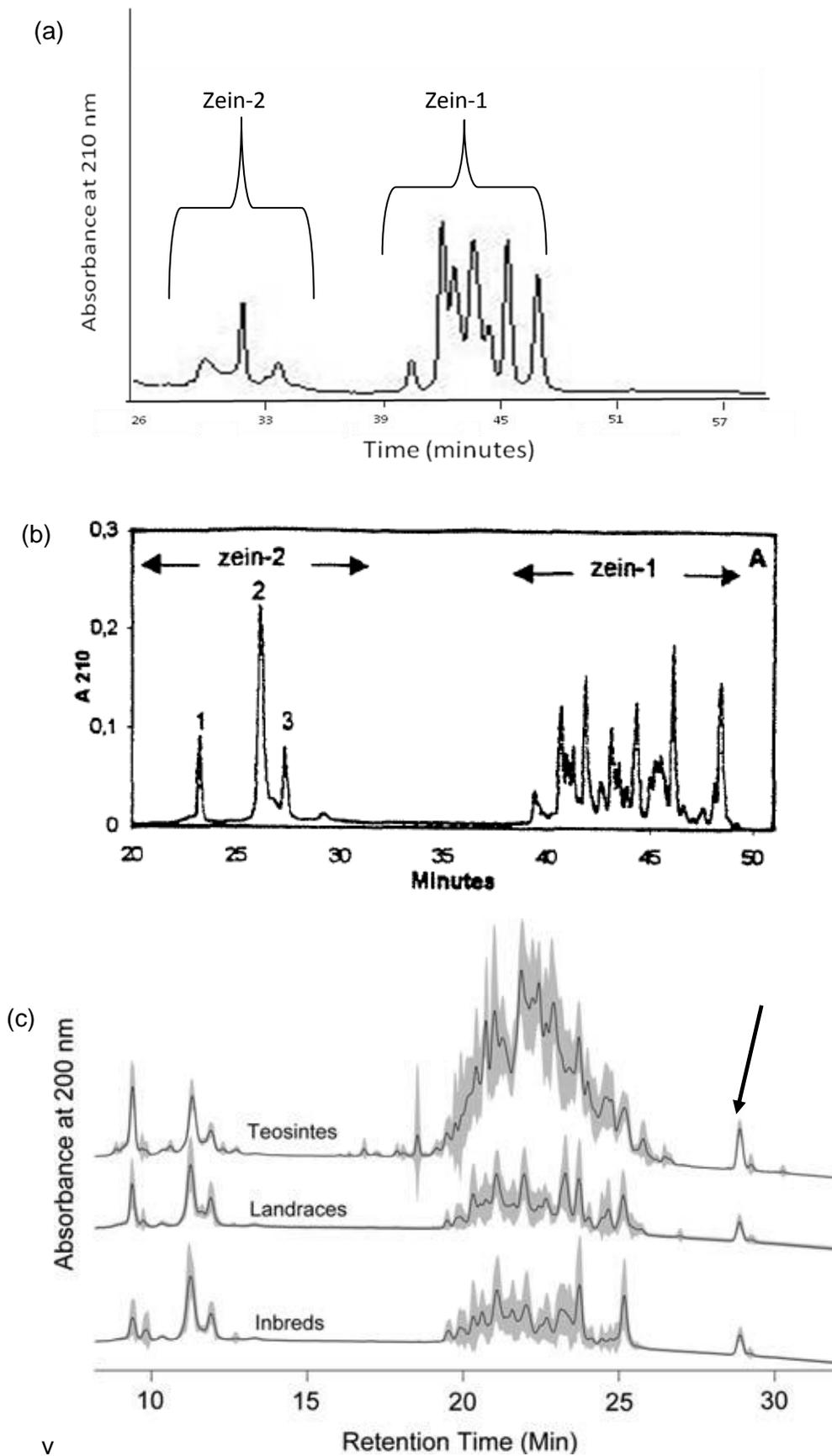


Figure 4.9 Chromatograms of zein from (a) the parent line, P7, (b) hybrid Constallno Colorado (CC) (Robutti *et al.*, 2000) and (c) teosintes, landraces, and inbred lines (Flint-Garcia *et al.*, 2009). The arrow indicates the position of 10 kDa δ -zein which is absent in (a) and (b).

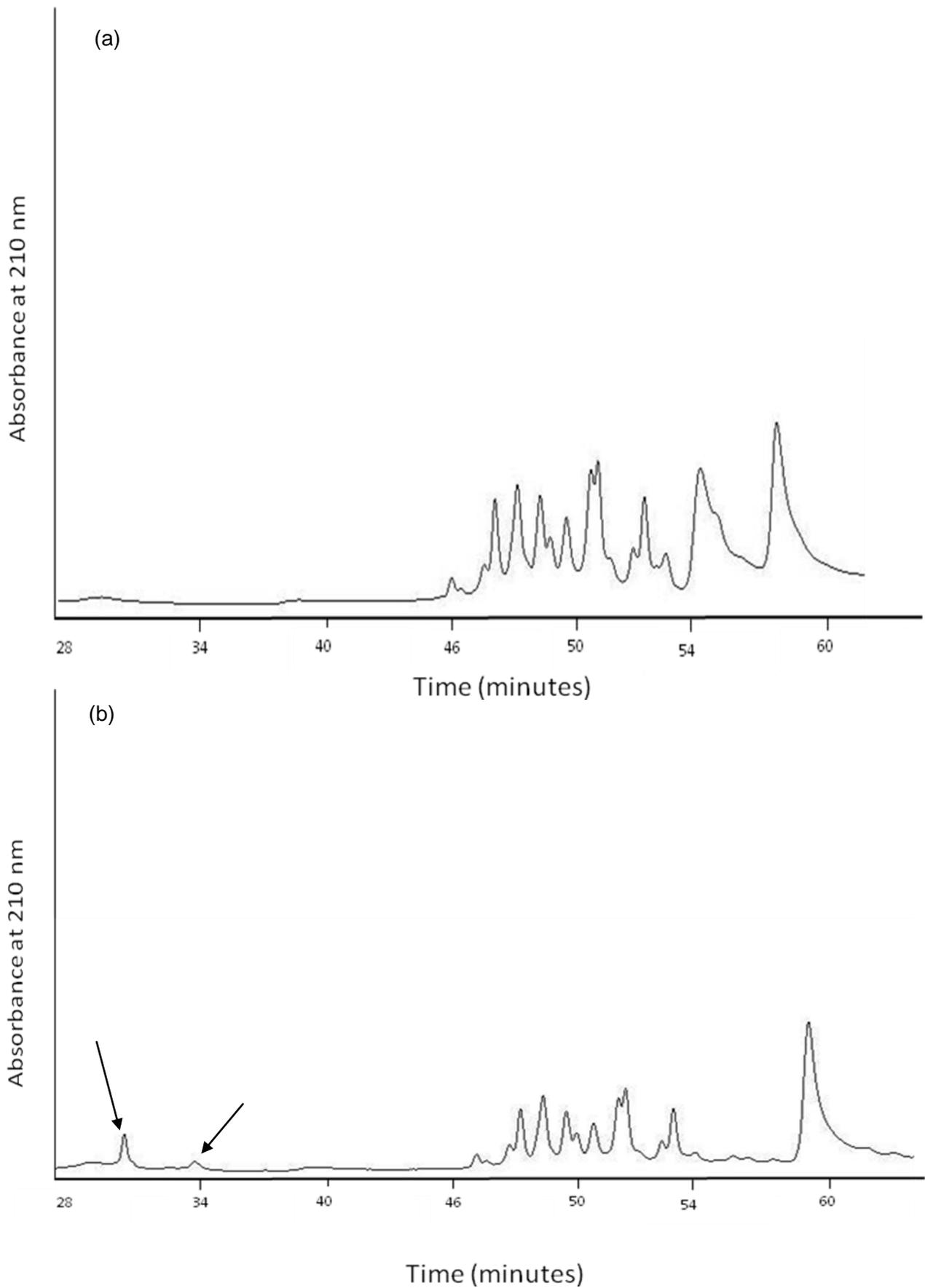


Figure 4.10 Chromatograms of zein from samples, (a) H10A2 and (b) the parent line P7, obtained while the column was operated at 60°C. Two peaks (indicated with black arrows) eluted in zein-2 region for P7 whereas no peaks eluted in this region for H10A2.

Less α -zein peaks were also observed compared to chromatograms from previous studies (Dombrink-Kurtzman & Beitz, 1993; Eyherabide *et al.*, 1996; Robutti *et al.*, 2000). This can be attributed to the column operating at ambient temperature and not at an elevated temperature of 55°C (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994) or 60°C (Eyherabide *et al.*, 1996; Robutti *et al.*, 2000). Zein from hybrid, H10A2, and the inbred parent line, P7, were subjected to duplicate RP-HPLC analysis with a column operating at a temperature of 60°C. Although more peaks were present in the α -zein region, peaks in the zein-2 region of the chromatograms were absent for hybrid H10A2 (Fig. 4.10a) and only two peaks were present for the parent P7 (Fig. 4.10b). The α -zein peaks also had longer retention times and large irregular peaks eluted later. This was true for both the duplicate samples. Therefore, an operating temperature at ambient temperature was used in this study to also obtain zein-2 peaks.

Identification of zein classes

(Detailed results in Appendix 2)

A total of 25 peaks (peaks 1-25) were obtained. Variation amongst hybrids existed in both zein-1 and zein-2 regions of the chromatograms. Zein-2 regions consisted of a total of 11 peaks and zein-1 region of 14 peaks. Nine of the 11 zein-2 peaks and 11 of the 14 zein-1 peaks are depicted in Figs. 4.11a&b.

Three prominent peaks (peaks 3, 5 & 10) were present in zein-2 regions of most hybrids. Retention times for each peak ranged from 28.9 to 29.4, 31.3 to 31.7 and 33.4 to 33.7 min, respectively. Several low abundance peaks (<2% of total area) in both zein-1 and -2 regions with retention times ranging between 28.0 and 28.8 (peaks 1 and 2), 30.9 and 31.1 (peak 4) 32.1 and 33 (peaks 6-8), 37 and 35 min (peaks 10 and 11), 40.7 and 40.9 peak 12 and 51.0 and 51.2 (peak 25) were observed for some hybrids. Low abundance peaks (<2% of total area) have previously been observed in chromatograms but not included in analysis of data (Paulis *et al.*, 1993; Mestres & Matencio, 1996; Robutti *et al.*, 2000). Peaks 3, 5 and 10 were initially labelled as 15 kDa β -zein, 27 kDa γ - and 16 kDa γ -zein, respectively (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994; Eyherabide *et al.*, 1996; Mestres & Matencio, 1996). Hybrids 1, 7 and 10, however, had larger peak areas (3.5-7.5%) for peak 6 (retention time ~31.9 min) compared to other hybrids which had peak areas <1.5% (Fig. 4.11 c, j & l). Peak 6 eluted as a doublet with peak 5. MALDI-TOF MS spectra (Chapter 3, p. 74, 80 and 83) of these hybrids (H1, H7 and H10) indicated an additional signal in the β -zein region with a molecular weight of ~17150 Da. It is likely this RP-HPLC peak, peak 6, corresponded to this signal in mass spectra.

Two retention times have been shown for β -zein from different inbred lines and it was suggested their genes behaved as if they were allelic at the same locus (Wilson, 1991). It has been observed β -zeins eluted as doublets followed by 27 kDa γ - and 16 kDa γ -zein. This indicated some hybrids presented both genes for the β -zein and the areas of these peaks were summed to obtain one area for β -zein (Mestres & Matencio, 1996).

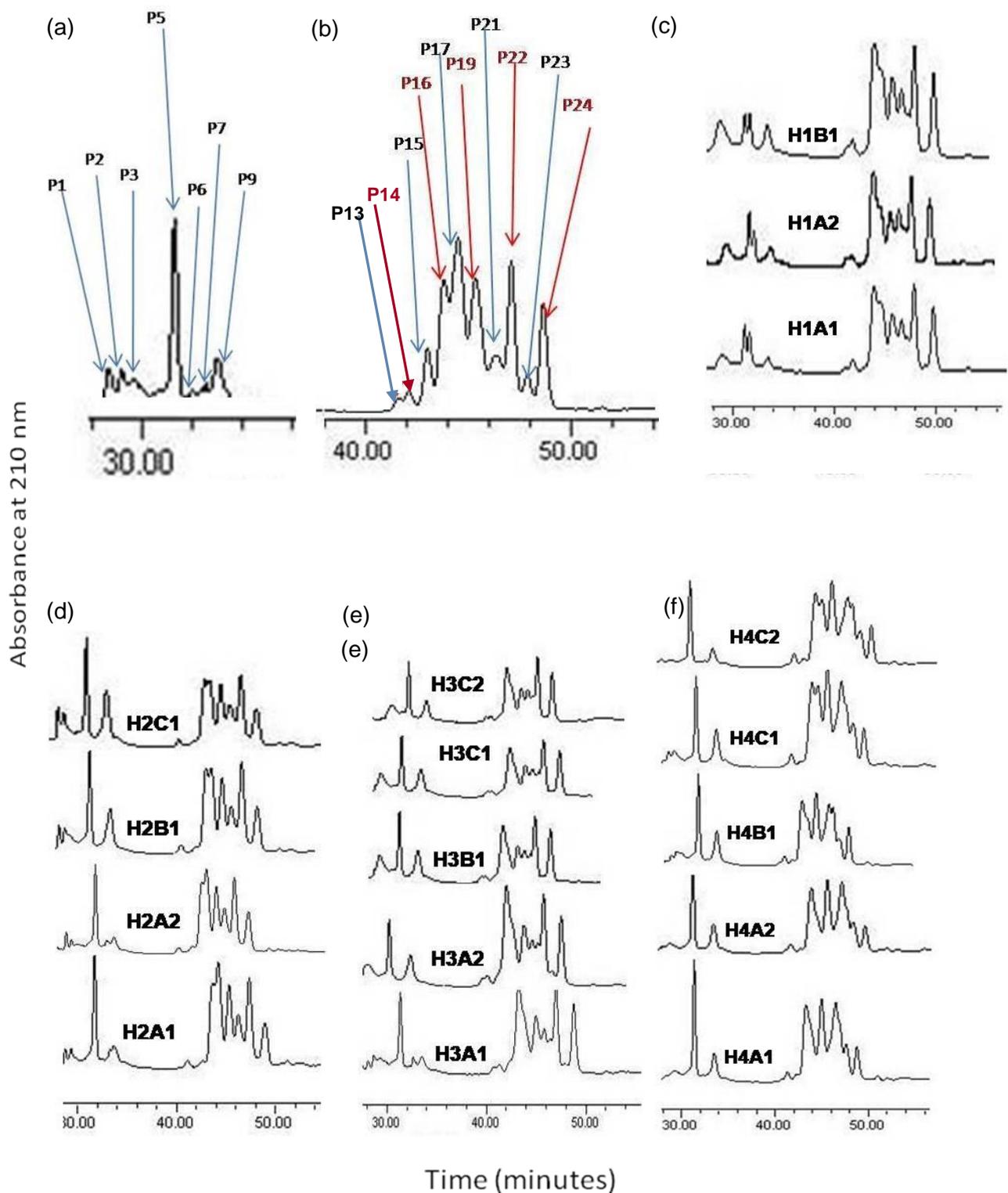


Figure 4.11 RP-HPLC chromatograms of (a) zein-2 region of H9C1, (b) zein-1 region of H9C2 (where peak numbers are indicated) (c) H1, (d) H2, (e) H3, (f) H4, (g) H5, (h) H6, (i) H7, (j) H8, (k) H9 and (l) H10. Localities and field replicates are indicated above each chromatogram (A=Greytown, B=Klerksdorp, C=Delmas).

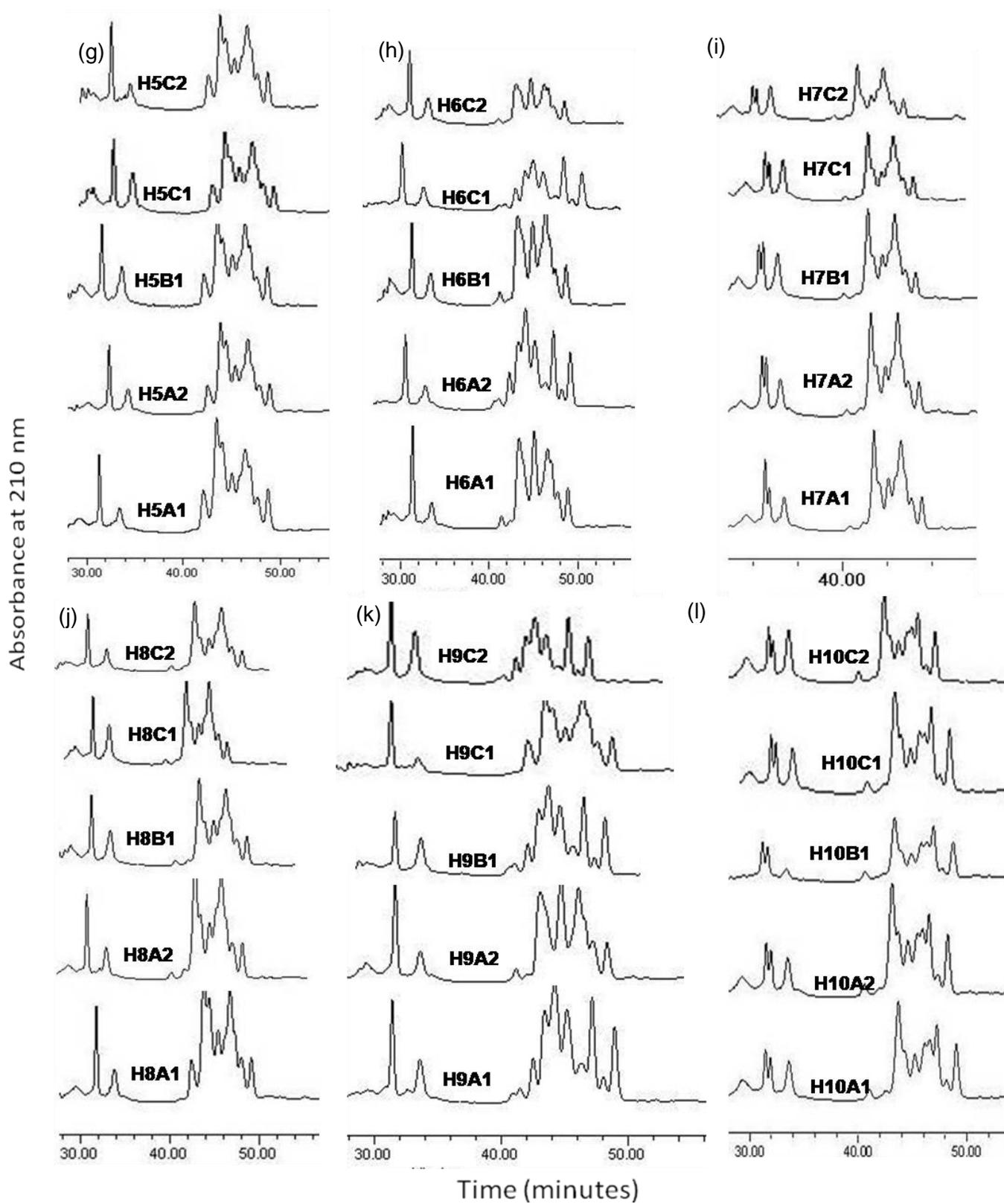


Figure 4.11 continued.

The doublet in the current study eluted after the first prominent peak (peak 3) followed by the third prominent peak (peak 10). This suggested the doublet did not contain the β -zeins, when compared to chromatograms indicating β -zeins eluted first (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994; Eyherabide *et al.*, 1996). Also, MALDI-TOF MS analyses of hybrid H2 had both peaks corresponding to the β -zeins (Chapter 3 p.75) but chromatograms indicated a doublet before peak 5 which coincided with literature (Mestres & Matencio, 1996). It was unlikely the second peak in the doublet after peak 3 was one of the other 27 kDa γ -zeins observed in MALDI-TOF MS spectra (Chapter 3, p. 74-83) of certain hybrids. Firstly, as MALDI-TOF MS analysis of hybrid H1 did not indicate a second 27 kDa γ -zein. Secondly, because this peak, at similar higher percentage areas, was not observed in chromatograms of the hybrids containing more than one 27 kDa γ -zein as indicated by MALDI-TOF MS analyses. Overall, this suggested peak 3 did not contain β -zein, peak 5 not 27 kDa γ -zein and the peak containing 16 kDa γ -zein was absent. The latter is not possible because MALDI-TOF MS analysis indicated all hybrids contained the 16 kDa γ -zein. Uncertainty of the identities of the peaks in zein-2 region, thus, exists and they will be referred to as peaks 3, 5 and 10 in the remainder of the chapter.

Zein-1 regions of the same hybrids, grown at the different localities, had similar chromatographic patterns, except for hybrid H6 and H9. Hybrids H6A2 and H6C1 had similar profiles but differed from the other profiles of hybrid H6 samples. Profiles of H9A2 and H9C1 also differed, from one another and other profiles of hybrid H9 samples. The zein from hybrids H6A2, H9C2 were extracted and analysed again and the same chromatographic patterns and comparable peak areas were obtained (data not shown). It is possible contamination of these samples occurred or zein proteins were differently expressed in these hybrids. It was previously reported the environment, thus locality, had minimal effect on RP-HPLC zein profiles (Paulis *et al.*, 1990).

Correlations between RP-HPLC peaks containing zein, protein content and PSI

Correlations between RP-HPLC data, protein content, PSI values and C/F ratio were determined. Peaks indicating significant correlations ($P < 0.05$, $P < 0.01$) are shown in Table 4.1. Total area, zein-1 and -2 regions will be discussed separately.

Total area

Total zein, expressed as total area, correlated positively with protein content. A negative correlation ($P < 0.05$), although not as strong, was observed between total area and PSI-2. Similarly low, but non-significant ($P > 0.05$), correlations have been reported between total zein and other hardness measurements; vitreousness ($r = 0.45$), specific density ($r = 0.36$), and regular and course grits yield ($r = 0.43$) (Mestres & Matencio, 1996). Higher correlations ($P < 0.01$) were observed between total zein and friability ($r = -0.65$), fine flour yield obtained from milling ($r = -0.66$) and flaking grits ($r = 0.64$, $P < 0.05$). High significant ($P < 0.01$) correlations have also been

reported between total zein, test weight ($r=0.917$) and abrasiveness ($r=0.894$) measured using the tangential abrasive dehulling device (Lee *et al.*, 2006), *albeit*, only six samples, three hard and three soft, were correlated and intermediate kernel texture types were not included. Lower but significant ($P<0.01$) correlations between total chromatographic area, density ($r=0.48$) and vitreousness ($r=0.41$) were reported for zein extracted from F2 and F3 generations of reciprocal crosses of two Quality Protein Maize (QPM) vitreous inbreds and two near-isogenic non-vitreous 02 maize inbreds (Paulis *et al.*, 1993). Overall, these trends and results from this study indicated total zein content plays a role in maize hardness.

Zein-2 region

Collectively, zein-2, expressed as percentage area, correlated ($P<0.01$) negatively with protein content. Non-significant ($P>0.05$) correlations with PSI measurements were observed but correlations indicated a negative relationship with PSI-1 and C/F ratio and positive relationship with PSI-2 and -3. Peaks 5 and 6 were grouped together due to eluting as a doublet. Negative correlations ($P<0.05$) were observed between percentage areas of prominent peaks 5, 5+6 and 10 and protein. Negative, non-significant ($P>0.05$), correlations between these peaks with PSI-1 and positive correlations ($P>0.05$) with PSI-2 and -3 were observed. An opposite relationship was observed for peak 6. It should be noted this peak was only present in three of the 10 hybrids. Although weak correlations were observed, these trends agree with a previous report using RP-HPLC analysis of zein, extracted from vitreous and floury endosperms. The authors observed a negative relationship between 27 kDa γ -zein, 16 kDa γ -zein, 15 kDa β -zein and endosperm texture (Dombrink-Kurtzman & Beitz, 1993). The vitreous and floury endosperm differed significantly in percentage areas, being higher in the floury endosperm, for 27 kDa γ -zein ($P<0.05$) and 16 kDa γ -zein ($P<0.01$). This indicated more of these classes were expressed in floury endosperm due to less α -zeins present. This was consistent with reports indicating the floury endosperm has less developed protein bodies (Lending & Larkins, 1989). When comparing actual amounts of the γ -zeins (based on integrated area of peaks), similar amounts were obtained from both hard and soft endosperm (Dombrink-Kurtzman & Beitz, 1993). In another study, a negative correlation ($P<0.10$) between 27 kDa γ -zein and vitreousness was obtained (Paulis *et al.*, 1993).

Positive correlations, although non-significant ($P>0.05$), were observed between zein-2 area (integrated area expressed as arbitrary units (AU)) and PSI-1 values, C/F ratio and protein content. Variation in correlations can be attributed to the fact hybrids can have a similar percentage area (of total area) of a given protein but the actual amount (area (AU)) of this protein can differ. Peaks 5+6, expressed as areas (AU), correlated ($P<0.01$) positively with PSI-1 and protein content and negatively ($P<0.05$) with PSI-2. Similar trends between peaks 5, 6 and 10 (AU), although non-significant ($P>0.05$), and PSI-1 and PSI-2 were observed. Peak 6, as seen when expressed as percentage area, correlated negatively with PSI-3 ($P<0.01$). Peak 5

(AU) correlated positively ($P<0.05$) with total protein. The correlations obtained were not strong but it did indicate a trend.

The correlations between areas (AU) of peaks and kernel texture from the current study agreed to a certain extent with studies where RP-HPLC peaks were correlated with degree of hardness of samples assessed with various hardness measurements (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006). Positive correlations ($r=0.33$; $P<0.01$) between 27 kDa γ -zein, expressed as percentage and peak area (AU), and grinding time have been reported (Paulis *et al.*, 1993). When expressed as area (AU), similar weak positive correlations ($P<0.01$) were observed with density and vitreousness whereas weak negative, non-significant ($P>0.05$), correlations were observed when expressed as percentage area. β -Zein, expressed as percentage area and peak area (AU), correlated ($r=0.43$; $P<0.01$) positively with grinding time, density and vitreousness (Paulis *et al.*, 1993). Stronger correlations were observed between 27 kDa γ -zein and vitreousness ($r=0.61$, $P<0.01$), specific density ($r=0.71$, $P<0.01$), regular and course grits yield ($r=0.50$, $P<0.05$), friability ($r=-0.64$, $P<0.01$), fine material obtained from milling ($r=-0.58$, $P<0.05$) and flaking grits ($r=0.61$, $P<0.05$) (Mestres & Matencio, 1996). The 15 kDa β -zein also correlated positively, although not significantly, with specific density ($r=0.18$) and regular and course grits ($r=0.31$) and negatively with friability ($r=-0.31$) and fine material obtained from milling ($r=-0.42$). Pratt *et al.* (1994) also indicated positive correlations between peak areas (AU) of 27 kDa γ -zein and density whereas 15 kDa β zein, correlated either negatively or positively, depending on the genotype.

Both β - and γ -zeins include sulfur containing amino acids, cysteine and methionine, responsible for disulfide binding. It is possible more of these proteins will result in increased binding resulting in an improved (higher) hardness level. In addition, vitreous endosperm has more mature protein bodies consisting of all classes with α -zein being most abundant and filling out the PB (Lending & Larkins, 1989). In the flourey endosperm less mature protein bodies are present due to α -zeins accumulating last and, thus, more γ - and β -zeins are present (Lending & Larkins, 1989). More protein bodies appeared to be present in the flourey endosperm for hybrid H4C2 (Fig 4.5b). This observation agreed with the positive correlations between the amount of γ - and β -zeins (expressed as area (AU)) and kernel texture.

Zein-1 region

Total zein-1 expressed as percentage area correlated ($P<0.01$) positively with total protein. Higher positive correlations ($P<0.01$) were observed with area (AU) of peaks. Positive correlations, although non-significant ($P>0.05$), between zein-1 (expressed as percentage area) and PSI-1 and C/F ratio was observed. Negative correlations ($P>0.05$) with PSI-2 and -3 was observed. Area (AU) of zein-1 correlated significantly ($P<0.05$) negative with PSI-2. Significant ($P<0.01$; $P<0.05$) correlations between certain PSI values, protein content and percentage peak areas of peaks 21, 22, 23 and 24 were observed. These peaks were observed for almost all

samples. Peaks 21 and 23, percentage area and area (AU), correlated ($P < 0.01$) positively with PSI-1. Negative correlations were obtained with PSI-2 ($P < 0.01$) and -3 ($P < 0.05$) values. Surprisingly, opposite trends were observed for peaks 22 and 24. Peaks 22 and 24, expressed as percentage area, correlated negatively ($P < 0.05$) with PSI-1 and peak 24 correlated positively ($P < 0.05$) with PSI-2. Area (AU) of peak 22 also indicated, a negative correlation ($P > 0.05$, $r = -0.20$) with PSI-1. Negative weak non-significant ($P > 0.05$) correlations between peak 20, expressed as percentage area ($r = -0.12$) and area (AU) ($r = -0.11$), and PSI-1 were also observed.

Due to unknown identities of the RP-HPLC peaks containing the α -zeins, they have been collectively correlated with traits in literature. To date, only a positive relationship between α -zeins and hardness has been reported (Paulis & Bietz, 1986; Dombrink-Kurtzman & Beitz, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Chandrashekar & Mazhar, 1999; Landry *et al.*, 2004; Lee *et al.*, 2006). It was expected individual α -zein peaks would all correlate positively with PSI-1 and negatively with PSI-2 and -3 due to the α -zeins collectively correlating positively and negatively, respectively. It was, however, not the case. The identity of these peaks is unknown and it would be beneficial to collect these RP-HPLC fractions in future. MALDI-TOF MS or other techniques can be used to identify peaks containing the various zein classes.

Principal component analysis of RP-HPLC zein peaks, protein content and PSI values

Percentage peak areas

PC1 and PC2 explained the largest part of the variation (Fig. 4.12). Although a low percentage (34.7%) of the variation was explained, trends were indicated. Zein-1, total area and protein content were differentiated from peak 24 and zein-2 peaks in the direction of PC1 (Fig. 4.12a). PSI-1, C/F ratio, peaks 21 and 23 were differentiated from peak 17, peak 15, peak 7, PSI-3, PSI-2 and peak 22 in the direction of PC2. The resulting score plot is indicated in Fig. 4.12b. Hybrids H4A2, H4A1, H4C1, H4C2, H6A1, H8A1, H8B1 H5A2 and H9A2 were positively associated. From the corresponding loadings plot it can be seen that these samples were high in protein content and had high PSI-1 values. Loadings plots also indicated hybrids H4A1, H4A2, H8C2, H7A1, H7A1 H7C1 and H7C2 had high percentages for peaks 21 and 23. Hybrids H6A2, H9A2, H5A1, H9B1, H5B2 and H5C1 for example had a higher total zein-1 content and total area. Interpretation of the left half of the loadings plot indicated positive associations between, total area, protein and zein-1. PSI-1, C/F and peaks 21 and 23 were closely associated. These variables were positively associated with protein content. Total area and zein-1 did not show strong associations with PSI-1 and C/F.

PSI-2 and -3 appeared to have no association with total area and zein-1. Weak negative correlations ($P > 0.05$) were observed between zein-1 and PSI-2 and -3 (Table 4.1). Due to peaks 1, 2 and -3 eluting closely they were grouped together. Zein-2 peaks 1+2+3, 3 6, 5+6, and 10 were associated and oppositely associated with total area and zein-1. Peak 6

associated negatively with PSI-3. Zein-2 peaks 3, 5+6, and 10 had negative associations with protein. This coincided with correlation data (Table 4.1). From the corresponding scores plot it can be seen hybrids H3C2, H3C1, H6C1 and H9C1 clustered together and had high PSI-2 and PSI-3 values and α -zein peaks 22 and 24 (Fig. 4.12b). Hybrids H9C1 and H10B2 had the highest percentage area of peaks 3 and peaks1+2+3 and this can be seen based on their position near these variables.

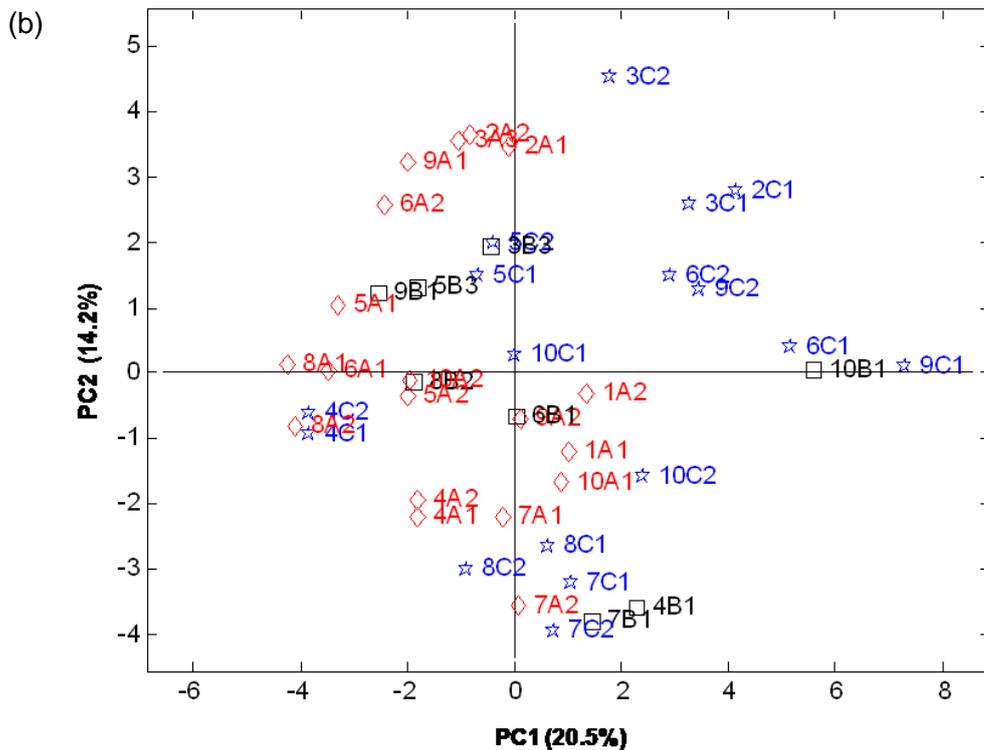
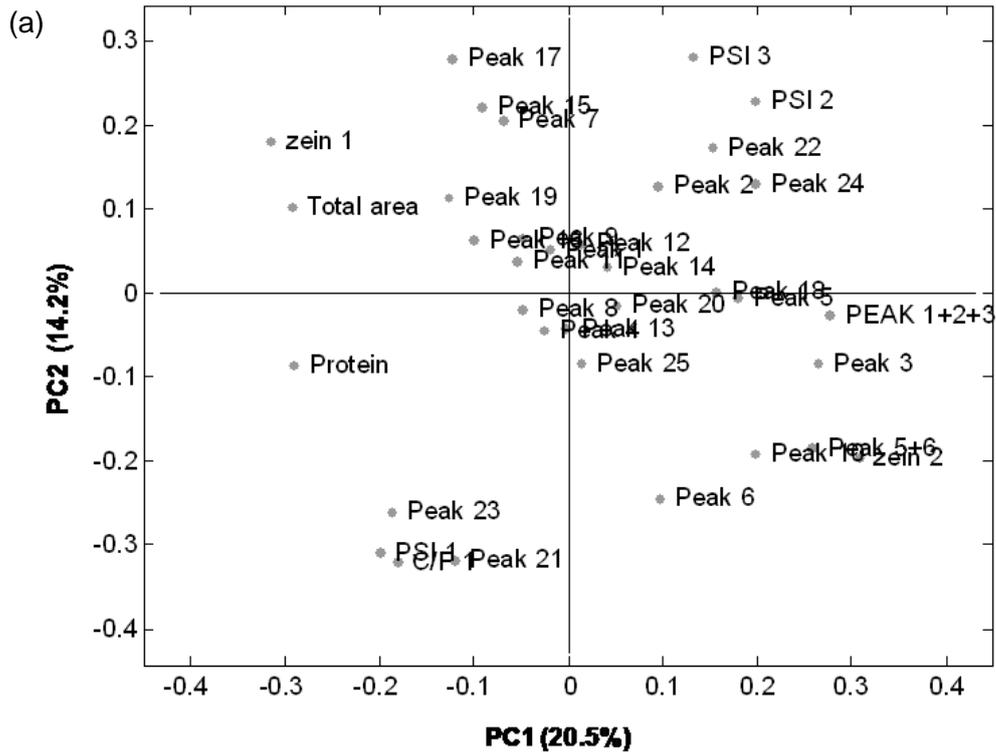


Figure 4.12 PCA loadings (a) and scores (b) plots, PC1 vs. PC2, of percentage area of zein peaks, protein content and PSI values. (A=Greytown, B=Klerksdorp, C=Delmas)

Peak areas given as AU units

The PCA loadings plots using peak areas (AU) differed from percentage peak areas. PC1 and PC2 (area AU) explained 36.4% of the variation. Total area, zein-1, zein-2, protein content and peaks 5, 5+6, 19 and 16 were not well differentiated from other variables in the direction of PC1 (Fig. 4.13a). Peaks 21 and 23, PSI-1, C/F ratio and peak 6 associated and were differentiated from PSI-2, PSI-3, and peaks 22 and 24 in the direction of PC2. Peak 6 and 10 associated positively with PSI-1 and negatively with PSI-2 and -3. Peak 22 also had a negative association with PSI-1. These trends were consistent with correlation data (Table 4.1). Peak 6 seemed to associate negatively with protein content and peak 10 positively and stronger with protein content. This was not consistent to correlation data but correlations were close to zero and thus the relationship between these variables was not well defined. The corresponding scores plots indicated a similar association of hybrids as previous PCA model using percentage peak areas (Fig. 4.13). Hybrids with high protein content and PSI-1 values were oppositely associated with hybrids that had high PSI-2 and -3 values.

Other principal component (PC3 and 4) of both PCA models (Figs 4.12&13) did not reveal any new information. Therefore, they were not discussed.

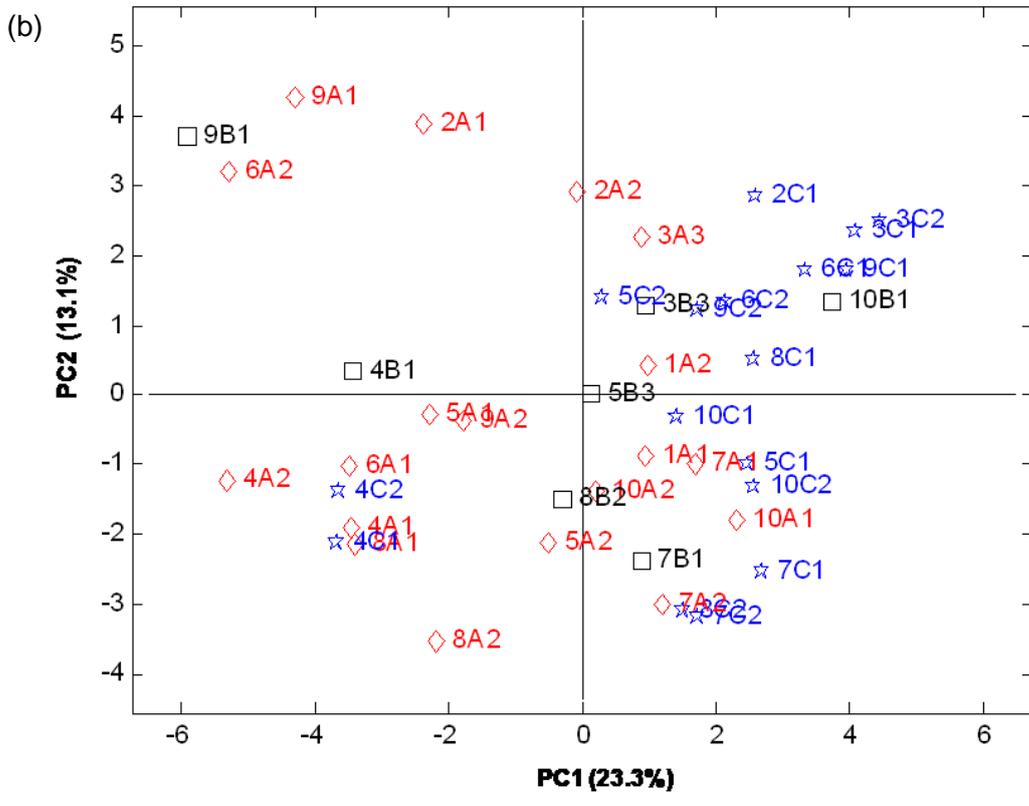
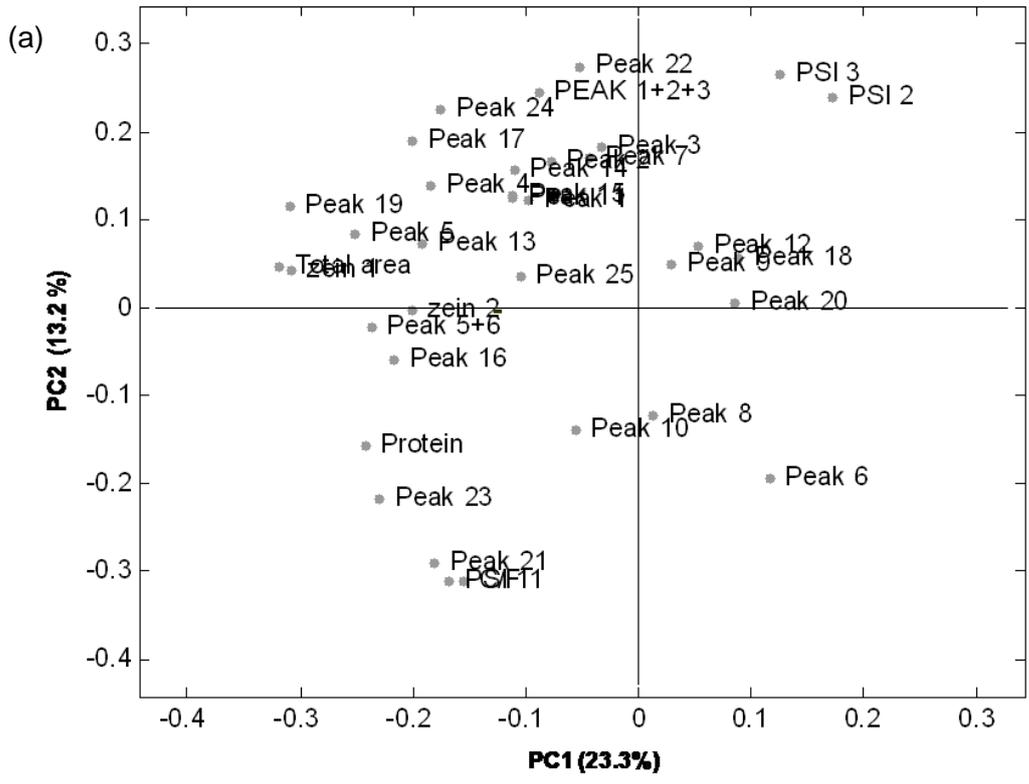


Figure 4.13 PCA loadings (a) and scores (b) plots, PC1 vs. PC2, of area (AU) of zein peaks, protein content and PSI values. (A=Greytown, B=Klerksdorp, C=Delmas)

Conclusion

Protein content was positively correlated to a harder kernel texture. Starch granule packing (evaluated using SEM) between flours of endosperms of hard and soft hybrids differed. Starch types should, thus, also be considered when evaluating kernel texture of maize. Total zein and α -zeins (zein-1) and most individual α -zeins correlated positively with protein and a harder kernel texture. Zein-2 (β - and γ -zeins) peaks, expressed as percentage peak areas of total area correlated negatively with a harder endosperm whereas opposite trends were observed when expressed as integrated area (AU). Therefore, the amount of these proteins influenced kernel texture.

PCA loadings plots using the first two principal components separated hybrids with a highest degree of hardness from softest hybrids in score plots. Loading plots indicated that hybrids with a higher degree of hardness were higher in protein content, total zein, zein-1 (area (AU)) and zein-2 (area (AU)). Thus, PCA can be used to associate various kernel texture related variables. It has the advantage of simultaneously associating variables and more information can be gained. Overall, results suggested total protein and zein proteins have an impact on kernel texture but correlations were not as strong and explained variation of PCA models low. Other methods and maize characteristics should also be considered to evaluate kernel texture.

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Chapter 5

General discussion and
conclusions

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Zein is the prolamin (alcohol soluble protein) of maize. It is the major storage protein and constitutes up to 70% of the total protein in conventional maize varieties and is located in protein bodies in the endosperm (Lending & Larkins, 1989; Prasanna *et al.*, 2001). Zein has been divided into four major classes, namely α -, β -, γ -, (Esen, 1987) and δ -zein (Kirihaara *et al.*, 1988; Woo *et al.*, 2001); each differing in abundance, molecular weights, solubility and pI values. Zein is important for its commercial application as an industrial polymer (Shukla & Cheryan, 2001), impact on the nutritional protein quality of maize (Gibbon & Larkins, 2005) and, together with total protein content, for their relationship to kernel texture (Dombrink-Kurtzman & Beitz, 1993; Mestres & Matencio, 1996; Blandino *et al.*, 2010). The latter formed the main focus of this thesis. A procedure for matrix assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) was optimised to characterise zein from three field replicates of ten white maize hybrids, grown at three localities, and their respective inbred parent lines. Zein from hybrids was quantified using reverse-phase high performance liquid chromatography (RP-HPLC). Kernel texture of hybrids was assessed with a particle size index (PSI) procedure. Total protein content was also determined.

In this study zein expression was characterised to differentiate between samples. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicate α -zeins comprise two (19 kDa and 22 kDa) γ -zeins comprise three (27 kDa, 16 kDa and 50 kDa) and δ -zein comprise two (10 kDa and 18 kDa) sub-classes (Woo *et al.*, 2001). The subclasses of α -zeins can further be divided into a family of homologous proteins with similar molecular weights. SDS-PAGE has classically been used to characterise zein proteins. This technique however, cannot distinguish between proteins with similar molecular weights. MALDI-TOF MS analysis of zein has been reported to provide better detection of zein classes (Adams *et al.*, 2004). The zein extraction and matrix preparation procedure for MALDI-TOF MS analysis was optimised in this research study.

Optimisation was predominantly based on the behaviour of the water soluble 27 kDa γ -zein with respect to two matrices [2-(4-hydroxyphenylazo)benzoic acid (HABA) and α -cyano-4-hydroxy-cinammic acid (CHCA)] and the pH of the matrix solution. Two extraction temperatures (ambient and 60°C) and extraction from defatted (DF) and non-defatted (NDF) maize meal was also investigated. Zein extracted from NDF maize meal at ambient temperature and the use of these matrices in combination dissolved in a 70% acetonitrile (ACN) solution (pH 2.9) gave optimal results in terms of signal-to-noise (S/N) ratio and detection of all major zein classes. The advantage of this procedure is that defatting of the maize meal prior to extraction and an elevated extraction temperature was eliminated. The CHCA matrix is a more water soluble

matrix and improved the detection of the 27 kDa γ -zein. Particle size of maize meal did not influence the quality of spectra and maize milled using a 1 mm sieve was sufficient for zein extraction. Using MALDI-TOF MS, seven of the possible nine α -zein peaks (Woo *et al.*, 2001) were observed for the hybrids and inbred maize lines in this study. Additional peaks corresponding to β -, 27 kDa γ - and δ -zeins were also observed. This could possibly be the result of allelic variation, but needs to be confirmed with detailed genetic studies.

Similarities in spectral profiles were observed between hybrids and their respective parent lines. Zein profiles of hybrids appeared to be a combination of their parent lines. Zein profiles of most hybrids did not differ between localities. Overall, these zein profiles could not have been gained using conventional SDS-PAGE and illustrated the value of MALDI-TOF MS analyses when assessing zein profiles.

Maize hardness is an important quality trait for many sectors in the maize industry. It is a positive trait for dry milling and a negative trait for wet milling. Maize endosperm constitutes up to 80% of the total kernel and consists of two types: vitreous (hard) and floury (soft). In South Africa dry milling is used for production of maize grits, samp, maize meal, and adequate hardness is necessary to obtain optimum yield and milling characteristics. Maize with good milling characteristics have a higher proportion of vitreous endosperm and will give a greater yield of larger maize grits, with a higher economic value. Thus, breeders aim to increase the proportion of the vitreous endosperm in newly released maize hybrids. At present maize kernel texture in breeding lines is assessed by visual inspection; protein and more specifically zein contents are not characteristics considered.

Results presented in this and other studies (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006; Blandino *et al.*, 2010) indicated total protein and zein proteins (assessed using RP-HPLC) play a role in kernel texture, and should be considered when evaluating kernel texture. In this study a two sieve method was used to assess kernel texture. Two sieves (150 μ m placed on top of a 75 μ m) fitted with a receiving pan were used to separate maize meal fractions after milling through a 1 mm sieve. Three PSI values were calculated, PSI-1 (fraction retained in 150 μ m sieve), 2 (fraction retained in 75 μ m sieve), and 3 (fraction obtained in receiving pan). A higher PSI-1 value indicated a harder kernel texture and a higher PSI-2 and PSI-3 the opposite.

The total protein content differed significantly ($P < 0.01$) between localities, implying environmental factors and/or genotypes played a role. Kernel texture also varied between localities, with a higher protein content correlating positively with a harder kernel texture. Although discrepancies existed between PSI values and protein content, visual inspection indicated kernels with a higher proportion of floury endosperm tended to have a lower degree of hardness (lower PSI-1 and higher PSI-2 and -3) and protein content.

Similar to MALDI-TOF MS zein profiles, RP-HPLC chromatographic zein profiles of most hybrids did not differ between localities, implying the environment did not have a big impact on

zein expression patterns. This was consistent with a previous study reporting the environment had minimal influence on chromatographic profiles (Paulis, 1990). Total area (zein) correlated positively with protein content. As mentioned protein content differed significantly ($P < 0.01$) between localities and, thus, total zein content was subsequently influenced. This implied environmental factors most likely influenced the amount of zein proteins expressed. Exposure of maize plants to elevated temperatures (Monjardino *et al.*, 2005) and variation in nitrogen fertilizer rates (Tsai *et al.*, 1980) have been shown to influence zein content.

Correlations and principal component analysis (PCA) indicated if β - and γ -zeins (zein-2) were collectively expressed as percentage area of total RP-HPLC peak area, negative correlations with PSI-1 was observed. When expressed as integrated area (proportional to amount of proteins) given as arbitrary units (AU), a positive correlation was obtained. Therefore, the amount of these proteins present, influences kernel texture. A similar trend was observed for the individual β - and γ -zeins peaks. This was in agreement with results obtained from scanning electron microscopy (SEM) micrographs. The floury endosperm of the harder hybrid appeared to have more protein bodies than that of a softer hybrid. It has been suggested the protein bodies of floury endosperm are less mature; the floury endosperm contains smaller protein bodies due to the presence of less α -zein and more β - and γ -zeins (Lending & Larkins, 1989). This can possibly explain the positive correlations obtained between β - and γ -zeins when expressed as amount present. Total zein and most individual α -zeins sub-classes contents, correlated positively, whereas certain α -zein sub-classes correlated negatively with a harder kernel texture.

Two hybrids that differed significantly ($P < 0.01$) in kernel texture, one having the highest PSI-1 value (hard kernel) and the other the lowest (soft kernel), were subjected to scanning electron microscopy. The vitreous endosperm between the two hybrids was similar; starch granules and proteins were densely packed forming a smooth surface. The starch granules in the floury endosperm of the softest hybrid were spherical and loosely packed whereas starch granules appeared to be more densely packed in the hardest hybrid. Therefore, indicating starch also plays an important role in kernel texture. Total starch content has been negatively linked to a harder kernel texture (assessed with various hardness measurements) whereas protein content was positively linked (Blandino *et al.*, 2010). This was expected; if starch content increases the protein content is diluted. A higher amylose:amylopectin ratio correlated positively with harder kernel texture and total protein content. Amylose has also previously been positively linked to a harder kernel texture due to its less crystalline structure, resulting in a higher degree of compressibility and subsequent denser packing of starch granules (Dombrink-Kurtzman & Knutson, 1997). Analyses of these starch types should, therefore, be considered in future studies.

Correlations obtained in this study between protein content, PSI values and RP-HPLC zein data, were not strong and/or significant. The explained variation by the first two principal

components was also not high. Stronger positive correlations for total protein content with other hardness measurements have been reported (Blandino *et al.*, 2010). Overall weak correlations ($r < 0.5$) have been reported between zein contents (assessed with RP-HPLC) and degree of hardness (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996). In these studies 70 % ethanol was also used as an extraction solvent. It is possible a higher alcohol (e.g. 2-propanol) can result in a more quantitative extraction of these proteins. Thus, higher alcohols should also be considered in future studies. More kernel texture assessment techniques should also be included to provide a better understanding of the relationship between protein content, zein content and kernel texture. Hardness assessment techniques should resemble parameters in industrial milling processes, such as milling time, milling energy, total grit yield and coarse-to-fine ratio (Blandino *et al.*, 2010). In addition, maize constituents and subsequently, kernel texture are influenced by the environment, genotype, maturity level of hybrid and duration of grain fill period. All these parameters should, thus, be considered when evaluating kernel texture in future studies to obtain a better understanding of maize hardness to, subsequently, breed suitable varieties.

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Appendices

Appendix 1

Table 1.1 Protein and moisture contents and PSI values of two field replicates of hybrids

Hybrid	Protein (%)	Moisture (%)	PSI 1 (%)	PSI 2 (%)	PSI 3 (%)
H1A1	10.90	11.76	74.24	14.35	10.88
H1A2	11.09	10.90	72.24	15.72	11.85
H2A1	9.75	10.75	65.39	18.37	16.64
H2A2	9.82	11.71	66.89	15.52	17.16
H3A1	10.33	11.32	68.10	17.13	14.69
H3A2	9.77	11.35	67.27	18.75	12.99
H4A1	10.35	11.99	72.24	14.68	12.01
H4A3	10.17	11.71	72.32	14.42	11.95
H5A1	11.96	11.59	70.52	15.36	13.72
H5A2	12.13	11.59	72.72	15.02	12.17
H6A1	10.57	11.68	71.85	14.37	13.6
H6A2	9.77	10.84	69.92	16.77	13.09
H7A1	10.13	11.77	70.38	18.19	11.81
H7A2	10.21	11.57	71.99	15.09	13.01
H8A1	10.65	11.22	71.66	14.79	13.4
H8A2	11.10	11.78	72.32	14.3	12.64
H9A2	11.51	11.36	65.98	13.35	20.36
H9A3	11.34	11.67	70.15	15.77	13.62
H10A1	10.03	11.35	72.11	14.62	12.25
H10A2	10.10	10.87	70.12	15.44	9.22
H1B1	10.80	10.69	72.34	13.85	12.46
H1B2	10.94	11.88	73.02	13.6	11.66
H2B1	7.95	11.23	65.39	17.88	16.64
H2B2	7.79	11.68	68.7	18.92	12.17
H3B1	10.23	10.76	67.05	19.27	12.96
H3B2	10.11	10.88	68.39	16.61	14.8
H4B1	9.92	11.30	72.16	16.17	6.94
H4B2	10.23	11.88	68.18	16.64	14.53
H5B1	9.75	11.34	67.43	17.31	14.59
H5B2	9.89	11.60	68.78	16.85	13.54
H6B1	10.00	11.73	70.7	16.64	12.71
H6B3	10.01	11.39	73.93	15.62	9.94
H7B1	10.06	11.27	70.74	15.35	13.07
H7B3	10.23	11.62	69.18	16.84	13.73
H8B1	10.6	11.89	68.32	16.35	14.97
H8B2	10.35	10.85	68.74	17.92	12.34
H9B1	10.70	10.55	72.86	15.77	10.45
H9B3	10.83	11.64	70.31	16.33	12.63
<u>H10B1</u>	<u>8.58</u>	<u>11.94</u>	<u>64.07</u>	<u>18.97</u>	<u>16.12</u>

Table 1.1 continued

H10B2	8.85	12.98	66.33	17.53	16.13
H2C2	7.34	11.59	61.42	20.06	18.41
H2C3	7.19	11.51	62.89	18.03	18.6
H3C1	7.81	11.76	61.85	17.79	18.89
H3C2	7.45	11.64	58.35	20.11	20.10
H4C1	11.71	11.23	72.82	14.04	12.69
H4C2	11.9	11.77	74.26	14.51	10.44
H5C1	9.33	11.96	69.71	17.07	13.12
H5C2	9.23	11.93	65.31	18.38	16.28
H6C1	7.85	12.17	63.03	18.37	18.94
H6C2	7.70	12.34	67.43	17.84	13.98
H7C1	8.66	11.99	70.37	16.21	12.96
H7C2	8.85	12.45	72.16	15.00	12.04
H8C1	9.02	11.34	71.37	14.02	13.7
H8C2	9.14	11.98	73.13	13.5	13.44
H9C1	7.38	12.23	67.59	17.5	14.4
H9C2	7.63	11.74	66.57	16.52	15.95
H10C1	9.15	11.98	70.19	16.69	12.27
H10C2	9.30	11.85	70.15	16.17	12.99

Appendix 2

Table 2.1 RP-HPLC zein-2 (β and γ -zeins) data expressed as percentage area

Hybrid	zein-2	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11
H1A1	20.11	0.00	0.00	4.71	0.00	6.90	5.83	0.00	0.00	0.00	2.66	0.00
H1A2	20.20	0.00	0.00	5.99	0.00	6.71	3.89	0.00	0.00	0.00	3.61	0.00
H1B1	24.34	0.00	0.00	10.49	0.00	3.89	4.15	0.00	0.00	0.00	5.81	0.00
H2A1	16.08	0.00	1.06	0.97	0.00	10.50	0.00	0.00	0.09	0.86	2.59	0.00
H2A2	13.05	1.57	0.63	0.00	0.00	9.07	0.00	0.23	0.00	0.00	1.54	0.00
H2C1	27.91	0.00	3.28	2.80	0.00	11.14	0.00	0.00	0.32	0.00	10.37	0.00
H3A1	11.29	0.65	0.73	0.00	0.00	7.69	0.00	0.49	0.00	0.00	1.73	0.00
H3B1	14.09	0.08	0.00	3.47	0.00	5.87	0.00	0.00	0.00	0.00	4.67	0.00
H3C1	18.19	0.00	0.54	4.97	0.00	9.46	0.00	0.00	0.00	0.00	7.21	0.00
H3C2	22.18	1.35	0.47	0.00	0.00	13.54	0.00	0.33	0.31	0.00	2.19	0.00
H4A1	19.14	0.00	0.00	2.83	0.00	12.45	0.00	0.00	0.10	0.00	3.76	0.00
H4A3	20.79	0.20	0.70	2.50	0.59	11.97	0.00	0.00	0.00	0.00	4.84	0.00
H4B1	33.85	1.31	0.00	6.01	1.19	16.54	0.00	0.00	0.00	0.00	8.80	0.00
H4C1	13.62	0.19	0.81	0.72	0.00	7.24	0.00	0.00	0.00	0.00	4.65	0.00
H4C2	13.02	0.42	1.57	0.00	0.00	8.88	0.00	0.00	0.00	0.00	2.16	0.00
H5A1	12.36	0.38	0.00	2.33	0.00	6.61	0.00	0.13	0.28	0.00	2.61	0.00
H5A2	20.34	0.56	0.00	0.00	0.00	11.96	0.00	0.00	0.00	0.00	7.81	0.00
H5B3	16.30	0.63	0.00	2.04	0.00	6.71	0.00	0.14	0.22	1.08	5.48	0.00
H5C1	22.72	0.00	0.00	0.02	0.00	13.98	0.00	0.55	0.54	1.18	6.45	0.00
H5C2	18.72	0.42	2.23	1.85	0.00	6.84	0.00	0.23	0.00	0.00	7.14	0.00
H6A1	12.69	0.64	0.46	0.00	0.00	8.25	0.00	0.16	0.00	0.00	3.17	0.00
H6A2	12.83	0.19	0.40	1.05	0.00	7.82	0.00	0.00	0.00	0.38	3.00	0.00
H6B1	22.74	0.81	0.00	1.85	0.00	12.19	0.00	0.00	0.00	0.00	7.88	0.00
H6C1	34.53	0.00	2.36	4.44	0.00	17.21	0.00	0.00	0.00	0.00	10.53	0.00
H6C2	26.00	0.00	0.00	4.16	0.00	15.17	0.00	0.00	0.11	0.00	6.56	0.00
H7A1	24.59	0.30	0.00	2.31	0.00	10.15	4.68	0.00	0.41	0.00	0.00	0.00
H7A3	26.27	0.00	0.00	2.70	0.24	7.93	7.15	0.00	0.15	0.00	8.10	0.00
H7B1	30.88	0.24	0.85	3.69	0.15	7.30	7.18	0.00	0.00	0.00	11.49	0.00
H7C1	27.79	0.00	0.15	0.97	0.00	7.93	5.90	0.00	0.00	0.00	12.84	0.00
H7C2	28.73	0.00	0.19	1.82	0.00	6.58	5.97	0.00	0.20	0.00	13.99	0.00
H8A1	10.83	0.00	0.21	0.66	0.00	6.05	0.00	0.00	0.10	0.00	3.85	0.00
H8A2	11.93	0.00	0.00	0.80	0.00	6.57	0.00	0.06	0.43	0.00	4.07	0.00
H8B2	15.57	0.00	0.62	0.85	0.00	7.27	0.00	0.00	0.05	0.00	6.72	0.00
H8C1	26.12	1.63	0.00	4.73	0.00	13.12	0.00	0.15	0.25	0.00	6.22	0.00
H8C2	24.22	0.00	0.67	0.87	0.00	10.47	0.00	0.00	0.28	0.98	10.95	0.00
H9A1	15.39	0.00	0.00	2.35	0.99	6.98	0.00	0.49	0.00	0.00	4.58	0.00
H9A2	25.33	0.81	0.00	1.89	0.29	16.47	0.00	0.00	0.00	0.00	5.86	0.00
H9B1	16.23	0.53	1.25	2.53	0.85	5.97	0.00	0.00	0.00	0.00	4.91	0.18
H9C1	32.47	0.43	0.00	10.84	0.00	15.63	0.00	0.00	0.00	0.00	5.57	0.00
H9C2	30.94	0.00	0.72	2.75	0.00	15.06	0.00	0.00	0.00	0.00	12.42	0.00
H10A1	22.86	0.00	0.00	2.09	0.00	6.64	5.21	0.00	0.00	0.00	8.92	0.00
H10A2	13.51	0.00	0.00	0.81	0.00	4.03	3.37	0.00	0.00	0.00	5.30	0.00
H10B1	33.75	0.00	1.87	5.45	0.00	7.42	5.75	0.00	0.00	0.00	13.26	0.00
H10C1	17.54	0.00	0.44	1.85	0.00	4.70	3.88	0.00	0.00	0.00	6.68	0.00
H10C2	28.60	0.00	0.08	1.59	0.00	7.66	5.27	0.00	0.00	0.00	13.99	0.00

Table 2.2 RP-HPLC data of zein-1(α -zeins) expressed as percentage area

Hybrid	zein-1	Peak 12	Peak 13	Peak 14	Peak 15	Peak 16	Peak 17	Peak 18	Peak 19	Peak 20	Peak 21	Peak 22	Peak 23	Peak 24	Peak 25
H1A1	79.89	0.39	2.48	0.00	0.00	12.87	0.00	0.00	11.09	10.65	0.00	25.36	0.00	17.05	0.00
H1A2	79.80	1.37	2.21	0.00	0.00	17.60	0.00	0.00	8.43	10.97	4.65	19.44	0.00	15.13	0.00
H1B1	75.66	1.07	3.05	0.00	0.00	14.30	0.00	0.00	10.28	12.03	0.00	18.84	0.00	16.09	0.00
H2A1	83.92	0.00	0.74	0.00	0.70	13.06	19.71	0.00	14.82	10.63	0.00	15.77	0.00	8.30	0.20
H2A2	86.95	0.00	0.56	0.00	0.69	13.26	19.64	0.00	15.18	11.42	0.00	16.68	0.00	9.51	0.00
H2C1	72.09	0.00	0.51	0.00	0.00	12.52	12.41	0.00	12.92	10.23	0.00	15.31	0.00	8.18	0.00
H3A1	88.71	0.82	1.44	0.00	1.17	33.51	0.00	0.00	13.60	8.10	3.88	14.55	0.00	11.64	0.00
H3B1	85.91	0.79	1.40	0.00	0.81	34.88	0.00	0.00	12.95	7.06	4.68	13.08	0.00	10.26	0.00
H3C1	78.47	0.00	1.63	0.00	0.00	27.34	0.00	0.00	10.71	7.82	4.17	14.77	0.00	11.37	0.00
H3C2	77.82	0.00	0.00	0.00	10.18	27.45	15.74	0.00	4.42	1.97	9.63	0.00	3.34	9.09	0.00
H4A1	80.86	0.00	0.86	0.00	0.65	24.27	0.00	0.00	17.08	0.00	26.23	0.00	6.37	5.12	0.29
H4A3	79.21	0.00	1.00	0.00	0.38	22.51	0.00	0.00	17.83	0.00	26.33	0.00	6.43	4.74	0.00
H4B1	66.15	0.00	1.53	0.00	0.13	10.46	0.00	0.00	13.71	0.00	15.75	10.98	5.00	8.15	0.44
H4C1	86.38	0.00	1.13	0.00	0.00	14.50	13.85	0.00	18.54	0.00	26.23	0.00	7.03	5.10	0.00
H4C2	86.98	0.00	1.29	0.00	0.74	14.94	12.55	0.00	18.58	0.00	18.56	8.00	6.53	5.78	0.00
H5A1	87.64	0.00	0.00	0.00	6.40	18.24	14.79	0.00	9.57	5.60	15.42	7.06	5.29	5.28	0.00
H5A2	79.66	0.00	0.00	0.00	7.69	26.61	17.91	0.00	5.53	0.00	11.65	0.00	3.34	6.93	0.00
H5B3	83.70	0.00	0.00	0.00	5.41	16.94	11.60	0.00	9.52	6.04	16.16	7.68	5.02	5.33	0.00
H5C1	77.29	0.00	0.00	0.00	9.10	25.42	15.06	0.00	4.49	1.53	9.60	0.00	3.58	8.51	0.00
H5C2	81.28	0.00	0.00	0.00	6.00	17.16	11.57	0.00	9.69	0.00	26.97	0.00	5.75	4.14	0.00
H6A1	87.31	0.00	1.48	0.00	0.98	26.93	0.00	0.00	19.24	0.00	19.19	7.69	6.07	5.73	0.00
H6A2	87.17	0.00	1.00	1.60	5.75	12.32	22.88	0.00	15.22	0.00	5.44	11.97	2.62	8.36	0.00
H6B1	77.26	0.00	2.12	0.00	0.17	38.79	0.00	0.00	13.19	0.00	11.75	0.00	2.46	8.78	0.00
H6C1	65.47	0.00	1.06	0.00	0.00	8.19	0.00	0.00	15.33	0.00	17.59	10.50	5.08	7.72	0.00
H6C2	74.00	0.00	1.90	0.00	5.35	11.86	20.37	0.00	11.34	0.00	0.00	11.99	1.00	10.18	0.00
H7A1	75.41	0.00	0.00	0.00	0.54	15.80	0.00	0.00	7.78	7.13	31.82	6.16	6.19	0.00	0.00
H7A3	76.43	0.00	0.89	0.00	0.50	15.70	0.00	0.00	6.60	7.20	33.37	0.00	6.23	5.93	0.00
H7B1	69.12	0.00	0.75	0.00	0.00	14.78	0.00	0.00	6.13	5.85	30.88	0.00	5.82	4.91	0.00
H7C1	72.21	0.00	0.66	0.00	0.00	14.78	0.00	0.00	4.97	7.75	32.12	0.00	5.75	6.18	0.00
H7C2	71.27	0.00	0.97	0.00	0.00	15.26	0.00	0.00	5.30	6.78	31.03	0.00	5.46	6.47	0.00
H8A1	89.17	0.00	0.00	0.00	5.73	18.61	14.85	0.00	10.04	0.00	29.23	0.00	6.06	4.66	0.00
H8A2	88.87	0.00	0.65	0.00	1.14	21.51	9.27	0.00	9.50	8.70	26.98	0.00	6.44	4.69	0.00
H8B2	84.43	0.00	0.54	0.00	0.40	20.64	7.72	0.00	10.26	7.86	26.13	0.00	5.89	5.00	0.00
H8C1	73.88	0.00	0.99	0.00	0.00	15.74	0.00	0.00	5.80	7.39	31.65	0.00	7.06	5.25	0.00
H8C2	75.78	0.00	0.80	0.00	0.00	15.68	0.00	0.00	6.48	7.08	33.47	0.00	7.58	4.70	0.00
H9A1	83.13	0.00	0.75	1.48	4.91	11.97	20.67	0.00	15.67	0.00	6.40	11.87	2.70	8.19	0.00
H9A2	74.67	0.00	0.00	2.04	0.27	33.74	0.00	0.00	18.43	0.00	9.54	0.00	3.47	7.18	0.00
H9B1	83.77	0.00	1.04	1.99	4.89	11.74	20.15	0.00	15.44	0.00	6.20	11.75	2.33	8.26	0.00
H9C1	67.53	0.00	0.00	0.00	0.00	10.56	0.00	8.79	8.69	0.00	4.75	19.44	0.00	15.30	0.00
H9C2	69.06	0.00	0.00	1.48	4.46	10.49	20.08	0.00	10.70	0.00	0.00	11.23	1.05	9.57	0.00
H10A1	77.14	0.00	0.00	1.96	0.50	16.67	0.00	0.00	6.07	9.56	12.62	15.28	2.30	12.18	0.00
H10A2	86.49	0.00	0.00	1.49	0.06	21.57	9.08	0.00	10.17	9.56	10.61	12.39	3.00	8.56	0.00
H10B1	66.25	0.00	0.00	2.06	0.00	15.38	0.00	0.00	4.58	8.27	11.21	13.65	0.00	11.10	0.00
H10C1	82.46	0.00	0.00	1.56	0.00	19.83	8.36	0.00	7.75	10.94	8.46	13.71	2.24	9.61	0.00
H10C2	71.40	0.00	0.00	2.41	0.00	15.83	0.00	0.00	4.72	8.31	11.85	14.76	1.82	11.70	0.00

Table 2.3 P-HPLC total area and zein-2 (β and γ -zeins) data expressed as arbitrary units (AU)

	Total area	zein-2	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9	Peak 10	Peak 11
H1A1	2608955	524595	0	0	122959	0	180092	152019	0	0	0	69524	0
H1A2	2981818	602292	0	0	178542	0	200197	115999	0	0	0	107554	0
H1B1	3600330	876469	0	0	377793	0	139991	149530	0	0	0	209155	0
H2A1	5716057	919025	0	60539	55362	0	600449	0	0	5151	49417	148106	0
H2A2	4173877	544832	65715	26354	0	0	378750	0	9740	0	0	64272	0
H2C1	2831875	790347	0	92901	79284	0	315420	0	0	8950	0	293792	0
H3A1	3690409	416813	24140	27093		0	283885		18000	0	0	63695	0
H3B1	3690713	520075	2930	0	127938	0	216742	0	0	0	0	172464	0
H3C1	1816530	330427	0	12019	111512	0	212225	0	0	0	0	161821	0
H3C2	2243026	497577	7327	0	184159	0	265523	0	0	0	0	94653	0
H4A1	4613575	882907	0	0	130433	0	574590	0	0	4485	0	173399	0
H4A3	5449575	1133190	10880	37968	136054	32212	652051	0	0	0	0	264024	0
H4B1	3492331	1181988	45649	0	209872	41492	577636	0	0	0	0	307339	0
H4C1	4870808	733435	10415	43880	38785	0	390031	0	0	0	0	250324	0
H4C2	5384622	634318	20337	76285	0	0	432581	0	0	0	0	105115	0
H5A1	5455062	673975	20880	0	127172	0	360834		7043	15507	0	142539	0
H5A2	3276156	666319	18485	0	0	0	391947	0	0	0	0	255887	0
H5B3	4130252	673142	26147	0	84335	0	277154	0	5660	9037	44509	226300	0
H5C1	2132269	484136	0	0	0	0	298070	0	11817	11602	25180	137467	0
H5C2	4005588	749989	16798	89486	74225	0	273928	0	9406	0	0	286146	0
H6A1	5219710	662178	33444	24125	0	0	430730	0	8314	0	0	165566	0
H6A2	6860330	880370	13019	27183	72031	0	536303	0	0	0	26315	205519	0
H6B1	2687691	611082	21799	0	49809	0	327715	0	0	0	0	211759	0
H6C1	2419549	639462	0	43726	82140	0	318659	0	0	0	0	194937	0
H6C2	1851877	629051	0	0	100532	0	367150	0		2554	0	158815	0
H7A1	2954857	726609	0	8916	68377	0	299892	138279	0	12118	0	199028	0
H7A3	3161023	745164	0	0	0	7455	250750	226074	0	4763	0	256121	0
H7B1	2893097	893473	6835	24481	106800	4242	211090	207641	0	0	0	332385	0
H7C1	2267506	630128	0	3495	21941	0	179818	133803	0	0	0	291071	0
H7C2	2362651	678815	0	4531	43012	0	155572	141124	0	3927	0	330648	0

Table 2.3 continued

H8A1	6234104	675023	0	12905	41102	0	377435	0	0	3851	0	239731	0
H8A2	5446770	606030	0	0	0	0	358042	0	2957	23187	0	221845	0
H8B2	4359874	679035	0	26842	37164	0	316782	0	0	5144	0	293103	0
H8C1	2552176	666594	41715	0	120834	0	334932	0	3930	6402	158780	0	0
H8C2	603037	603038	0	16763	21783	0	260558	0	0	6897	24410	272627	0
H9A1	5805319	893637	0	0	136438	57506	405116	0	28424	0	0	266153	0
H9A2	4149176	889843	28504	0	66250	10311	578757	0	0	0	0	206021	0
H9B1	5785345	938785	30602	72568	146490	49001	345469	0	0	0	0	284065	10589
H9C1	1699234	551662	24490	8546	0	0	245966	0	5962	5649	0	39814	0
H9C2	2610577	807774	0	18876	71703	0	393088	0	0	0	0	324106	0
H10A1	2236109	511162	0	0	46686	0	148446	116464	0	0	0	199566	0
H10A2	4206510	568221	0	0	33942	0	169623	141705	0	0	0	222952	0
H10B1	1511879	691648	0	38257	111727	0	152114	117824	0	0	0	271727	0
H10C1	3531377	619533	0	15661	65312	0	165857	136864	0	0	0	235839	0
H10C2	2282940	652851	0	1769	36204	0	174976	120424	0		0	319478	0

Table 2.4 RP-HPLC total area and zein-1(α -zeins) data expressed as arbitrary units (AU)

Hybrid	zein-1	Peak 12	Peak 13	Peak 14	Peak 15	Peak 16	Peak 17	Peak 18	Peak 19	Peak 20	Peak 21	Peak 22	Peak 23	Peak 24	Peak 25
H1A1	2084361	10244	64627	0	0	335784	0	0	289368	277868	0	661743	0	444726	0
H1A2	2379527	40760	65786	0	0	524892	0	0	251470	327008	138673	579680	0	451256	0
H1B1	2723861	38657	109734	0	0	514814	0	0	370259	433026	0	678177	0	579194	0
H2A1	4797032	0	42279	0	39862	746639	1126668	0	847001	607477	0	901267	0	474315	11525
H2A2	3629046	0	23576	0	28983	553482	819633	0	633757	476482	0	696120	0	397013	0
H2C1	2041529	0	14535	0	0	354663	351457	0	365995	289695	0	433502	0	231682	0
H3A1	3273596	30212	53147	0	43092	1236565	0	0	502059	298819	143237	536998	0	429467	0
H3B1	3170638	29128	51685	0	30060	1287167	0	0	477943	260550	172693	482863	0	378550	0
H3C1	1486103	0	36593	0	0	613319	0	0	240184	175467	93636	331203	0	255047	0
H3C2	1745449	0	0	0	0	179399	0	0	149278	147743	80730	330399	0	260022	0
H4A1	3730669	0	39556	0	29834	1119768	0	0	787813	0	1210229	0	293759	236319	13391
H4A3	4316385	0	54405	0	20503	1226591	0	0	971574	0	1434995	0	350213	258104	0
H4B1	2310344	0	53297	0	4445	365305	0	0	478895	0	550010	383546	174604	284797	15444
H4C1	4651188	0	61026	0	0	780987	745653	0	998179	0	1412270	0	378514	274558	0
H4C2	4236490	0	62989	0	36123	727848	611333	0	905159	0	903880	389663	318102	281394	0
H5A1	4781088	0	0	0	348980	995065	806620	0	522037	305595	841413	385190	288336	287851	0
H5A2	2609837	0	0	0	251813	871834	586662	0	181312	0	381745	0	109416	227056	0
H5B3	3457111	0	0	0	223619	699661	478980	0	393262	249503	667566	317150	207228	220142	0
H5C1	1648133	0	0	0	194053	542110	321168	0	95799	32645	204666	0	76256	181436	0
H5C2	3255600	0	0	0	240222	687538	463522	0	387949	0	1080311	0	230399	165659	0
H6A1	4557532	0	77180	0	51406	1405415	0	0	1004068	0	1001840	401422	317085	299117	0
H6A2	5979960	0	68285	110002	394270	845071	1569935	0	1044439	0	373166	821396	179551	573845	0
H6B1	2076609	0	57028	0	4603	1042518	0	0	354540	0	315697	0	66183	236040	0
H6C1	1212415	0	19564	0	0	151719	0	0	283978	0	325686	194436	94023	143008	0
H6C2	1790499	0	46022	0	129399	287001	492935	0	274381	0	0	290223	24163	246374	0
H7A1	2228249	0	0	0	15956	466765	0	0	0	229851	210754	940217	181898	182808	0
H7A3	2415860	0	28052	0	15893	496201	208723	0	0	227453	1054933	197059	0	187546	0
H7B1	1999624	0	21567	0	0	427700	0	0	177462	169171	893311	0	168404	142010	0

Table 2.4 continued

H7C1	1637379	0	15068	0	0	335063	0	0	112628	175740	728343	0	130457	140080	0
H7C2	1683837	0	22853	0	0	360542	0	0	125289	160193	733122	0	128919	152918	0
H8A1	5559081	0	0	0	356928	1160322	925456	0	626051	0	1822279	0	377598	290446	0
H8A2	4840741	0	35417	0	62351	1171537	504906	0	517471	473626	1469565	0	350653	255215	0
H8B2	3680839	0	23565	0	17274	899746	336375	0	447478	342620	1139361	0	256591	217828	0
H8C1	1885583	25314	0	0	401598	0	0	147917	188635	807750	0	180254	134115	0	0
H8C2	1886529	0	19834	0	0	390273	0	0	161279	176343	833159	0	188689	116952	0
H9A1	4911683	0	43772	85676	285266	694772	1199939	0	909735	0	371291	689003	156867	475362	0
H9A2	2623214	0	0	71810	9575	1185469	0	0	647474	0	334971	0	121816	252100	0
H9B1	4846560	0	60050	115101	282617	679082	1165505	0	893464	0	358581	679586	134684	477889	0
H9C1	1147572	0	0	0	185008	498617	285838	0	80220	35850	174936	0	60595	165038	0
H9C2	1802804	0	0	38543	116445	273928	524136	0	279272	0	0	293257	27437	249784	0
H10A1	1724948	0	0	43925	11133	372745	0	0	135719	213859	282253	341596	51427	272289	0
H10A2	3638289	0	0	62713	2680	907202	382124	0	427740	402057	446389	521178	126211	359995	0
H10B1	1357821	0	0	42243	0	315161	0	0	93959	169531	229673	279783	0	227469	0
H10C1	2911844	0	0	55049	0	700124	295242	0	273723	386415	298678	484088	79237	339287	0
H10C2	1630090	0	0	55011	0	361376	0	0	107829	189678	270512	336888	41622	267174	0