PROJECT TITLE

Analysis of zein from South African maize of variable endosperm texture

Progress Report

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APPLICANT
Dr Marena Manley
Department of Food Science
Faculty of AgriSciences
Stellenbosch University (SU)
Private Bag X1
Matieland 7602

Tel: 021 808 3511
Fax: 021 808 3510
Email: mman@sun.ac.za

COLLABORATORS
Dr Glen Fox
Department of Primary Industries and Fisheries
Queensland Grains Research Laboratory
PO Box 2282
Toowoomba
Queensland 4350
Australia

and

Kim O'Kennedy (MSc Food Science student)
Department of Food Science (SU)

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1. Objective

The objective of this study is to characterise and quantify zein from a range of South African maize hybrids and their respective inbred parents.

2. Introduction

Maize is an important crop produced worldwide and has many uses as food and livestock feed. These uses are influenced by yield, harvest and storage characteristics of the maize kernels. Development of kernels with a sufficiently hard endosperm texture is important for optimum yield, harvest and storage conditions. Variability in endosperm texture can be influenced by protein and starch composition. Maize contains around 8% protein and, although relatively low, it has been linked to endosperm texture.

Cereal proteins have been studied for many years. The complexity of these proteins is vast and various classification systems have been developed. Cereal proteins can be separated according to similarities/differences in solubility, function, composition and structure. Possible systems include classification based on solubility, morphology, biological functions or chemical composition (Lasztity, 1984). T.B. Osborne, who is regarded as the father of plant proteins, developed a classification system which differentiated cereal proteins based on their solubility (Osborne, 1908). Four protein classes have been identified, i.e. albumins (water soluble), globulins (salt soluble), prolamins (alcohol soluble) and glutelins (soluble in dilute alkali). Zein is the prolamin of maize and is morphologically found in the endosperm and its biological function is to serve as a storage protein.

Zein has been suggested to influence endosperm texture (Dombrink-Kurtzman & Beitz, 1993; Dombrink-Kurtzman, 1994) and can account for up to 50-60% of the total protein of maize (Lending *et al*., 1988; Lasztity, 1984). The name, zein, is derived from the Latin name for maize, i.e. *Zea maize* (Shewry & Tatham, 1990). Four main zein classes have been identified based on difference in solubility, molecular mass (Mr), pI value and amino-acid sequences. These classes are referred to as α-, β, γ, and δ-zein with some divided further into sub-groups. The α- & γ-zeins are divided into two main sub-groups namely the 19 and 22 kDa α-zeins and the 16 and 27 kDa γ-zeins. The two α-zein sub-groups can be divided into even more sub-classes. The β-zein is usually referred to as the 15 kDa β-zein and the δ zein as the 10 kDa δ-zein; based on their Mr obtained from SDS-PAGE results. These masses obtained are, however, not accurate in comparison with their calculated masses. **Table 1** summarises the characteristics of the respective zein classes. Zein proteins are located in protein bodies within the maize. **Fig. 1** illustrates where the zein protein classes are located within these protein bodies and the protein bodies within the endosperm.

Table 1 Characteristics of zein classes

Characteristics	α-zein	β -zein	γ – zein	δ-zein
Solubility (%) (Esen, 1987)	50-95 ethanol / 2-propanol	30-85 2-propanol*	0-80 2-propanol* / water*	Soluble in alcohol*
Molecular mass (kDa)** (Esen, 1987)	19-25	17-18	27 & 16	10
Relative abundance (%)	75-85	10-15	5-10	<5
Amino-acid composition	High in alanine and leucine	High in methionine	High in proline and cystein	High in methionine

*In presence of a reducing agent (2-mercaptoethanol/dithiolthreitol)

** Based on SDS-PAGE results

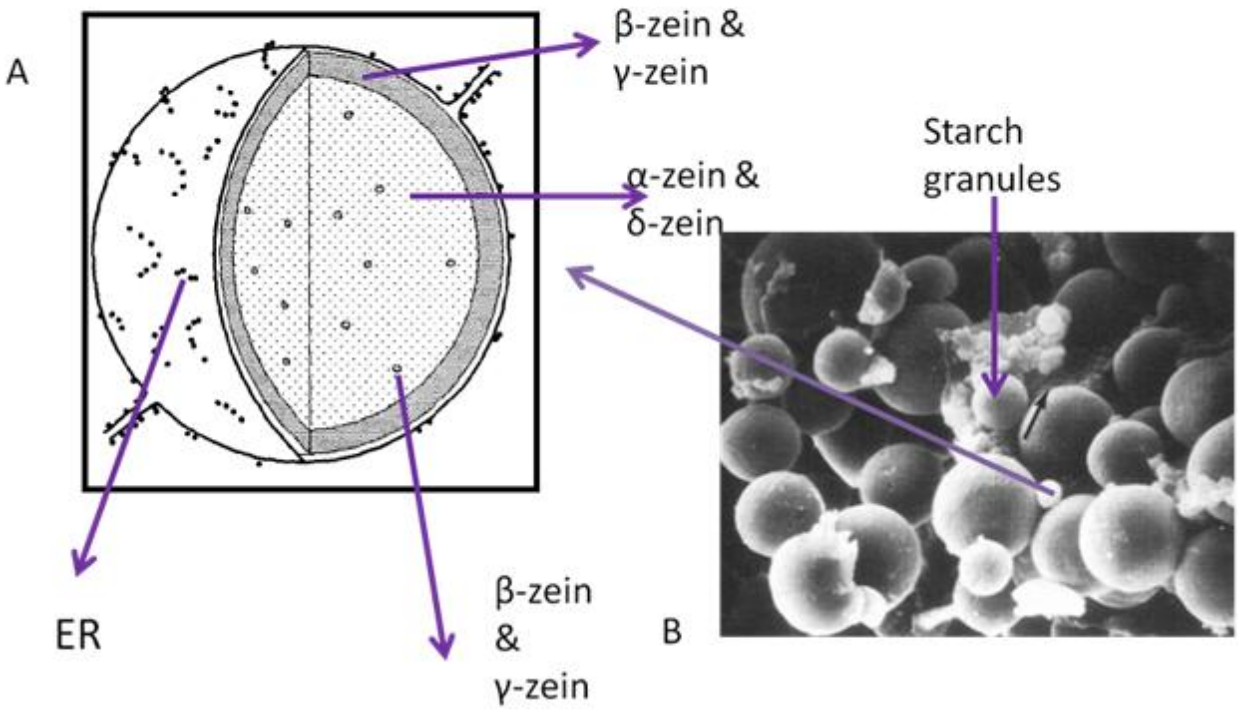

Figure 1 (A) Illustrative representation of the process of protein body development showing localisation of α-, β-, γ- and δ-zein proteins in the protein bodies (Lending & Larkins, 1989) and (B) localisation of protein body within the endosperm (Dombrink-Kurtzman, 1994).

Over the years various methods have been used to separate, indentify and/or quantify the various zein classes. These methods include Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Reverse Phase–High Performance Liquid Chromatography (RP-HPLC), Size Exclusion Chromatography (SEC), Ion Exchange Chromatography (IEC), Iso-electric Focusing (IFE), Enzyme-Linked Immunosorbent Assay (ELISA) and more recently Matrix Assisted Laser Desorption Ionization – Time-of-Flight Mass Spectrometry (MALDI-ToF MS).

Hard maize endosperm is often referred to as vitreous, glassy or horny endosperm whereas soft endosperm is referred to as chalky or floury endosperm (Dombrink-Kurtzman & Beitz, 1993). Results of separation and quantification of zein using RP-HPLC indicated the presence of up to three times more α-zein in vitreous endosperm compared to that of more floury endosperm (Dombrink-Kurtzman & Beitz, 1993). These type of results are not as yet available for South African inbred maize parents or commercial hybrids.

Opaque-2 maize was discovered in the 1960's and was found to contain less zein, and higher lysine and tryptophan contents (Gibbon & Larkins, 2005). It was, however, not ideal due to its soft endosperm texture. Subsequently a modified Opaque-2 maize (also referred to as Quality Protein Maize (QPM)) was developed to obtain a harder endosperm texture. When QPM was compared to that of vitreous maize it was found to contain more γ-zein than α-zein (Dombrink-Kurtzman, 1994). Thus γ-zein has now also been linked to endosperm texture.

The objective of this study is to characterise and quantify zein from a range of South African maize hybrids and their inbred parents. This progress report, however, only covers method development performed thus far for optimisation of the RP-HPLC and MALDI-ToF MS methods. Once optimised RP-HPLC will be used to separate and quantify the respective proteins whereas MALDI-ToF MS will be used to characterise the proteins.

3. Materials and Methods

Throughout the various stages of method development only one maize hybrid sample has been used; no comparisons between samples differing in endosperm texture have thus been made during these stages. The MALDI-ToF MS method development is based on protocols obtained from literature (Wang *et al.*, 2003; Adams *et al.*, 2004). Modifications of the method were based on availability of equipment and advice from experienced researches in this field of research. Similarly the RP-HPLC method development was conducted based on advice from experienced researchers.

3.1 MALDI-ToF MS

MALDI-ToF MS is a soft ionisation technique for mass spectrometry analysis (Karas & Hillenkamp, 1988; Hillenkamp *et al.*, 2008). Various matrices and ionisation sources are used, depending on the sample. The sample which exhibits moderate absorption is embedded in the matrix consisting of high absorbable species. During ionisation the matrix protect the sample and absorbs energy from the laser that causes part of the target sample to be vaporised. Once vaporised and ionised they are transferred to a time-of-flight mass spectrometer where they are separated from the matrix ions and individually detected according to their mass(m)-to-charge(z) (m/z) ratios by measuring the time it takes for ions to travel through a field free region known as the flight or drift tube. The heavier ions are slower than the lighter ones. **Figs. 2 and 3** illustrate the mechanism of the MALDI-ToF MS.

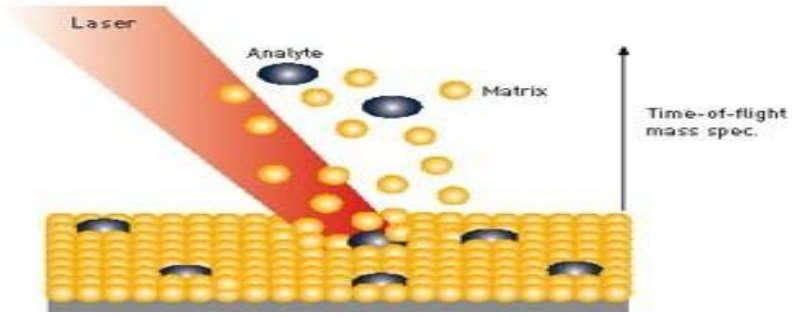

Figure 2 Ionisation process of analyte/sample (http://www.eurogentec.com/EGT/Images/madi_tof.jp).

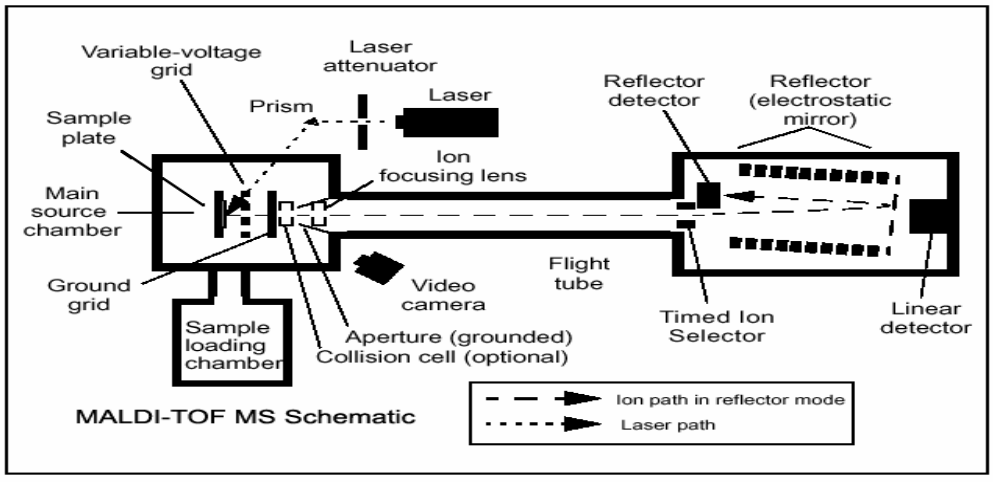

Figure 3 Schematic layout of MALDI-ToF MS system.
(http://cbsu.tc.cornell.edu/vanwijk/images/massspec/massspec_schem_maldi.gif)

Two matrices were tested namely 2,5 dihydroxyl benzoic acid (DHB) and 2-(4-hydroxyphenalazo)benzoic acid (HABA). Maize meal was defatted by continuous extraction in a Soxhlet apparatus with 150 ml of petroleum ether as solvent.

All MALDI-TOF MS analyses were performed on a Voyager-DE STR system (Applied Biosystems). The operating conditions used in all analyses were 337 nm pulsed nitrogen laser; a positive ion linear mode; and a 25 kV accelerating voltage.

3.1.1 MALDI-TOF MS analysis using DHB as a matrix

3.1.1.1 Zein extraction

Three 10 g maize samples were defatted separately, washed with 50 ml hexane and air-dried for 3 days. The zein proteins were extracted from 10 g defatted maize meal with 45 ml 55% 2-propanol in a 100 ml glass beaker and stirred on a magnetic stirrer overnight. The extract was filtered and mixed with an equal volume of 2% NaCl solution. The zein proteins was subsequently precipitated at 4ºC overnight. The precipitate obtained was then filtered, washed with distilled water and dried at room temperature for two days and the filtrate discarded. Sample preparation of commercial zein, which was also analysed, was the same as that of extracted zein.

3.1.1.2 Matrix preparation - DHB

The Matrix solution was prepared by adding 10 mg of DHB to 1 ml of 55% aqueous-2-propanol.

3.1.2 MALDI-ToF MS analysis

Precipitated zein was re-dissolved in 55% 2-propanol to a final concentration of 5 mg mL^{-1}. Formic acid (5%) was added to promote dissolving and 1 ml of the final solution was centrifuged at 4000 g for five minutes. Supernatant (10 µL) obtained from centrifugation was mixed with 90 µL of the matrix. A 1:1 ratio of DHB matix solution:zein was also prepared. Two spotting techniques were tested. In the first technique approximately 0.6 µL of the matrix and zein mixture were spotted in triplicate. In the second spotting technique 0.3 µL of the zein solution only was spotted in triplicate. After drying, 0.3 µL of the matrix solution was spotted on the zein spot.

3.1.2 MALDI-ToF MS analysis using HABA as a matrix

3.1.2.1 Zein extraction

Maize meal was defatted as mentioned above. Various meal: extraction solvent ratios were tested. Zein proteins were extracted with 1 mL 70% ethanol in a 1.5 ml eppendorf tube. Zein was extracted with 20, 30, 40, 50, 60, 100 mg maize meal:mL extraction solvent at room temperature for 2 hours. Continuous shaking kept the sample in solution to ensure efficient extraction. Samples were centrifuged at 2000 *g* for five minutes. The supernatant was kept for analyses.

3.1.2.2 Matrix preparation - HABA

HABA (10.4 mg) was dissolved in 1 mL of 70% acetonitrile (ACN) and 0.3% triflouroacetic acid.

3.2.3 MALDI-ToF MS analysis

Supernatant (10 µL) was added to 90 µL of the matrix solution. Approximately 0.6 µL was spotted on the sample plate.

3.2 RP-HPLC

HPLC is a form of column chromatography used to separate, identify, and quantify compounds. RP-HPLC utilises a column that holds non-polar chromatographic packing material (stationary phase) and a pump that moves the polar mobile phase(s) through the column. The stationary phase is situated in the column and consists of silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. A detector, in this case a UV-VIS absorbance detector, shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analysed, and the solvent(s) used. Two solvents are used and are referred to as A and B in literature. During analysis these eluants/solvents are mixed to prescribed concentrations to elute various molecules at certain retention times (Bidlingmeyer, 1992).

3.2.1 Zein extraction

The same extraction procedure was followed for all RP-HPLC analysis. Maize was defatted as for Maldi-ToF MS extractions and zein was extracted with 55% 2-propanol and 1% 2-mercaptoethanol; 1:5 meal to extraction solvent. Continuous shaking at room temperature kept the sample in solution to ensure efficient extraction.

3.2.2 RP-HPLC analysis – Waters System

A Phenomenex column was used (250 mm x 4.6; 5 µm pore size) as described by Dombrink-Kurtzman (1994). Three protocols in terms of varying the solvent and gradient were followed. The same solvents, i.e. (A) 100% acetonitrile (ACN) containing 0.1% trifluoro-acetic acid (TFA) and (B) 100% ACN solvents were used in all three protocols.

Protocol 1

A linear solvent gradient was used: 25-50% (B), 0-40 min, 50-60% (B) 25-55 min, 70% (B) 55-60 min and 70-25% (B) 60-70 min.

Protocol 2

A linear solvent gradient was used: 25-60% (B), 0-25 min, 60-70% (B) 25-55 min, 70% (B) 55-60min, 70-25% (B), 60-61 min and 25% (B), 61-70 min.

Protocol 3

A linear solvent gradient was used: 25-50% (B), 0-25 min, 50-65% (B) 25-55 min, 65-70% (B) 55-60min, 70-25% (B), 60-61 min and 25% (B), 61-70 min.

3.2.3 RP-HPLC analysis - Sumatza system

RP-HPLC analysis was performed with a defatted as well as a non-defatted sample. Two solvents used were: (A) 95% ACN containing 0.1% TFA; (B) water containing 5% ACN and 0.1% TFA. This work was kindly performed with the support of the Department of Plant Breeding, University of the Free State.

A linear solvent gradient was used: (B) 58-42%, 0-40 min, 42 -58% (B) 40-60 min.

4. Results and Discussion

4.1 MALDI-TOF analysis

4.1.1 MALDI-ToF MS analysis using DHB as a matrix

Fig. 4 shows a Maldi-ToF MS spectrum with DHB as matrix from one of the spots that was ionised. Of the three identified peaks, peaks 2 and 3 correspond to Mr of 19 kDa α-zeins. The smaller peak observed to the right of peak three (indicated with circle) corresponds to 22 kDa. This peak's intensity was much higher in the other spectra obtained (data not shown). The Mr of peak one corresponds to that of β-zein. Although β-zein is mostly observed with the addition of a reducing agent a small amount is dissolved in 55% 2-propanol. Peak 1 was only observed with one of the three spots that were ionised. No peaks were observed for the commercial zein. A possible reason for this could be that too little zein was dissolved for analysis. Commercial zein also still contains other components such as fat and carotenoids, which was not taken into consideration when the solution was prepared.

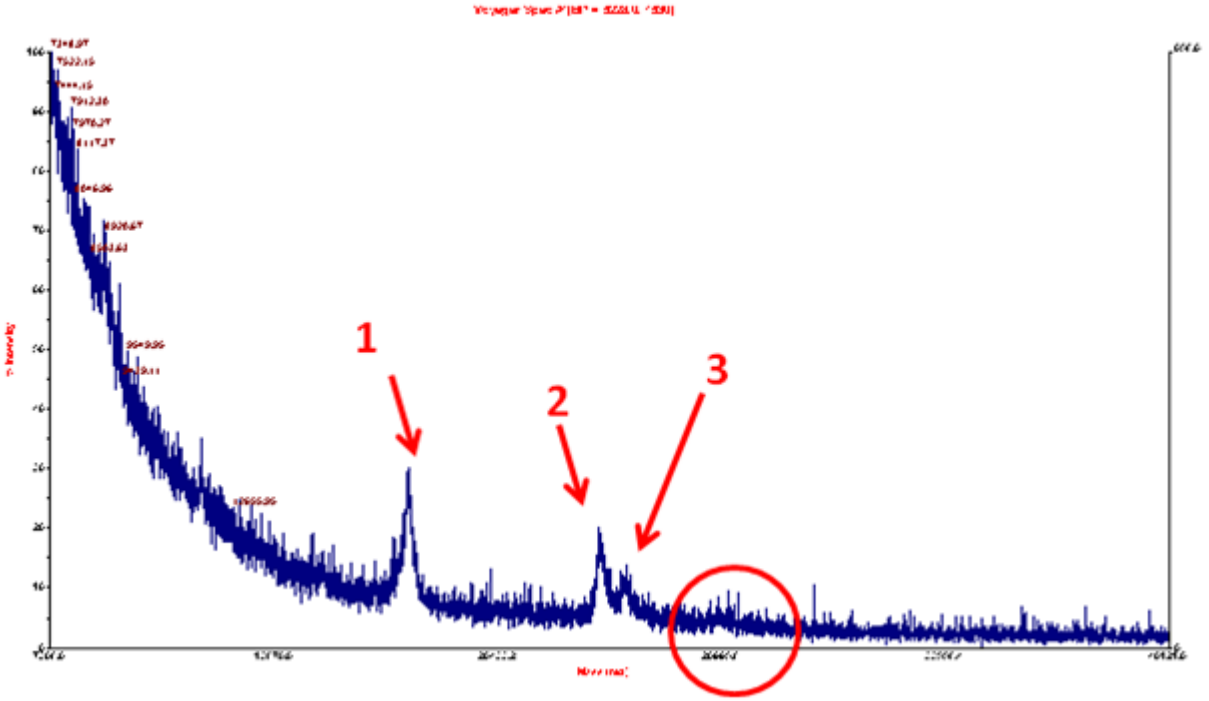

Figure 4 MALDI-ToF MS spectrum using DHB as matrix of one of the ionised spots.

4.1.3 MALDI-TOF MS analysis using HABA as a matrix

Different maize meal-to-extraction solvent ratios were compared to establish the sufficient amount of zein needed for desorption/ionisation that would result in the highest signal-to-noise ratio. If the concentration of zein is too high, desorption/ionisation will be inhibited and vice versa. The spectra obtained were not, at this stage, processed further for baseline correction. A meal-to-extraction solvent ratio of 30 mg mL^{-1} (**Fig. 5**) resulted in the best signal-to-noise ratio.

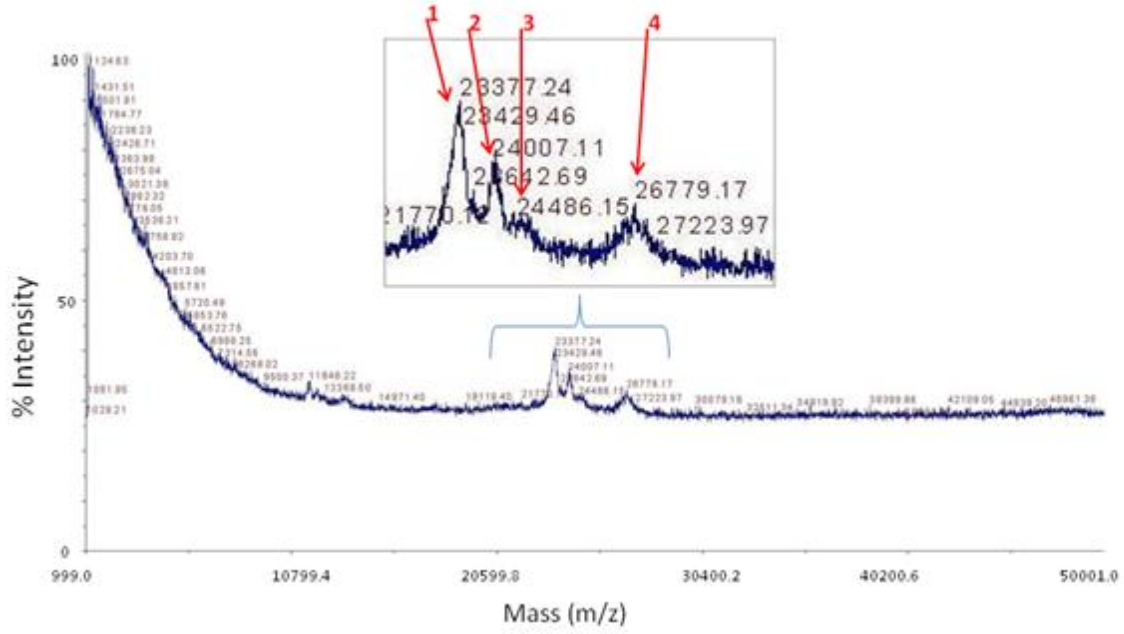

Figure 5 MALDI-ToF mass spectrum when meal-to-extraction solvent ratio of 30 mg mL^{-1} was used.

Four peaks were observed in all spectra (**Fig. 4**). These peaks correspond to the calculated true Mr of α-zeins. Peaks 1, 2 and 3 are 19 kDa α-zeins and peak 4 is the 22 kDa α-zein. The Mr of peak 1 corresponds to 19 kDa, i.e. the B1 sub-group of α-zein. The Mr of this 19 kDa α-zein

ranged from 23314-23369 Da in the respective spectra. The second 19 kDa α-zein (peak 2) corresponds to the B3 subgroup and peak 3 to the D2 subgroup. Peak 4 corresponds to the Z3 sub-group of 22 kDa α-zein. This comparison is based on Mr of mature zein of B73 inbred line obtained from Genbank Accessions (Adams *et al.*, 2004). The results were as expected since no reducing agent was used. When 2-mercaptoethanol was used in earlier attempts no results were obtained. This could have been due to its oily nature which can inhibit desorption/ionisation. If a reducing agent should be used, dithiothreitol (DTT) would be more desirable due to its more crystalline form.

4.2 RP-HPLC

4.2.1. RP-HPLC analysis - Waters System

It is expected that in RP-HPLC chromatograms of zein the α-zein proteins would show a series of peaks (due to all the sub-classes of the two main classes); γ-zein proteins two peaks (due to two sub-classes) and β-zein one peak (Dombrink-Kurtzman & Beitz, 1993) as indicated in **Fig. 6**. Using **Protocol 1**, this was, however, not observed. Only one broad peak eluted around 25-30 min in the chromatogram (results not shown). This could possibly have been α-zeins that were not separated efficiently. **Protocol 2** also did not result in satisfactory results as again the series of peaks towards the end of the chromatogram did not separate well and eluted five minute earlier. Using **Protocol 3** (**Fig. 7**) improved separation was achieved due to the decrease in solvent B (100% ACN) between 25-55 min which increases the retention of the proteins in the column. The series of peaks towards the end of the chromatogram was likely α-zein and peaks 3 and 4 β- and γ-zeins, respectively. This, however, needs to be confirmed during further optimisation of this method. The presence of possible contaminants also needs to be investigated. The peaks eluted at 5 min were confirmed to be the solvent when it was analysed without zein proteins (results not shown). Further optimisation is also still needed in terms of concentration of gradient solvents.

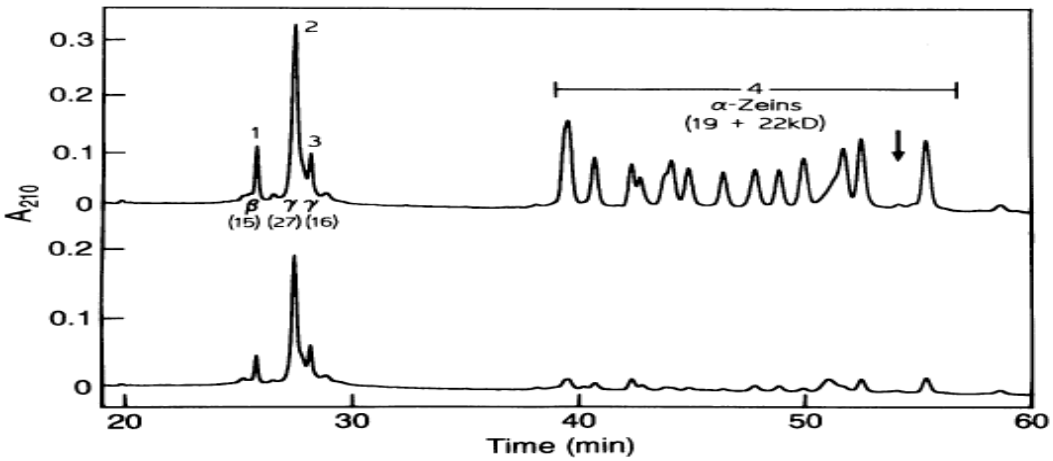

Figure 6 Typical RP-HPLC chromatograms of zein of hard (top) and soft (bottom) maize, respectively (Dombrink-Kurtzman & Beitz, 1993).

4.2.2 RP-HPLC analysis - Sumatza system

No differences were observed between the defatted and non-defatted samples. Thus samples will not be defatted before analysis in future work.

5. Conclusion

Optimisation of the MALDI-ToF MS and RP-HPLC methods, to efficiently characterize and quantify the zein proteins, has not as yet been achieved but protocols investigated thus far show promise. MALDI-ToF MS protocols will be further optimised by the addition of a more suitable reducing

agent such as DTT and by adjusting the pH to be more alkaline. The RP-HPLC method will be further optimised by increasing the retention times to enable sufficient separation of the zeins. Once this method has been optimised the respective zein fractions will be collected and characterised by MALDI-TOF MS.

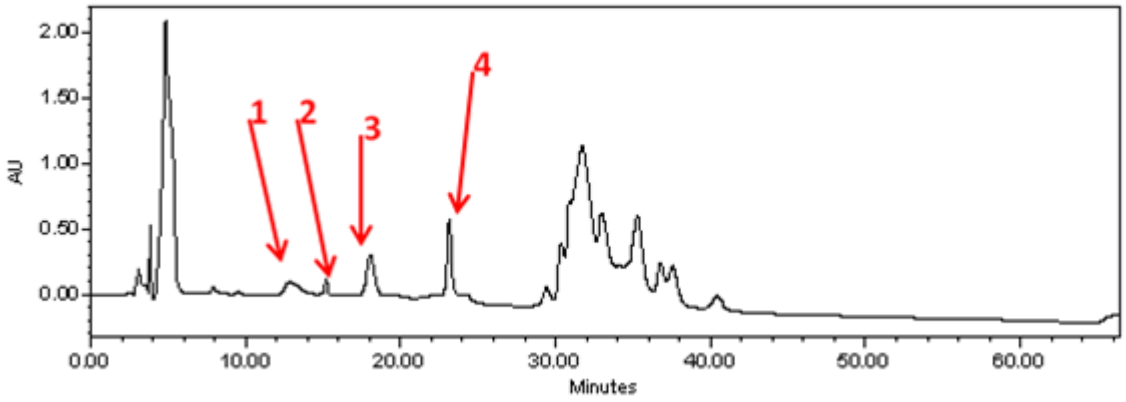

Figure 7 A RP-HPLC chromatogram of zein using Protocol 3.

6. Future Research (2010)

Maize kernels with known difference in endosperm texture, i.e. hard, intermediate and soft will be analysed to establish the difference in zein composition. This will be followed by comparing inbred lines and their respective hybrids to determine a possible relationship in terms of endosperm texture as interpreted by means of zein protein composition.

7. References

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