THE MUTAGENESIS OF *Sorghum bicolour* (L.) Moench TOWARDS IMPROVED NUTRITION AND AGRONOMIC PERFORMANCE

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PREFACE

The experimental work described in this thesis was carried out at the Agricultural Campus and Ukalinga Research Farm at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Prof MD Laing of the discipline of Plant Pathology.

These studies represent original work by the author that have not been submitted in any form for any degree or diploma to any University. Where use was made of the work of others it is duly acknowledged in the text.

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I, the undersigned, confirm that I supervised the candidate, EM Brauteseth, in the reading of this thesis.

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ABSTRACT

In the breeding of grain sorghum (*Sorghum bicolour* L. Moench) towards improved nutrition and agronomic performance, new methodologies are required to increase genetic diversity and lower the inputs required to track and screen breeding populations. Near-infrared calibration models were developed by partial least squares (PLS) and test-set validation on 364 sorghum samples to predict crude protein and moisture content on whole-grain and milled flour samples. Models using milled flour spectra were more accurately predictive than those from whole grain spectra for all constituents (eg. Protein: R^2 = 0.986 on flour vs R^2 = 0.962 on whole grain). Discriminant calibrations were established to classify grain colour using partial least squares discriminant analysis (PLS-DA) based upon CIE L*a*b* reference values and visual ranking. Preliminary calibrations were developed for quantities of 18 amino acids, fat and apparent metabolisable energy (AME) on 40 samples using cross-validation, highlighting potential for reliable calibration for these parameters in sorghum. An investigation into the potential of $^{12}C^{6+}$ heavy-ion beam mutagenesis of sorghum seed was undertaken by treatment at RIKEN Accelerator Research Facility (Saitama, Japan) and subsequent breeding at Ukulinga research farm and analysis at the Department of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Dosage rates of 75, 100 and 150 Gy were compared in seven sorghum varieties to establish optimal dose treatments as determined by germination and survival rates, visible morphological changes and field data over two seasons of field trials. Crude protein variation within the M_2 generation was analysed to compare dose rate effects. The need for higher dose rates was indicated by few quantified differences between treatments and control although good correlations between protein deviation and treatment dose rate were elucidated. Differences in varietal response suggest a need to optimize dose rate for specific varieties in future endeavours. In addition, all mutagenized populations were screened for crude protein content using near-infrared spectroscopy (NIRS). Significant differences in protein levels and standard deviations were observed between treated self-pollinated M_2 generations and untreated control populations. Individual plants displaying significantly different protein levels were isolated.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

THESIS INTRODUCTION

There is a strong need to develop agricultural resources in the developing regions of the world. Widespread hunger and malnutrition are compounded by poverty at a stage where the availability of agricultural land is under threat from a rapidly increasing population. There is thus a need to increase agricultural productivity from all available resources. A potential solution to combat these challenges is to develop and breed crop varieties which are capable of delivering increased yields of enhanced nutritional quality in more marginalised areas. Sorghum (*Sorghum bicolour* L. Moench) is a crop which is inherently high in protein and yields well in a small-scale or subsistance farmer environment, whilst capable of tolerating lower inputs and more marginal environments. It is the 5th most consumed staple cereal globally and over 80% of its cultivation lies in developing areas. As such, development of this crop could have significant impacts, especially considering it is relatively unexplored genetic capacity.

Plant breeders require a range of genetic diversity in order to manipulate sexual crossing to develop hybrids and open-pollinated varieties (OPV's) capable of rendering significantly increased performance. Classical breeding approaches have become limited by the increasing complexity of crop characteristics required, whilst available genetic diversity remains the same. As such, new methodologies to increasing genetic diversity are needed. The advent of biotechnology has increased such methodologies through tissue culture, recombinant DNA technology, marker assisted selection and more specific mutation induction techniques. Unfortunately many of these technologies are expensive and thus inaccessible to plant breeders and research institutes in developing regions. Additional limitations in breeding for increased nutrition include the expense of analysis. As such, very little breeding material is screened and selected for nutritional characteristics. There is thus a need for cheaper and more accessible screening methodologies in order to speed up breeding programs and produce nutritional varieties.

Several technologies developed over the past few decades hold potential to address these limitations. Firstly, cyclotron generated irradiation is being used to cheaply increase genetic diversity through mutagenesis with increased frequency and fewer deleterious effects than those caused by chemical and physical methods employed in the past. Secondly, non-destructive analysis of agricultural products has been performed inexpensively in other crops by the calibration of near-infrared spectroscopy for nutritional parameters. The general objective of this thesis was thus to investigate the potential of these technologies with the ultimate aim of using them together to track induced change in breeding populations.

The work presented in this thesis investigates these technologies and their potential in the development of grain sorghum. Chapter One has comprehensively introduced the technologies and motivated their potential through citing examples in the literature. Chapter Two aimed to investigate cyclotron induced heavy-ion beam mutagenesis for sorghum improvement. Specifically it aimed to induce mutagenesis and establish an optimum dosage for sorghum seed through tracking mutagenized populations over successive generations of self-pollinating. It also aimed to identify specific mutants displaying novel characteristics such as increased crude protein, abnormal flowering dates, recessive dwarfing or different grain characteristics. Chapter Three aimed to investigate the potential of NIR analysis of sorghum grain and flour, and its reliability and suitability for use in sorghum breeding. This was completed through the development of several calibrations for parameters such as crude protein and moisture, which were tested during routine analysis. Additionally this chapter aimed to investigate the potential for calibration development on several other parameters such as several amino acids, fat, phytic aicd, and several colour characteristics through developing preliminary calibrations. A thesis overview is reported in Chapter Four which draws generalised conclusions and reports on the secondary aim of the thesis, which was to isolate potentially beneficial material using NIR from cyclotron treated material, as well as identifying future areas of research and limitations discovered whilst performing this work.

CHAPTER ONE

LITERATURE REVIEW

1.1 CROP DEVELOPMENT IN AFRICAN CEREALS

Despite continual research and discussion, the world currently faces the familiar problems of widespread hunger and malnutrition (van Braun, 2005). In addition, it is becoming clearer that "hidden hunger" due to micronutrient deficiencies is also prevalent (Conway, 1997; van Braun, 2005). Although common to all developing nations, the progress in reducing hunger has been uneven across regions and countries (FAO, 2005). Africa remains most direly affected, with increasing numbers of underweight children and high levels of micronutrient deficiencies (Micronutrient initiative and UNICEF, 2005). The number of hungry people in Sub-Saharan Africa has increased by 20 % since 1990 (FAO, 2005).

Poverty has been climbing to the top of the global development agenda since the adoption of the Millennium Development goals (MGDs) in September 2000 (van Braun, 2005). The United Nations and its Millennium Project task force on hunger issued a report in 2005 entitled: Halving hunger: It can be done (UN Millennium Project, 2005), outlining an agenda for the highest priorities in action to reduce hunger by half. This report consists of a broad range of strategies including policy reforms; the creation of enabling environments; production safety nets; increasing incomes and making markets work for the poor (UN Millennium project, 2005). In particular, the report stressed the need to take measures to increase the agricultural productivity of food-insecure farmers and to improve nutrition for the chronically hungry and vulnerable (UN Millennium project, 2005).

Achieving these goals will require consideration of three principal factors. First, the world's population is increasing dramatically (Swaminathan, 1995; James, 1997). Second, agriculture in the developing world is dominated by small farmers and will

continue to be so (Smale et al., 2003). Third, poverty remains the root cause of hunger and malnutrition (van Braun, 2005). Despite admittance that no single technological, economic or political approach will offer an instant, dramatic solution (IFPRI, 2005), targeted steps at global, country and local levels are required to improve health and nutrition, and science-based bio- and info- technological innovations for the poorest and marginalized will be critical (van Braun, 2005).

Agricultural resources need to be developed in order to increase productivity in Africa (Dysan, 1997). The increasing world population, most of which is in developing nations, places a limitation on the availability of suitable agricultural land for agriculture as well as a higher demand on food production (Swaminathan, 1995; FAO, 1997). Other factors may also impinge on the availability of land for agriculture such as the increasing trend to replace fossil fuels with biofuels (Henniges, 2005; Jacobsen, 2008). The establishment of new crop varieties and breeding of varieties with more favourable agronomic characteristics and greater productivity is a potential solution to these problems which needs to be investigated thoroughly (Slabbert et al., 2001). Genetic improvement through biotechnology is one way to ensure crop productivity increases (Villalabos, 1995). Biotechnology research is well established in South Africa, nevertheless lags behind developed countries (Rybicki, 1999; Cloete et al., 2006).

Over this last century, research and development of maize, wheat and rice has increased their productivity sufficiently to supply the world in its current state of overpopulation (NRC, 1996; Conway, 1997). It is highly improbable that future population increases can be buffered through redoubling the production of these three crops (NRC, 1996; Conway et al., 1999). A significant cause of the low subsistence food production in developing regions involves the selection of inappropriate food crops, such as maize, which are grown in environments too marginal for reliable production (Eicher et al., 2005). Development of food crops which are more suited to these marginal areas would practically help to alleviate hunger and malnutrition, especially in sub-Saharan Africa (Slabbert et al., 2001).

1.2 SORGHUM

1.2.1 Suitability for cultivation in developing regions

Sorghum (*Sorghum bicolour* (L.) Moench) is an African cereal which remains vastly untapped in terms of agronomic performance through breeding and biotechnological advancement (Doggett, 1988; NRC, 1996). Sorghum is currently the fifth most important cereal on the globe as the dietary staple of more than 500 million people in over 30 countries (NRC, 1996). Of the total world area devoted to sorghum, over 80% lies in developing nations (FAO, 1995). The inherent high nutritional value, photosynthetic efficiency, ability to perform in marginal areas, versatility in production and use along with underutilized and underdeveloped genetic potential compel further research and development of sorghum as a staple crop in developing regions across the globe (NRC, 1996; Conway et al., 1999). In Sub-Saharan Africa, correlations between a change in diet from sorghum to maize and an epidemic of oesophageal cancer highlight potential medical benefits to the promotion of sorghum as a staple crop (Isaacson, 2005). The high similarity between sorghum and maize genomes suggest that its usefulness as a crop can be vastly improved through breeding and biotechnology, considering that many limitations in sorghum resemble those previously encountered in maize, such as poor resistance to several pests and diseases including ergot and *Fusarium spp.* Stalk rot, poor tolerance for abiotic stresses and low yield potential (York, 1989; Binelli et al., 1992).

1.2.2 Origin and cultivation

Sorghum is believed to have been initially domesticated in central Africa (Ethiopian region) with secondary origins in India, Sudan and Nigeria (Devries et al., 2001). Cultivation has subsequently stretches through the rest of Africa, Asia and eventually to the Americas and Australia (Doggett, 1988). In Africa, it is predominantly grown in a large belt that spreads from the Atlantic coast to Somalia and Ethiopia, bordering the Sahara in the north and the equatorial forest in the south (FAO, 1995). It is widely cultivated in the drier regions of eastern and southern Africa where rainfall levels are too low to support maize production (FAO, 1995). Five species of cultivated sorghum

are in common use amongst sorghum breeders including: *bicolor*, *durra*, *kafir*, *guinea* and *caudatum* (Doggett, 1988; Devries et al., 2001). All five of these are believed to have originated in Africa and continue to be cultivated there, often in conjunction with one another towards different uses (Devries et al., 2001). *Kafir* types originate from eastern and southern Africa (NRC, 1996). *Durra* sorghums are spread across a wide area of Nigeria and west-African savannah, although they developed primarily in Ethiopia and the African horn (Devries et al., 2001). *Guinea* varieties are cultivated heavily in west and central Africa, although some land races have spread as far south as Mozambique (Devries et al., 2001). *Caudatum* sorghums were developed in Kenya and Ethiopia while *bicolor* varieties are sparsely spread across east Africa and form the least important cultivated races in Africa (Devries et al., 2001).

1.2.3 Production

Sorghum has typically been produced for either feed or human consumption but there is increasing cultivation for bioethanol production (FAOSTAT, 2005; National Sorghum Producers, 2006). Sorghum ranks fifth in cereals for global production totalling just under 57 million MT for 2005 (FAOSTAT, 2005). Global trends in production are split between developed and developing countries, rendering sorghum the second most important cereal (after maize) in sub-Saharan Africa (FAO, 1995). Due to decreases in global production between 1995 and 2001, as much as 90% of the world's area under sorghum cultivation lies in developing nations, mainly in Africa and Asia (Devries et al., 2001; FAO, 1995). Of total world sorghum production approximately 50% is used for human food (National Sorghum Producers, 2006). World sorghum production peaked over the period 1979-1981 at 66 million MT, although the decline to present levels has largely been due to two countries, the United States and China, which accounted for 6.2 million MT between 1981 and 1990 (FAO, 1995). This decline has been attributed to farmers planting more profitable crops in the east (such as pulses and oilseeds), and policy interventions and the availability of more drought-tolerant maize varieties in the United States, resulting in the expansion of the maize belt further west into traditional sorghum areas (FAO, 1996). In the period from 1980 to 2000, the growth in demand for this crop exceeded its production growth, the imbalance being most pronounced in Africa and its least developed countries (FAO, 1995).

Improvements in production, availability, storage, utilization and consumption of this crop will greatly contribute to the household food security and nutrition of the inhabitants of these areas (FAO, 1995).

1.2.4 Utilization

Sorghum is perhaps the world's most versatile crop (NRC, 1996). Aside from the use of grain for human consumption sorghum has a variety of potential uses: Whole plants are used for forage, hay or silage; stems of some varieties are used for firewood, building, fencing, weaving or broom making; stems and grains of others are used for liquid biofuels production; whole living plants serve as windbreaks and are used for staking heavy climbers such as yams; seeds are used for animal feed; and a variety of products can be produced including industrial alcohol, vegetable oil, adhesives, waxes, dyes and starches for lubricating oil-well drills (NRC, 1996). Important food uses of sorghum include: leavened and unleavened bread; bread from alkali cooked grain; thick and thin porridge; boiled grain; noodles; alcoholic and non-alcoholic beverages; and popped and sweet sorghum snacks (House, 1984). Examples of these foods include: porridges such as *tô* (west Africa), *bogobe* (Botswana), *sankati* (southern Africa) and *ogi* (Nigeria); leavened breads such as *injera* (Ethiopia) and *kisra* (Sudan); unleavened breads such as *roti* (India), *chapatti* (south Asia) and *tortilla* (Latin America) and fermented beverages such as *umkhombothi* (South Africa) (Friedereksen, 1986; Doggett, 1988; NRC, 1996).

1.2.5 Production constraints in developing regions

Sorghum cultivation in developing regions, and especially in Africa, is dominated by subsistence farmers who seldom produce excess to sell, thus limitations in production vary from those common to commercial scale production (NRC, 1996). Africa's farmers in particular face several, interlocking constraints which vary in degree and combination from one region to another (NRC, 1996).

Sorghum cultivation is hampered by biotic and abiotic stresses, especially in the semi-arid tropics (House, 1984). Sorghum can be distinguished from other cereals by the broad range of diseases to which it is susceptible (Frederiksen, 1986). Both pathogen

related disease and abiotic stress arise from the wide range of environments in which it is cultivated (Frederiksen, 1986). In fact sorghum plants grown in traditional areas may be under stress from as many as six foliar pathogens; one or more viruses; a host of soil borne organisms; a mycoplasma-like organism; and at least two systemic fungal diseases (Frederiksen, 1986). Of great importance to sorghum cultivation is resistance to insects such as headbugs, molds and bird pests (NRC, 1996).

Drought severely hinders sorghum production in the semi-arid regions of the world; a problem which is compounded by management, variable climates, soil characteristics, pests and in some cases socio-economic political aspects (NRC, 1996). Soil fertility, fertilizer use, heavy-metal and salinity presence are particularly significant constraints in sub-Saharan Africa (Doggett, 1988). The inherently low fertility of tropical soils, in conjunction with resource poor management practices, results in drastically low yields in these regions (NRC, 1996). In addition, sorghum production is limited by a host of weed species both monocotyledonous and dicotyledonous. In particular, *Striga* species (most notably *Striga hermonthica* (Del.) Benth and *Striga asiatica*) are prevalent throughout Africa, vastly reducing yields over a wide variety of regions (Rodenburg et al., 2004). In 1998 it was estimated that *Striga* species have the potential to invade 48 million ha of grain cultivating areas in Africa (Watson and Kroschel, 1998).

1.2.6 Constraints in sorghum cultivation

In addition to constraints in production, sorghum possesses several inherent characteristics which lower its nutritional value (FAO, 1995). Sorghum is typically high in prolamine protein fractions which are highly cross-linked and thus indigestible by human enzymes (NRC, 1996). The amino acid composition of sorghums is typically deficient in some essential amino acids namely: lysine, tryptophan, methionine and threonine (FAO, 1995). These deficiencies arise from the amino acid sequences of kafirins, the proteins responsible for up to 80% of the total grain proteins (Taylor et al., 1984). Sorghum also possesses several antinutritional factors which decrease the nutritional bioavailability (FAO, 1995). High tannin levels in grain may bind proteins and fiber rendering them less usable in human digestion, although they are a good source of antioxidants (Awika et al., 2004). Tannins comprise a diverse range of

phenolic molecules, thus the degree of nutritional inhibition is related to the relative ratios and concentrations of these (Rooney et al., 2004). Of greater concern are high levels of phytic acid in sorghum grain as these bind mineral cations, specifically zinc, calcium and iron, and reduce their bioavailablilty (Zhou et al., 1995).

1.3 CONSTRAINTS IN BREEDING APPROACHES AND METHODOLOGIES

1.3.1 Limitations in classical breeding

Classical breeding approaches have progressively become less effective as the complexity of crops required increases, whilst available genetic diversity within breeding populations remains the same (Singh, 2003). Traditional breeding tends to incorporate more undesirable characteristics into early progenies than sought after traits; increasing both the time and space required by the necessity for back-crosses to remove them (Slabbert et al., 2001). Reliance on diversity and novel gene combinations generated through homologous recombination is limiting due to the degree of genetic similarity required between species, the low degree of segregation often shown in early progenies and the need to develop inbred lines prior to crossing, which may take as long as seven generations of self-pollination (Briggs et al., 1967). Nevertheless, classical plant breeding has been highly successful in increasing yields and is imperative to combining novel characteristics generated through other means into existing breeding populations (Morandini et al., 2003).

1.3.2 The development of biotechnology tools

A more comprehensive understanding in biochemistry, physiology, recombinant technology and plant genetics over the last 50 years has facilitated the development of a host of biotechnology tools available to breeders, able to increase genetic variation for selection as well as improve selection stringency and speed (Cassels et al., 2003). The formulation of a complete plant growth media and advanced understanding of plant growth regulators has led to the advent of *in vitro* propagation, facilitating the mass production of clonal, disease-free plants, genetic transformation and a reduction in space and time required for experimental research and breeding (Murashige et al., 1962;

El-Sharkawy, 1989). Plants unable to introduce genetic diversity through cross-pollination can be coerced in laboratories *in vitro* through protoplast fusion, or through manipulating ploidy levels through exposure to chemicals such as colchicines (Foolad, 2004). Mutagenesis through inducing somaclonal variation *in vitro* or exposure to chemical or physical mutagens can induce genetic change in breeding populations, and is widely cited in the literature (Larkin and Snowcroft, 1981; Slater et al., 2003). Molecular techniques are also able to target highly specific areas for knockout mutations, provide selection criteria at the gene level and randomly insert foreign genetic material into plant genomes (Gale, 2003).

Genetic transformation through biollistics or *Agrobacterium*-mediated transfer has become a popular strategy for producing crops with novel characteristics (Goure, 2004). The success of this technology is evident in the increase in global area under cultivation of these crops: in the period from 1996 to 2000 this grew from 1.7 to 42.4 million hectares (Cockburn, 2002). This further increased from 2000 to 2006 to total 102 million hectares (James, 2006). It has often been claimed that transformation approaches are a faster and more accurate way to develop new crop varieties (NRC Committee on Genetically Modified Pest-Protected Plants, 2000; Prakash, 2001). These claims, however, are not always accurate as cultivar development results from overlapping cyclical processes of which assembling and generating new genetic diversity is only the first phase (Allard, 1960; Simmonds, 1979; Mayo, 1987; Brown et al., 1990; Stalker et al., 1992; Bos et al., 1995; Comstock, 1996; Hill et al., 1998; Charrier et al., 2001). The ability to use transformation approaches is further limited by the following factors: The need for special technological requirements (eg. tissue culture facilities and protocols for the crop of interest); accessibility to different plant tissue; expense (ie. for laboratories operating on low budgets); availability of specialized laboratory equipment; and patent clearance (Hansen et al., 1999).

1.3.3 Screening in breeding programs

Selection methodologies and their efficiency are critical to both conventional and biotechnology-based breeding programs (Singh, 2003; Mayo et al., 1987; Bos et al.,

1995). In the past, selection criteria were based more heavily upon observable characteristics related to performance and phenotype (Bos et al., 1995). Whilst these criteria are still important, more comprehensive screening at a genetic and biochemical level is crucial to effective plant selection in terms of modern breeding objectives (Bos et al., 1995). Whilst a range of informative tests are available and in use, these are often rate limiting in breeding programs for several reasons including: time required; large numbers of plants involved in breeding programs (especially mutation-based programs); and cost of labour, equipment and reagents (Givens et al., 1997).

The limitations mentioned in this chapter advocate the need for more suitable, low-input approaches to enhancing conventional breeding in resource-poor developing nations.

1.4 NEAR-INFRARED SPECTROSCOPY

1.4.1 Introduction

Agriculture produces food and fibre to sustain human existence (Shenk et al., 2001). These agricultural products have been marketed, traded and fed based on their quantitative characteristics all through recorded history (Shenk et al., 2001). It has become apparent that quality and nutrient characteristics are equally if not more important than quantity (Fontaine et al., 2002). Subsequent research has led to the development of many techniques and methods for food analysis, yet these are often expensive, time intensive, destructive and require repetition for every different constituent requiring analysis (Givens et al., 1997). Chemical analysis of foods occurs in many disciplines including: quality and safety analysis; dietetics research; and feed and forage evaluation amongst others (Givens et al., 1997). Plant breeders often use quality traits to select the best candidates in breeding programs (Bos et al., 1995). Due to large numbers of plants, a need to reuse seed following screening, wide ranges of constituents and time constraints, wet-chemical procedures are rate limiting factors in plant breeding (Frenzel, 2003).

The “increasing demand for product quality and production rationalization” in the food and agriculture industries has led to the development of more rapid and environmentally friendly analytical methods (Kawano et al., 2002). Through this, near-infrared

spectroscopy (NIRS) has emerged as an extremely powerful tool for quality control and process monitoring (Kawano et al., 2002).

1.4.2 The potential of NIRS analysis in plant breeding

Near-infrared spectroscopy (NIRS) is a powerful, non-destructive tool able to indirectly estimate levels of chemical entities in a range of environments following careful calibration (Shenk et al., 2001). It emerged predominantly for use in quality control and process monitoring; however is becoming progressively more popular amongst plant breeders, providing qualitative and quantitative information on all the constituents of a sample in a single spectral reading (Frenzel, 2003). Bulk grain samples can be rapidly screened by NIRS, without prior preparation, to determine multiple constituents in a sample, allowing further analysis at a later stage, or even propagation (Velasco et al., 1999; Orman et al., 1991; Baye et al., 2004). Unfortunately bulk grain analysis does not allow the identification of individual grains whose constituents deviate from the population mean, which is highly desirable to plant breeders (Baye et al., 2006). Single grain NIRS analysis has been used to sort individual maize kernels for fungal infections (Dowell et al., 2002; Pearson et al., 2001and 2004) and to identify genetically modified kernels (Munck et al., 2001). These studies suggest that NIRS may be used to detect the levels of nutritional compounds in whole single grain sorghum. Such non-destructive methods are invaluable to mutation breeding programs where variation exists between individual seeds, and the goal is often to isolate those outlying individuals without the need to plant them for an additional season (Baye et al., 2006). The incorporation of NIRS in a breeding program may decrease the number of generations of self-pollination required prior to screening for significant novel mutations in terms of constituent levels, thus saving time, and aid the isolation of candidate lines in a non-destructive manner.

1.4.3 NIRS principles

Light absorption at particular wavelengths within the near infrared region of the light spectrum correlates with the presence of particular molecular bonds. Unlike conventional spectroscopy, this spectral information is repeated several times throughout the NIR spectrum, requiring multiple wavelengths to determine the presence

of a particular molecule. In addition to absorbencies at a variety of wavelengths, spectral information in complex molecules is also obtained from wave overtones and combinations according to the number and nature of molecular bonds constituting the molecule (Reeves, 1997; Givens et al., 1999).

There are a range of instrument models available for NIR analysis, yet despite their differences they are all based upon the same essential principles (Workman et al., 2001). A primary light beam is produced from a source of radiant energy and discriminated into particular wavelengths to obtain more specific information (Workman et al., 2001). In older models, interference filters were used to obtain several fixed wavelengths entered into the NIR instrument prior to scanning. This is extremely limiting as knowledge of the necessary wavelengths for the analysis would be required prior to analysis. Newer models contain monochromators or interferometers that facilitate scanning across the entire NIR spectrum, thus allowing spectral information to be obtained for unknown and previously unstudied samples. Interferometers (eg. ft-NIR) have been indicated to solve issues of calibration transfer and sample perparation and generate cleaner spectra. This spectral information may then be disseminated through multivariate statistical techniques (chemometrics) in conjunction with appropriate wet chemistry reference methods (Workman, 2001). Following discrimination the light beam is directed onto the sample and a photoelectric detector collects and determines the resultant reflected or absorbed radiant energy (Workman et al., 2001). Loss of transmitted or reflected radiant energy is proportionate to the distance between the sample and the detector, thus placing them close together provides more accurate data (Workman et al., 2001).

Near infrared spectroscopy utilises light in the interval between 4000 and 12500 cm^{-1} (800nm – 2500nm), which has in the past most often been provided by Tungsten halogen lamps (Frenzel, 2003). More recent instrumentation also uses diode array displays (DAD) as a light source and interferometers as grating (Wolf et al., 20007). The basic relationship between light absorption and the concentration of a solute is described by the Beer-Lambert law $C_x = A_x/e.l$ where:

Cx = concentration of the test solute

Ax = absorption of the test solution

e = molar absorptivity of the test solute

l = the path length travelled by the light through the solution (Frenzel, 2003)

Where infrared light is incident on a solid sample it possesses a variety of fates (Dryden, 2003). Some light will be reflected from the surface of the sample (specular reflectance), some will enter the sample and possibly be absorbed within it, and the remainder will either be transmitted through the sample or be reflected from it (diffuse reflectance) (Hruschka, 1987). Where NIRS is applied to a solid sample (eg. whole grain or seed) the Beer-Lambert law becomes limiting, as path length cannot be predicted (Dryden, 2003). In addition, variations within diffuse reflectance spectra result from non-specific radiation scatter, variable path lengths and ranging chemical compositions of the sample (Barnes et al., 1989). Accordingly, any mathematical relationship between transmitted or reflected radiant energy and analyte content cannot be described, thus predictive ability for each application must be established through calibration (Givens et al., 1997).

There are three essential types of scanning options available for NIR analysis: Reflectance, transmittance and transflectance (Baer et al., 1983). Reflectance utilises diffuse radiant energy bounced off the surface of a solid, opaque sample (Baer et al., 1983). Transmittance is a measurement of the absorption of light at different wavelengths as it passes through a transparent sample (Ciurczak, 2001). Similarly to transmittance, transflectance is also performed on transparent samples although spectral information is collected in the same manner as with reflectance. Light thus passes through the sample and is reflected back through the sample (Ciurczak, 2001). All radiation is recorded, including that reflected within the sample, providing a more reliable measure of light scattering than transmittance where back-scattered radiation is not recorded. In all three cases the light beam is passed across the optics window of the instrument at set distance intervals determined by the instrument. Multiple readings are typically performed and averaged to eliminate inaccuracy generated through the presence of uneven particle sizes and inconsistent composition (Workman et al., 2001). Fibre optic cables are now available to facilitate scanning outside of the instrument

itself (Workman et al., 2001). A suitable method of scanning must be selected according to the characteristics of the sample to be scanned, and the sample preparation required (Williams, 2001).

Baye et al (2006) report that near-infrared transmission spectroscopy of single maize kernels generate levels of noise too high to calibrate reliable models. Similar conclusions have previously been drawn, strongly recommending the use of near-infrared reflectance spectroscopy on samples of a more solid composition (Orman et al., 1992; Cogdill et al., 2004). Reflectance has been shown to be more accurate than transmittance on solid samples due to the inability of light to pass through the sample (Williams et al., 1992).

1.4.4 Advantages and considerations

NIRS exhibits a wide range of advantages over traditional wet-chemistry methodologies (Givens et al., 1997). These include: Reduction in time as reflectance or transmission spectra can be measured in seconds, elimination of toxic reagents or waste products, investigation into a range of constituents in a single non-destructive scan and facilitating formulation of a complete fingerprint of a sample providing both qualitative and quantitative information (Osborne et al., 1993; Ciurzack et al., 2002).

The greatest limitations in NIR analysis revolve around the need for high-precision spectroscopic instruments, dependence on time consuming and laborious calibration procedures, complexity in the choice of data treatment and lack of sensitivity for minor constituents (Norris, 1989; Workman, 2001). In addition to these, calibrations may not necessarily be transferred between instruments (Givens, 1997). Baye et al. (2006) suggest that NIRS is not highly predictable for single seed analysis in maize considering external factors (such as seed mass) may dramatically contribute to the relative composition changes which are not proportionally reflected in the NIR spectra. They recommend robust calibrations to account for variations in seed weight and also advise using absolute quantities over percentages of constituents within single grains (Baye et al., 2006).

1.4.5 Calibration

The accuracy of NIRS is highly dependent upon sample-specific calibration and an accurate reference method (Ciurczak, 2001). Even in cases where instruments have been supplied with calibration equations in place they still require adjustments for local conditions and instrument bias (Hymowitz et al., 1974, Osborne et al., 1982, Shenk et al., 1991). Despite rigorous study, it is widely accepted that calibrations for complex systems cannot be reliably transferred between NIR machines (Feudale et al., 2002). Accurate calibration of NIRS instrumentation requires selection of a sample/spectral library representative of the entire population to be analysed (Shenk et al., 2001). Statistical analysis of the data set can be performed to verify the global nature of the calibration (Burns, 2001). Mahalanobis distances, using the standardized H statistic, have been used to predict how different a sample is from the average sample in the calibration set. Known as the Global H (GH), this value has the ability to control the linearity of the relationship between reference sample chemistry and NIR spectrum, as enlarged domains can cause this relationship to become non-linear (Shenk et al., 2001). The distance from a sample to the closest neighbouring sample is known as neighbourhood H (NH), and can be used to control the closeness of samples in the calibration set. It has been defined as "the space around the sample where another spectrum would have a distance to the sample less than a specified limit value" (Shenk et al., 2001). These statistical limits aid the selection of a data set that covers the entire analysis domain and reduces the number of samples required for accurate representation to a minimum, excluding none providing significant input (Shenk et al., 2001).

Due to the complexity of NIR calibration a wide variety of calibration methods and associated software packages have become available (Coetzee, 2003). These aim to eliminate overlapping, redundant and interfering peaks from those of interest (Lee et al., 1997; Givens et al., 1999) and reduce the strong interference caused by sample scattering, especially where particle size varies greatly. The most appropriate calibration modelling method needs to be selected, although all should be able to compute multiple wavelengths (Shenk et al., 1991). Examples of these methods include partial least

squares (PLS), multiple linear regession (MLR) or principal components analysis (PCA) (Boysworth et al., 2001).

A calibration experiment must be performed by acquiring spectral information of the sample set under constant, controlled conditions and relating them to separate reference analysis methods using a suitable multivariate technique (Workman, 2001). Multivariate analysis will isolate any pattern with which it can predict constituents. Naturally the nature and size of the data set are critical to the predictive ability of the equation developed (Workman, 2001).

The predictive ability of equations generated through calibration experiments are typically indicated by the goodness of fit shown through standard deviations and correlation coefficients (Shenk et al., 1991). The standard error of calibration (SEC), or residual standard deviation, is a root mean square average of the errors about the fitted line, representing variation from the prediction, and is usually an underestimate of errors likely to occur whilst trying to predict further samples using the model (Lee et al., 1997). This is because the calibration is developed specifically for the calibration data set and not for the population as a whole (Shenk et al., 1991). Ensuring a large, evenly distributed sample set helps to keep this error to a minimum (Coetzee, 2003).

1.4.6 Reference methods

Reliable calibration is dependent on accurate reference techniques (Workman, 2001). Crude protein can be determined through several indirect techniques such as the Micro- or macro-Kjeldahl method (Chibber, 1978), Dumas combustion method and use of autoanalysers (eg Technicon or Leco) (Jambunathan et al., 1984; AOAC, 2005). These methods assay for nitrogen levels, which are assumed to be a relative indication of protein amino groups, and are multiplied by the universal constant 6.25 in order to obtain a crude protein value (AOAC, 2000). Lysine analysis can be performed using standard acid hydrolysis followed by HPLC amino acid analysis. Typically this is performed using 6N HCl at 110° C for 24h and determined using classical Moore-Stein ninhydrin reagent in an amino acid analyser such as a Beckman 6300 (Moore et al., 1948; Applications data, 1983; Fontaine et al., 2002). Tryptophan, similarly to cysteine,

cannot be analysed using acid hydrolysis methodology, as it is highly susceptible to acid and will be destroyed by the reaction (West et al., 1996). The use of one of the following reagents is thus required: dodecanethiol, methane sulfonic acid, mercaptoethane sulfonic acid and thioglycolic acid (Jones et al., 1981; West et al., 1996). Alkaline hydrolysis using barium hydroxide was used in successful NIRS calibration for a range of amino acids in feedstuffs (Fontaine et al., 2002). Phytate P has been assayed using several methods including: a modified spectrophotometric evaluated colorimetric method described by Young (1936) and modified by Haug et al. (1983); a colorimetric method described by Chen (1956); and anion-exchange HPLC (Harland et al., 1986) amongst others. Moisture analysis is critical to ensuring reliable calibrations and is typically performed through oven drying and recording mass differential (ISTA, 1996; AOAC, 2000; AOAC, 2005).

Many NIR spectrometers contain detectors which cover the visible range of light thus calibrations for colour have also been reported (Black and Panozzo, 2004). Colour classifications performed by a trained eye might be sufficient for crude discriminant colour modelling, however, a visible light colorimeter, standardised to ceramic reference tiles, would be needed to quantify the colour parameters of a sample accurately enough for regression purposes (Hunter, 1975; ASTM, 1996; Black and Panozzo, 2004).

1.4.7 Validation, assessment and prediction

The aim of validation is to determine the predictive ability of the model generated during calibration. This is accomplished through the calculation of the standard error of performance (SEP), which is the standard deviation for the residuals arising out of differences between actual and NIRS predicted values for samples excluded from the calibration set (Workman et al., 2001). This is also known as standard error of analysis (SEA) or performance (SEP) and is most reliable when computed on samples not included in the calibration sample set (Wetherhill et al., 1987; Velasco et al., 1998; Velasco et al., 1999; Workman et al., 2001; Esbensen, 2006).

The chosen mathematical model is required to meet the following criteria as according to Shenk et al. (2001):

1- Lowest SEC value and fewest number of wavelengths to avoid over-fitting
2- No wavelength must have a partial F-stat value of less than 10 for its corresponding regression coefficient to prevent over-fitting
3- Regression coefficients must not exceed approximately 10000 in order to minimise problems in equation transfer due to instrument noise and other inter-instrument variations.

The most accurately predictive model can then be selected where the slope of the regression line relating SEP to the primary reference value is closest to 1 (Shenk et al., 2001). In addition, the number of principal components upon which the model is based can best selected by choosing that which results in the smallest difference between SEC and SEP (Esbensen, 2006).

1.4.8 Applications

NIRS has been used to asses a wide variety of characteristics in plant material to date. Total and phytate phosphorous levels have been predicted using NIRS in a range of cereals including: maize, wheat, barley, sorghum and oats (De Boever et al., 1994). Protein, fibre, ash, fat and starch quantities have been reliably measured in 22 different types of feeds (Gerlach, 1990). In some cases it has been reported that crop varieties (such as wheat, oats and field beans) have been screened for limiting amino acids such as lysine, threonine, tryptophan and methionine (Williams et al., 1984; Biston, 1987; Shenk, 1995). Essential amino acid calibrations have been obtained using NIRS in soy, rapeseed meal, sunflower meal, peas, fishmeal, meat meal products and poultry meal (Fontaine et al., 2001). In fact, NIRS was shown to be superior to other methods for estimation of amino acid distribution (Fontaine et al., 2001; Fontaine et al., 2002). NIRS has even been used as a tool for classifying endosperm genes and gene combinations in milled barley seeds (Jacobsen et al., 2005).

Crude protein, acid detergent fibre (ADF) and neutral detergent fibre (NDF), amongst other digestion measures, have been accurately predicted in sorghum silage (Budiongo et al., 1996). Whole seed determination of single grain kernels has previously been

attempted in a range of crops, with particular focus on maize kernel analysis (Orman et al., 1992; Velasco et al., 1998; Velasco et al., 1999; Cogdill et al., 2004; Baye et al., 2006). Sorghum has been shown to exhibit high variation in amino acid composition and crude protein levels which aids establishment of good NIRS calibration equations (Fontaine et al., 2002). It has been reported that highly informative and accurate predictions can be obtained for sorghum on milled and sieved samples of more than one seed (Fontaine et al., 2002). Successful calibration of NIRS for a wide range of sorghum parameters would be of great benefit to plant breeders and other researchers of the crop.

1.5 MUTAGENESIS

1.5.1 Methods of mutagenesis

In 1928, Herman Nilsson-Ehle and Ake Gustaffson started mutation experiments on barley, reporting mutants with a compact head type (Medina et al., 2005). Much research has subsequently been performed and a wide variety of methodologies have been developed. All mutagenic agents may be categorised as either being physical or chemical in nature. Chemical mutagens include: diethyl sulphate; ethyl methane sulphonate; isopropyl methane sulphonate; ethylamine; and sodium azide amongst others (Medina et al., 2005). Physical mutagens include: ultra violet radiation; electromagnetic radiation (eg. x-rays or gamma rays); corpuscular radiation (fast neutrons or beta particles); and ion or electron beams (Medina et al., 2005). In addition, mutants have been generated through somaclonal variation in callus cultures, and grown out and introduced into breeding programs (Cassels et al., 2003).

A wide variety of plant tissue has been treated successfully including seeds, pollen, cuttings, bulbs, tubers, corms, stolons, *in vitro* cultured cells and even whole plants (Medina et al., 2005).

At a genetic level there are several phenomena which cause mutations, some of which occur naturally without exposure to mutagenic agents (Medina et al., 2005). They can be categorised as spontaneous or induced, somatic or genetic, chromosomal or extra-chromosomal (Medina et al., 2005). The type and frequency of mutation is dependent upon the mutagenic agent, and its dose rate (Medina et al., 2005). Most mutagenic

agents used by plant researchers and breeders in the past have caused large genetic changes resulting in high mortalities and the need for large populations for treatment (Micke, 1999). Induced mutations occur more or less randomly in the genome thus only one of the two or more alleles of a locus is affected, resulting in the need for breeders to ensure homozygosity through self-pollination before the trait will be properly expressed (Micke, 1999).

1.5.2 Heavy-ion beams in plant breeding

The use of mutagenesis in breeding has been limited by high plant mortalities and the scale of operation required, often using thousands of plants, all of which require screening (Singh, 2003). Chemical mutagenesis have been widely used has been favoured in the past and still remains popular, although exposure dose plays a critical role in mutation frequency (Medina et al., 2005). Physical methods are typically less destructive and simpler to perform as they do not require cautious wet chemistry procedures (Medina et al., 2005). Most physically induced mutants have previously been obtained through radiation with X-rays or gamma-rays, which are classified as low-Linear energy transfer (LET) irradiations (Abe et al., 2002). Such low-LET irradiations show a wide range of variation and frequency, causing large deletions, translocations and various rearrangements in plant genomes (Redei et al., 1992; Abe et al., 2002).

On the other hand, high-LET radiations such as those generated through the use of heavy-ions, can be controlled to deposit high energy at precise locations (Abe et al., 2002). AVF cyclotron particle accelerators have the potential to generate a range of Carbon, Neon or Cobalt heavy-ions for radiation treatment of biological material (Abe et al., 2002). The use of heavy-ion beam irradiation generated by cyclotron particle accelerators is, however, relatively unexplored and there is currently no literature on this technology having been used to develop sorghum (Abe et al., 2002). Although most studies on heavy-ion irradiation induced mutations at the molecular level have been performed on mammalian cells (Chan et al., 2002; Morimoto et al., 2003), it has been reported that the frequency of deletion mutations is higher than mutations generated

through gamma-ray irradiation (Thacker et al., 1986; Kagawa et al., 1995). Research on mutation breeding using cyclotron generated radiation shows the induction of significantly fewer deleterious mutations than with other treatment methods, although there is a very limited amount of research on this subject available (Vorojtsov et al., 2001).

There is a need to perform further basic and practical research in order to make the use of ion beams more efficient for breeders in the future (Medina et al., 2005). Access to cyclotron facilities, however, is difficult for South African plant breeders to obtain, let alone breeders in less developed regions of Africa.

1.6 HERBICIDE TOLERANCE

1.6.1 Striga problem

Sorghum production is hampered by several insect and weed parasites against which breeding programs have attempted to develop resistance with varying degrees of success (Hausman et al., 2000; Kumari et al., 2000; Mehta et al., 2005; Kumar et al., 2006). In particular, sorghum yield losses are greatly reduced by the prevalence of *Striga* (most notably *Striga hermonthica* (Del.) Benth and *Striga asiatica*), or "witchweed", which is an obligate out-crossing hemi-parasitic weed species affecting most tropical Gramineae (Rodenburg et al., 2004). *Striga* damages its host by withdrawing water, nutrients and assimilates and inducing enzyme and hormone changes which disrupt host-water relations and carbon fixation (Press et al., 1996). Studies have been performed to ascertain the relationship between tolerance and resistance to the parasite and the associated yield losses, helping to identify screening procedures yet rendering little success in producing varieties unaffected by *Striga* (Rodenburg et al., 2004). This approach to combating the parasite is limited by the complexity of the science, the need for highly heritable resistance, significant genotype x environment interactions in the field, putative *Striga* variability and the time required performing trials and breeding programs (Omanya et al., 2004). Strategies developed in Africa over the last 50 years have focused on agronomic practices, host plant resistance and herbicide applications yet while effective have not been widely adopted by farmers due to their benefits only becoming evident in the medium to long-term; the

requirement of an understanding of the parasites life-cycle; inadequate and ineffective grain under high levels of infestation and expensive conventional herbicide applications (Kanampiu et al., 2003). The development of imidazolinone herbicide resistant varieties of maize has proved to be an effective means of combating *Striga*, avoiding complications in breeding for tolerance or resistance (Newhouse et al., 1991; Kanampiu et al., 2002). As yet there remain no varieties of sorghum resistant to herbicides able to control *Striga* (Dembele et al., 2004).

1.7 PHYTIC ACID

1.7.1 Structure, biosynthesis and role in nutrition

Phytic acid, or *myo*-inositol 1,2,3,4,5,6-hexakiphosphate, is an abundant component of sorghum seeds which is typically deposited in protein bodies as a mixed salt of mineral cations (eg. K, Mg, Ca, Zn and Fe) (Guttieri et al., 2004). Diets high in phytic acid thus decrease the bioavailability of essential micronutrients (Jacobsen et al., 1983; Kies, 1985; Sandstrom et al., 1987; Brune et al., 1992). This is of great concern where sorghum is a staple diet, especially considering the prevalence of hidden hunger due to micronutrient deficiencies (von Braun, 2005). In order to ensure lower levels phytic acid in commercially available varieties, it is critical that breeders ascertain levels in the varieties used in their breeding programs and design programs to generate new varieties with low phytic acid phenotypes, such as the generation of low phytic acid (lpa) mutants in maize (Raboy, 2008).

1.7.2 Induced low phytic acid (lpa) mutants

Mutation breeding for low phytic acid varieties has been met with success in several varieties (Guttieri et al., 2004), although not through the use of cyclotron irradiation. These include: maize (*Zea mays* L; Raboy et al., 1996; Pilu et al., 2003); barley (*Hordeum vulgare* L; Larson et al., 1998; Hatzack et al., 2001); rice (*Oryza sativa* L.; Larson et al., 2000); and soybean (*Glycine max* (L.) Merr.; Wilcox et al., 2000; Hitz et al., 2002). Although a wide range of phytic acid concentrations have been identified in various sorghums, no low phytic acid mutants have been isolated and characterized to date.

Heritability studies on low phytate (lpa) maize has shown that the distribution of P as inorganic P relative to phytic acid P is a quantitative trait determined by seed characteristics (Gutteiri et al., 2004). Genetic similarity between maize and sorghum (Binelli et al., 1992) provides strong motivation towards successful isolation of lpa mutants in sorghum.

1.8 PROTEIN QUALITY

1.8.1 Aspects of protein quality in nutrition

Protein quality and quantity limits sorghum as a staple crop (NRC, 1996). Large variation in total protein and amino acid profile exists between the many different sorghum varieties and varieties (Jambunathan et al., 1984). This variation can probably be attributed to the diverse range of agroclimatic conditions under which the crop is cultivated (Waggle et al., 1967). Sorghum is typically deficient in the amino acids lysine, tryptophan and methionine, although lysine is almost always the most limiting (FAO, 1995). Amino acid scores are related to the ratios of protein fractions in the seed (FAO, 1995). The alcohol-soluble prolamine fractions are reportedly low in lysine thus a mechanism to increase lysine content in grains is to generate mutants or breed for varieties with low prolamine (van Scoyoc et al., 1988). An increase in protein quantity alone will not resolve issues relating to protein quality and limiting amino acids (FAO, 1995).

1.8.2 Previous attempts to breed quality protein crops and sorghum

High lysine mutants have been isolated and studied in several cereal crops species including: maize (Mertz et al., 1964); barley (Munck et al., 1969; Doll, 1970; Ingversen, 1972); and sorghum (Singh et al., 1973). The biological value and protein efficiency ratio (PER) of high lysine sorghum mutants surpassed those of wildtype controls (Singh et al., 1973; Axtell et al., 1976). Although high lysine mutants have previously been isolated in sorghum, there is nevertheless a need to develop protein quality in existing varieties, and especially in varieties adapted for the marginal areas of the African subsistence farmer.

1.9 DISCUSSION AND OBJECTIVES

This review of the literature highlights the current use and future potential of sorghum as a grain crop, and draws attention to its limitations in terms of inherent nutritive capacity and production. A need for the improvement of existing germplasm, as well as generation of new material for breeding, was raised and several methods of achieving this end were discussed. Out of this, the potential of heavy-ion beam mutagenesis in introducing new genetic diversity was illuminated. In addition, a need for rapid screening techniques became evident, particularly for screening nutritional characteristics. The past success and future potential of near-infrared spectroscopy was discussed in addition to the methodology of reliable calibration development.

Accordingly, the objectives of the study reflected in the following chapters were to investigate cyclotron induced heavy-ion beam mutagenesis for sorghum improvement and the tracking of sorghum mutagenesis breeding programs through the development and use of NIR calibrations. In addition to investigating the potential of the methodologies used, there was a secondary objective of isolating improved or diverse material for potential inclusion in breeding programs.

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CHAPTER TWO

THE INFLUENCE OF HEAVY-ION IRRADIATION DOSES ON MUTATIONS IN SORGHUM

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ABSTRACT

In the breeding of grain sorghum, new low-input methodologies are needed to increase genetic diversity in breeding populations. Heavy ion beam mutagenesis is an emerging technique promising to overcome some of the limitations of classical mutagenesis methods through high linear energy transfer (LET) interactions. The aim of this study was to investigate its potential in seven sorghum varieties and to establish an optimal dose rate for maximum relative biological efficiency (RBE). Varieties were treated with 75, 100 and 150 Gy $^{12}C^{6+}$-ions accelerated to 135 MeV u^{-1}. M_1 generation was scored for germination, plant survival, morphological characteristics and chlorophyll mutations, and comparisons were made between dose rates. Field trials were performed over two generations from 2006 to 2008 and individual plants were self-pollinated to render the M_2 and M_3 generation seed. Developmental stage (DVS), stigma formation, chlorophyll mutations and protein variability was also measured and compared. No consistent trends were observed between all varieties across all treatments for germination or survival. ZSV3 and N249/R9019 (77) exhibited no difference in germination or survival between treatment and control samples. Kari Mtama, Serena and Seredo showed significantly improved germination and survival following

treatment at 100 and 150 Gy. Germination of ICSV3 improved after treatment, but plant survival decreased. 02MN5453 showed decreased germination and survival after treatment. Visible morphological and growth differences were clearly evident between treated and untreated seedlings and more severe in some varieties than others. These differences were not replicated in the field although slight differences were observed between treated and control varieties with respect to stigma formation and DVS. The M_1 generation showed only one chlorophyll mutation whereas 30 were visible in the M_2 field trials of 75 Gy treated N249/R9019 (77). No significant mean difference in M_2 crude protein content was induced by the treatment of the four varieties tested. Strong positive regressions between M_2 crude protein variability and treatment were shown in three of the four varieties analysed. Despite evidence of effects of the treatments, an optimal dose rate was not established because the range of treatments was not wide enough. Large differences in varietal responses suggested a need to establish an optimal dose, or dose range, for specific varieties or material from common backgrounds. The methods used to track such high-LET mutagenesis methods need to be more informative and able to track more subtle genetic change at a biochemical or molecular level.

2.1 INTRODUCTION

Sorghum (*Sorghum bicolour* (L.) Moench) is a widely grown crop, cultivated for a variety of uses (NRC, 1996). It forms the staple diet of more than 500 million people in over 30 countries and is currently the 5th most important cereal after rice, wheat, maize and barley (NRC, 1996). Sorghum is a primary source of protein and energy for many of the world's poorest people because it is cultivated largely under subsistence conditions for home consumption in Africa and Asia (Klopfenstein and Hoseney, 1995). In addition it has a range of non-food uses including the production of: sugar, syrup, fuel, fodder, bedding, roofing, fencing and paper (Schaffert, 1992). Sorghum has been suggested as a source for fuels (Schaffert and Gourley, 1982) and with current trends to look for alternatives to fossil fuels, it is becoming an important crop for biofuels production (Vermerris et al., 2007; Yuan et al., 2008). Sorghum's inherent high nutritional value, photosynthetic efficiency, ability to perform in marginal areas, versatility in use and production, combined with underutilized and underdeveloped

genetic potential, compel further research and development of sorghum as a staple crop (NRC, 1996; Conway et al., 1999).

Classical breeding approaches have progressively become less effective as the required complexity of crop characteristics increases, whilst available genetic diversity within breeding populations remains the same (Singh, 2003). Advances in molecular biology and plant genetics over the last 50 years have led to the development of a host of biotechnology tools available to plant breeders (Cassels et al., 2003). These include: *in vitro* propagation (Murashige et al., 1962; El-Sharkawy, 1989), protoplast fusion and ploidy manipulation (Foolad, 2004), gene knockouts (Gale, 2003) and genetic transformation (Goure, 2004), amongst others. These so called "high-technology" approaches are limited by the need for technological facilities and specialized laboratory equipment, patent clearance and expense (Hansen et al., 1999). There is thus a need to investigate "low-input" methods of enhancing and introducing new genetic diversity for breeding programs. The need to generate novel sorghum mutants for inclusion in breeding programs has been explained in greater detail (Vermerris et al., 2007).

Mutagenesis is a well established method of introducing genetic diversity and developing novel crop characteristics (Medina et al., 2005). A wide variety of methodologies have been cited using either physical (eg. UV, x-rays or γ-ray) or chemical (eg. Methyl methanesulfonate or ethyl methyl sulfonate) mutagens (Micke, 1999). Physical methods are typically less destructive than chemical approaches and are simpler because they do not require cautious wet chemical procedures (Medina et al., 2005). Most physically induced plant mutants have previously been induced through radiation with X-rays or γ-rays, which are classified as low-linear energy transfer (LET) irradiations (Abe et al., 2002). Such low-LET irradiations show wide range of variation and frequency, causing large deletions, translocations and various rearrangements in plant genomes characterising low relative biological efficiency (RBE) (Redei et al., 1992; Abe et al., 2002). There has been increased use of heavy-ion beam irradiation for mutagenesis in medicine research and plant breeding (Kobayashi et al., 2004). It has been claimed that the radiation effects of heavy-ion beams differ from those of X-rays

and γ-rays due to high-density ionization along narrow ion tracks in a target (Oikawa et al., 2007).

Heavy-ion beams are generated through ring or avf-cyclotrons and are characterised as being high-LET radiations which reach deeper penetration depths than X-rays and γ-rays (Oikawa et al., 2007). It is well established that high-LET radiations are more effective than low-LET radiations at causing biological effects and therefore increased mutagenicity (Suziki et al., 2006). The cause for this is that high-LET irradiation results in a few ion tracks crossing a single cell, thus causing higher levels of deletion mutations and fewer deleterious effects (Jäkel, 2008). Research on the nature of ion-beam induced mutations has shown some of the differences between low-LET and high-LET irradiations including: the induction of a higher fraction of deletion mutations resulting from high-LET (Kronenburg et al., 1995; Stoll et al., 1996; Zhu et al., 1996; Schmidt and Kiefer, 1998; Kiefer et al., 1999); higher numbers of mutations induced by high-LET (Suziki et al., 2006); and different types of DNA damage caused by high-LET ion-beams (Kronenburg et al., 1995; Zhu et al., 1996; Schmidt and Kiefer, 1998; Kiefer et al., 1999). The use of mutagenesis in plant breeding has been limited in the past by high plant mortalities due to low-LET approaches and the requirement for large trials using thousands of plants (Singh, 2003). Thus new methodologies that address these limitations would be of great value.

Heavy-ion beams for mutagenesis have been widely applied to medical research in mammalian tissues (Suzuki et al., 2006). However, research on a number of agricultural, ornamental and model plants has also been reported (Yu et al., 1991; Abe et al., 2002; Zhang et al., 2008). These include: *Arabidopsis* (Hase et al., 2000; Tanaka et al., 2002); wheat (Zhang et al., 2008); maize (Mei et al., 1994a; Mei et al., 1994b); ornamental flowers (Abe et al., 2002; Zhou et al., 2006) and rice (Liu et al., 2008). More recently heavy-ion beam mutagenesis has been used to improve sweet-stem sorghum by Dong et al. (2008), who have published several additional papers on sorghum mutagenesis in 2007 in Chinese (Zhang et al., 2008). This research highlights the potential of ion-beam mutagenesis, and motivates the need for further investigation.

The aim of this work was to investigate the potential of heavy-ion treatment on several sorghum varieties with respect to dose rate and varietal differences. As access to cyclotron heavy ion-beam facilities is difficult for South African plant breeders, was important to establish clear treatment protocol for optimum RBE in respect to current breeding targets in commercially viable varieties.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Grain samples of 7 varieties of sorghum (*Sorghum bicolour* (L.) Moench) were hand selected for mutagenesis from the African Centre of Crop Improvement[1] (ACCI) breeding program (Table 2.1). Selections were made based on variety performance and suitability towards established ACCI breeding targets (Table 2.2). Seven varieties were selected for a dose-rate trial based on their diverse genotypes and plant type (Table 2.1). Grain was hand-cleaned and counted into three replicates of 30 grains for each treatment and control. These were packed into plastic containers for transport (Fig. 2.1).

Figure 2.1 Cleaned grain packaged for transport to RARF[2] for treatment.

2.2.2 Heavy-ion treatment

Samples were sent to the RIKEN Accelerator Research Facility[2] (RARF) for mutagenesis in 2006. Sorghum grain was exposed to $^{12}C^{6+}$-ions accelerated to 135 MeV u^{-1} with a dose of 75, 100 and 150 Gray (Gy) (Table 2.1), and returned to ACCI[1].

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[2] RIKEN Accelerator Research Facility, Saitama, Japan

Table 2.1: Varieties selected for mutagenesis dose rate trial and their attributes from African Centre for Crop Improvement (ACCI) field trial records (unpublished)

Variety	Treatment (Gy)			Grain colour	Glume colour	Grain size	Head L x W (cm)	No. of heads	No. of tillers	Yield	Avg. height (m)	Heading	Bird tolerance	Other	Origin	Cultivated
	75	100	150													
Kari Mtama	X	X	X	W	B	L	130x80	4	4	G	1.5	medium	5		Kenya	Kenya
ZSV3	X	X	X	RB	R	L	100x70	4	4	H	2.2	early	4	GSS	Zambia	Zambia
Serena	X	X	X	B	BR	M	90x60	5	5	G	1.5	late	4	HT	Uganda	South & East Africa
Seredo	X	X	X	B	RB	M	90x60	5	5	G	1.5	late	4	HT	Uganda	South & East Africa
ICSV3	X	X	X	YB	LB	L	150x50	4	4	G	1.8	late	4	GPT	India	Kenyan breeding line
N249/R9019 (77)	X	X	X	Y	LB	S	125x55	3	3	G	1.7	medium	4		USA, Kansas	Breeding line
02MN5453	X	X	X	WY	LB	L	140x75	5	5	P	1.4	early	5		USA, Texas	Breeding line

Colours: C = clear; R = red; B = brown; LB = light brown; RB = red/brown; W = white, Y = yellow; YB = yellow/brown; WY = white/yellow; D = dark; DR = dark red

Bird tolerance: 1= birdproof/no damage; 2 = highly tolerant/minimal damage; 3 = tolerant/significant damage; 4 = slight tolerance/major damage; 5 = intolerant/total destruction

Other: CT = cold tolerant; GSS = prone to grain storage insects; GPT = good plant type; HPH = high plant health; HT = herbicide tolerant; PS = photoperiod sensitive; WA = wide adaption; WS = worker selection

Yield: A= acceptable; G = good; H = high; P = poor Grain size: L = large; M = medium; MS = medium/small; S = small Origin: SALR; South African landraces

Table 2.2: List of sorghum limitations and ACCI targets in mutagenesis breeding program

Category	Limitation	Breeding target
Drought	Susceptible	Tolerance
Heat	Susceptible	Tolerance
Diseases	Downy mildew	Resistance
	Ergot	Resistance
	Leaf spot	Resistance
	Rust	Resistance
Pests	Stalkborer	Resistance
	Weevils	Resistance
Nutrients	Low tryptophan and lysine	Enhance
	Low protein	Enhance
	Stem sugar	Enhance
Agronomic	Late seed set	Earliness
	Lodging	Dwarfism
Other	Birds	High tannin varieties
	Striga	Herbicide resistance

2.2.3 Greenhouse germination trials

All M_0 treated and control seeds were germinated in 2006 according to the speedling system (Speedling Inc.[3]) (Anonymous, 1983) modified and described by Smith (1986). Seedlings were cultivated in standard white polystyrene trays with 200 plug cavities, inverted pyramid in shape, 30 mm square at the top, 60 mm deep and 36 ml in volume. Seedling trays were placed on racks 1 m above the ground (Fig. 2.2). Gromor[4] seedling mixture was used as a media. Nutrient solution containing 200 mg l^{-1} N, 25 mg l^{-1} P and 150 mg l^{-1} K was applied via microjets from a nutrient injector system. Observations were made for atypical seedling morphologies and chlorophyll mutations as suggested by Sree Ramulu (1970; 1972). Trays were scored daily for radical protrusion as an indication of germination. Final germination counts were made at Day 10. Survival was scored at Week 3 prior to transplanting. Total survival was expressed as the percent (%) of surviving seedlings of the total planted. Relative survival was calculated as the

[3] Speedling Inc., Sun City, Florida, USA

[4] Gromor, P.O. Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa

percent of germinated seeds which survived to seedling stage for transplantation (Medina et al., 2005).

Figure 2.2 Greenhouse germination trials using the Speedling[3] system (Anonymous 1983).

2.2.4 Field trials and breeding

All M_1 seedlings raised in the greenhouse were transplanted into the field at Ukulinga research farm[5] after 3 weeks in 2006 (Fig. 2.3 and 2.4). Seedlings were planted at an inter-row spacing of 60 cm with and intra-row spacing of 20 cm. 2.3.4 (28%) NPK fertilizer (AFGRI[6]) was applied to the field 1 week prior to planting at approximately 250 kg Ha^{-1}. Limestone ammonium nitrate (LAN) (48%) fertilizer (AFGRI[6]) was applied as a side dressing over the growth season at an application rate of approximately 150 Kg Ha^{-1}. Once flowering had commenced, the main heads were covered with brown paper pollination bags (Highfield Packaging[7]) to ensure self-pollination (selfing) and production of M_1 seed (Fig. 2.5). These were removed after 2 weeks, once seed set had begun, and covered with fine-mesh bags (Victoria Packaging[8]) to protect them from bird damage and thus ensure seed retention. Fine mesh bags also served to increase aeration and thus decrease grain loss by fungal infection. M_1 seed was harvested in

[5] Ukulinga research farm, University of KwaZulu-Natal, Private Bag X01, Pietermaritzburg, 3209, KwaZulu-Natal, South Africa

[6] AFGRI Ltd., 33 Sloane St, Bryanston, 2060, Gauteng, South Africa

[7] Highfield Packaging , 3 Chesterfield Rd, Willowton, 3201, KwaZulu-Natal, South Africa

[8] Victoria Packaging, 289 Victoria Rd, Pietermaritzburg, 3201, KwaZulu-Natal, South Africa

individual heads in 2007 and performance field data was used to select heads to plant for 2007/2008 M_2 trial from each treatment. Such selections were critical due the large number of seed produced from the M_0 generation (approximately 6 500 000).

Figure 2.3 Transplanting M_1 seedlings from germination trials into the field at Ukulinga[5] research farm.

Figure 2.4 Field trials at Ukulinga[5] research farm.

Figure 2.5 Pollination bags for selfing (top) and fine-mesh bags[8] to protect from bird damage (bottom).

2.2.5 Field data collection

Routine observations were made on all plants and data was collected for the occurrence of albinism and other atypical morphologies (M_1 and M_2). The date of stigma formation was scored, in addition to height, diseases, number of heads per plant, numbers of tillers, head size, grain size, weight and number of heads per tiller per plant. These data were used for selection of seed to plant in the M_2 2007/2008 trial. M_2 plants were scored for developmental stage (DVS) at 78, 98 and 167 days after planting (DAP) by the decimal code for the growth stages of cereals method (Zadocks et al., 1974; Tottman et al., 1979).

2.2.6 Protein variability

Main heads were selected from ten M_2 plants of each variety from each treatment and control. These were threshed and three 15 g subsets of M_2 seed were taken from each head using the spoon method, by which all grain was spread evenly on a tray and composites of at least ten evenly spaced samples were collected with a small spoon and pooled to form a subsample (ISTA, 1996). These were milled using a Kenwood CG100

coffee grinder (Kenwood[9]) to pass through a 0.5 mm mesh. Samples were analysed for total nitrogen by the Dumas method according to Official Method 990.03 of the AOAC International (2005), using a LECO FP2000 nitrogen analyser **(LECO** Corporation[10]) at the Department of Animal and Poultry Science[11], UKZN. Nitrogen values were multiplied by the constant 6.25 to approximate crude protein values. These values were pooled to calculate the mean for each head. The mean and standard deviation of crude protein for each treatment in the M_2 generation was calculated by pooling the means for each head.

2.2.7 Data analysis and statistical methods

All data was subjected to analysis of variance (ANOVA) using Genstat® Release 11.1 statistical analysis software (Anonymous, 2008) to determine significance between treatments, varieties and treatment-variety interaction effects.

[9] Kenwood, Highway Business Park, Park St (south), Centurion, 0061, South Africa

[10] LECO Corporation, 3000 Lakeview Ave, St. Joseph, Michigan, USA

[11] Department of Animal and Poultry Science, University of KwaZulu-Natal, Private Bag X01, Pietermaritzburg, 3209, KwaZulu-Natal, South Africa

2.3 RESULTS

2.3.1 Effect of heavy-ion treatment on sorghum seed germination and survival

2.3.1.1 Germination

Table 2.3: Germination (%) of treated and control M_1 seedlings 10 days post planting

Variety	Germination (%)			
	Treatment (Gy)			
	0	75	100	150
Kari Mtama	37.78 ± 8.7^{a}	95.56 ± 3.1^{hij}	94.44 ± 1.6^{hij}	92.22 ± 5.7^{hij}
ZSV3	76.66 ± 4.7^{def}	75.55 ± 4.2^{def}	65.55 ± 1.6^{bcde}	64.44 ± 8.3^{bcde}
Serena	56.66 ± 12.5^{bc}	96.66 ± 2.7^{ij}	93.33 ± 2.7^{hij}	97.77 ± 3.1^{j}
Seredo	61.11 ± 4.2^{bcd}	81.11 ± 3.1^{efg}	86.67 ± 8.2^{fgh}	96.66 ± 4.7^{ij}
ICSV3	77.78 ± 1.6^{def}	62.22 ± 24.7^{bcd}	95.56 ± 1.6^{hij}	92.22 ± 1.6^{ghi}
N249/R9019 (77)	62.22 ± 5.7^{bcd}	62.22 ± 1.6^{bcd}	62.22 ± 5.7^{bcd}	56.67 ± 7.2^{bc}
02MN5453	70.00 ± 7.2^{cde}	48.89 ± 3.1^{ab}	64.44 ± 3.1^{bcde}	53.33 ± 2.7^{ab}
Treatment x variety interactions:				
F probability	<0.001			
s.e.d	5.465			
l.s.d	10.648			
cv%	10.8			

The values are the average of triplicate experiments ± standard deviation
Means with the same letter do not differ significantly at P<0.005

ANOVA confirmed significant differences between treatments across varieties, varieties across treatments and most significantly, it highlighted treatment x variety interactions effects (all P<0.001) (Table 2.3). However, no consistent trends were observed between treatments in all varieties. Treatment effects varied significantly between varieties. No significant differences were observed between treatments of ZSV3 and N249/R9019 (77). In the majority of varieties,

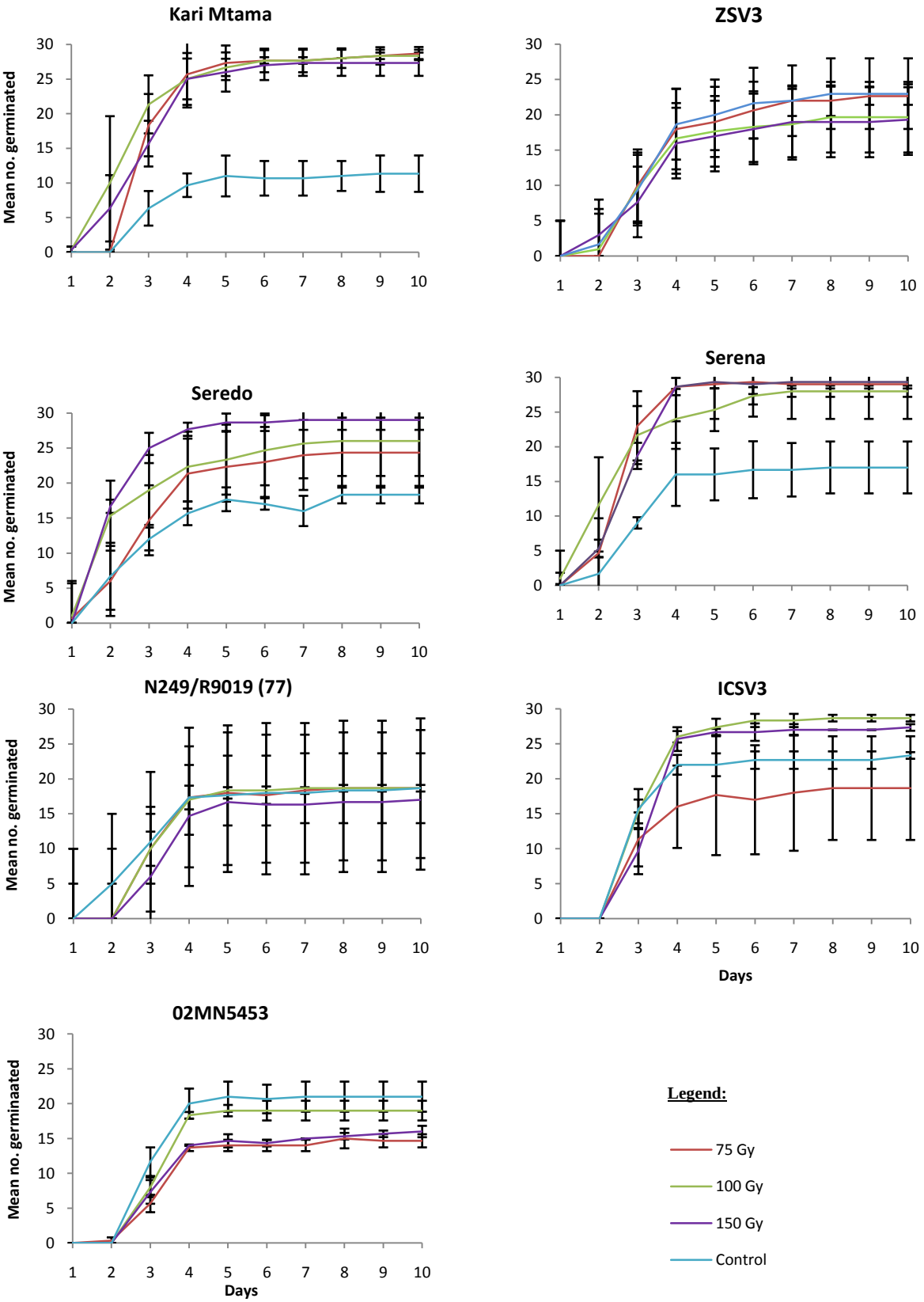

Figure 2.6: Mean germination profile of 30 M_1 sorghum seeds in triplicate over ten days treated with 0, 75, 100 and 150 Gy.

including Kari Mtama, Serena, Seredo and ICSV3, at least two treatments of 100 Gy and 150 Gy showed significantly increased germination rates in comparison to the untreated control. On Kari Mtama, Serena and Seredo, all three treatments showed increased mean germination. Different treatments of Kari Mtama and Serena did not affect germination significantly. Treatment of 150 Gy on Seredo significantly increased mean germination above the germination levels resulting from 75 and 100 Gy treatments by approximately 10 %. Germination of ICSV3 after 100 and 150 Gy treatments did not differ from each other but had a significantly improved germination rate relative to the control and 75 Gy treatments. The largest mean increase in germination of approximately 54.4 % was observed in Kari Mtama when comparing the control and 150 Gy treatments. Conversely on 02MN5453, treatments of 75 and 150 Gy significantly decreased mean germination relative to the control. Curiously, treatment of 100 Gy did not result in a germination level significantly different from the control.

These trends are visible in graphs of the germination profile over ten days (Figure 2.6) where the control rate of germination over the first 4 days was significantly decreased as a result of treatments of Kari Mtama, Serena and Seredo. In the Kari Mtama variety, 100 and 150 Gy treated seed germinated one day earlier than the control and the 75 Gy treated seed. No clearly significant differences were observed as a result of treatments in the germination profile graphs of ZSV3, ICSV3 and N249/R9019 (77) (Figure 2.6). In N249/R9019 (77), the control and 75 Gy treatments showed identical germination profiles, only differing in standard deviation about the mean. Control treatment of 02MN5453 exhibited a significantly higher germination rate over the first three days than seed from the 75 and 150 Gy treatments.

2.3.1.2 Total survival

Table 2.4: Percent total survival of treated and control M_1 seedlings 10 days post planting

Variety	Total Survival (%)			
	Treatment (Gy)			
	0	75	100	150
Kari Mtama	35.56 ± 10.3^{a}	93.33 ± 4.7^{ijk}	92.22 ± 3.1^{hijk}	87.78 ± 6.3^{ghij}
ZSV3	75.55 ± 4.2^{defg}	75.55 ± 4.2^{defg}	57.77 ± 10.3^{abcd}	58.88 ± 8.7^{abcd}
Serena	54.44 ± 12.3^{abc}	96.66 ± 2.7^{jk}	93.33 ± 2.7^{ijk}	96.66 ± 4.7^{k}
Seredo	60 ± 2.7^{abcd}	80.0 ± 2.7^{efgh}	82.22 ± 8.7^{fghi}	95.56 ± 4.2^{jk}
ICSV3	75.56 ± 4.2^{defg}	57.78 ± 27.3^{abcd}	83.33 ± 2.7^{fghi}	63.33 ± 8.2^{abcde}
N249/R9019 (77)	60.00 ± 66.7^{abcd}	62.22 ± 1.6^{abcde}	61.11 ± 4.2^{abcd}	53.33 ± 9.4^{abc}
02MN5453	68.89 ± 6.8^{cdef}	47.78 ± 4.2^{ab}	63.33 ± 4.7^{abcde}	46.67 ± 5.4^{ab}
Treatment x variety interactions:				
F probability	<0.001			
s.e.d	5.876			
l.s.d	11.772			
cv%	12.2			

The values are the average of triplicate experiments ± standard deviation
Means with the same letter do not differ significantly at P<0.005

Percent total survival is shown in Table 2.4.

Total survival data showed almost identical trends to germination levels. ANOVA yielded significant differences between treatments across varieties, varieties across treatments and most significantly highlighted treatment x variety interactions effects (P<0.001). Similarly to germination levels, no consistent trends were observed as a result of treatments of all varieties. Again, no significant differences in total survival were observed between treatments in ZSV3 and N249/R9019 (77). Kari Mtama, Serena, Seredo and ICSV3 again showed significantly increased total survival from at least two treatments of 100 Gy and 150 Gy in comparison to the untreated control. 02MN5453 also showed significantly decreased total survival following treatments of 75 and 150 Gy when contrasted to the untreated control. The only significant difference

between results for germination and total survival was with ICSV3 where the 150 Gy treatment did not differ significantly from the control and 75 Gy treatment for total survival.

2.3.1.3 Relative survival

Table 2.5: Percent relative survival of treated and control M_1 seeds germinated 10 days post planting

Variety	Relative survival (%)			
	Treatment (Gy)			
	0[x]	**75**[x]	**100**[xy]	**150**[y]
Kari Mtama[b]	93.33 ± 9.4[¥]	97.62 ± 1.7[¥]	97.62 ± 1.7[¥]	95.14 ± 1.8[¥]
ZSV3[b]	98.61 ± 2.0[¥]	100 ± 0.0[¥]	87.80 ± 13.9[¥]	91.28 ± 6.2[¥]
Serena[b]	95.92 ± 3.2[¥]	100 ± 0.0[¥]	100 ± 0.0[¥]	98.81 ± 1.7[¥]
Seredo[b]	98.33 ± 2.4[¥]	98.66 ± 1.9[¥]	94.80 ± 3.7[¥]	98.89 ± 1.6[¥]
ICSV3[a]	97.10 ± 4.1[¥]	89.26 ± 9.1[¥]	87.23 ± 3.2[¥¤]	68.69 ± 8.8[¤]
N249/R9019 (77)[b]	96.08 ± 5.5[¥]	100 ± 0.0[¥]	98.41 ± 2.2[¥]	93.61 ± 5.1[¥]
02MN5453[b]	98.48 ± 2.1[¥]	97.62 ± 3.4[¥]	98.15 ± 2.6[¥]	87.48 ± 4.8[¤]
Treatment:				
F probability	0.004			
s.e.d	2.662			
l.s.d	5.333			
cv%	10.6			
Variety:				
F probability	0.002			
s.e.d	3.522			
l.s.d	7.055			
cv%	10.6			

The values are the average of triplicate experiments ± standard deviation
Treatments and variety names with the same letter do not differ significantly at P<0.005
Means with the same symbol within a variety do not differ significantly at P<0.005

Percent relative survival is shown in Table 2.5.

No significant treatments x variety effects were evident following ANOVA of relative survival data. However, overall significant differences were observed between varieties

and between treatments. Across all varieties 150 Gy treatments were significantly more effective than the untreated control and 75 Gy treatments, whilst no differences were shown between 100 Gy treatments and any other treatment or control. Across all treatments variety ICSV3 was shown to be significantly different to all other varieties. The only significant differences as a result of the treatments were evident in ICSV3 and 02MN5453. In ICSV3, treatments of 150 Gy significantly decreased relative survival compared to the untreated control and 75 Gy treated seeds whilst treatment of 100 Gy did not cause any significant difference on seed performance relative to any other control or treatment. Similarly, in 02MN5453, treatment of 150 Gy significantly decreased relative survival in comparison to all other treatments and control.

2.3.1.4 General observations

Visible differences were noted between seedlings of the untreated control and treated seedlings that were not reflected in the presented data on germination and survival. For example, in Figure 2.7, the difference in size between seedlings of the untreated control and 150 Gy treated seed of ICSV3 was clearly evident, although the reported germination was significantly higher in the 150 Gy treated seed (Table 2.3), the total survival was not significantly different (Table 2.4) and the relative survival was significantly lower than the untreated control (Table 2.5). There were less clearly visible differences between treated in seedlings than between treated and untreated control seedlings (Fig. 2.8). In some varieties no visible differences were present at all.

Chlorophyll abnormalities (Fig. 2.9) were observed in both the M_1 and M_2 generations and were scored in Table 5.6. Interestingly, all chlorophyll mutations were observed as a result of 75 Gy treatment, and the variety N249/R9019 (77) exhibited the most (33). Chlorophyll deficient mutants observed at the M_1 stage in the greenhouse did not survive to the M_2 generation.

Figure 2.7: Visible differences between 150 Gy treated and untreated ICSV3 at two weeks.

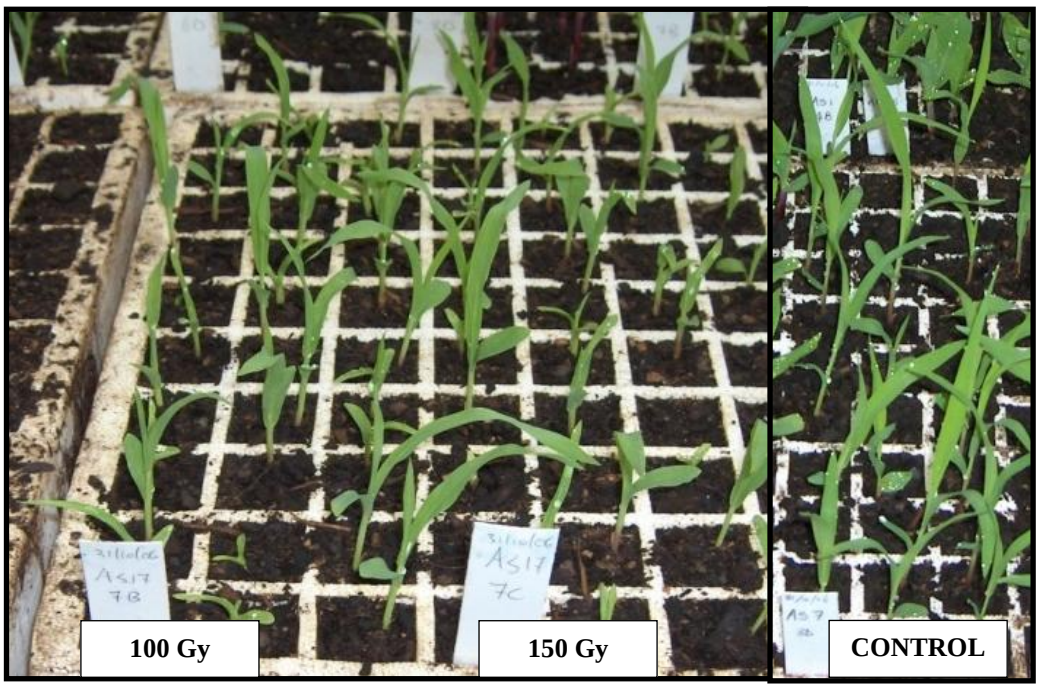

Figure 2.8: Untreated control, 100 Gy and 150 Gy treatments of Seredo.

Figure 2.9: A chlorophyll deficient Serena M_1 seedling as a result of 75 Gy treatment.

Table 2.6: Varieties and treatments in which chlorophyll mutants were observed over 2006/2007 (M_1) and 2007/2008 (M_2) trials and the number of mutants observed

Variety	Control	Treatment (Gy)			Generation	Trial
	0	75	100	150		
Kari Mtama	-	-	-	-		
ZSV3	-	-	-	-		
Serena	-	1	-	-	M_1	greenhouse
Seredo	-	-	-	-		
ICSV3	-	-	-	-		
N249/R9019 (77)	-	33	-	-	M_2	field
02MN5453	-	1	-	-	M_1	greenhouse

2.3.2 Effect of heavy-ion treatment on field performance

2.3.2.1 Field data and observations

Despite visible differences between seedlings as a result of treatments at the seedling stage, there were few observable differences between plants of treated varieties in the field (M_1 and M_2). The development of plants in the field differed slightly in some varieties at different stages but no consistent trends were observed (Table 2.7). In some of the treated varieties flowering was delayed whilst in others it was hastened when compared to the untreated control (Table 2.8). Stigma formation was inconsistent as a result of treatments of 100 and 150 Gy in Kari Mtama, when compared to untreated plants (Table 2.8). 100 Gy treated plants formed stigmas 6 days before controls whilst 150 Gy treated plants formed stigmas three days after untreated controls.

Table 2.7: Developmental stage (DVS) comparison of M_2 treated and control untreated sorghum varieties during 2007/2008 field trials

Variety	DVS	DAP*	Control	Treatment		
			0	75	100	150
Kari Mtama	DVS1	78	29-37	29-37	29-41	29-41
	DVS2	98	65-80	65-80	65-80	65-80
	DVS3	167	99	99	99	97-99
ZSV3	DVS1	78	37-41	37-41	15-37	29-33
	DVS2	98	55-70	55-70	55-65	55-65
	DVS3	167	94-99	94-99	94-99	94-99
Serena	DVS1	78	29-33	29-33	29-33	29-33
	DVS2	98	30-45	30-45	35-50	35-50
	DVS3	167	87-99	91-99	91-99	92-99
Seredo	DVS1	78	29-33	29-33	29-33	29-33
	DVS2	98	35-50	35-50	35-50	35-45
	DVS3	167	93-99	93-99	93-99	87-99
ICSV3	DVS1	78	15-33	15-33	15-29	15-33
	DVS2	98	55-70	55-70	55-70	65-75
	DVS3	167	94-99	94-99	94-99	94-99
N249/R9019 (77)	DVS1	78	15-29	15-33	15-29	15-33
	DVS2	98	60-80	60-80	65-80	65-80
	DVS3	167	99	99	99	99
02MN5453	DVS1	78	15-29	15-33	15-33	15-29
	DVS2	98	60-70	60-70	55-70	50-65
	DVS3	167	94-99	94-99	93-99	94-99

*DAP = days after planting

Table 2.8: Stigma formation of treated M_2 sorghum varieties during 2007/2008 field trials expressed in days before/after untreated controls

	Treatment (Gy)		
Variety	**75**	**100**	**150**
Kari Mtama	0	-6	+3
ZSV3	+3	+3	+3
Serena	0	0	0
Seredo	0	0	0
ICSV3	+4	+3	+3
N249/R9019 (77)	0	0	0
02MN5453	0	0	0

"+" indicates stigma formation after control
"-" indicates stigma formation before control

2.3.2.2 Crude protein variability

Due to damage by vervet monkeys during field trials, M_3 grain was only collected from four of seven varieties treated from the M_2 generation for crude protein analysis. Crude protein means of ten heads from each treatment and the standard deviation is shown in Figure 2.10. No significant mean increase or decrease in grain crude protein was evident as a result of treatments.

Regressions of crude protein standard deviation in M_2 plant grain (M_3 seed) versus treatment dose showed positive correlations in all four varieties, although these were strong only in Kari Mtama, Serena and Seredo (Figure 2.11). In all varieties the standard deviation of grain crude protein levels from plants treated with the highest dose of 150 Gy were approximately double that of grain from untreated control plants.

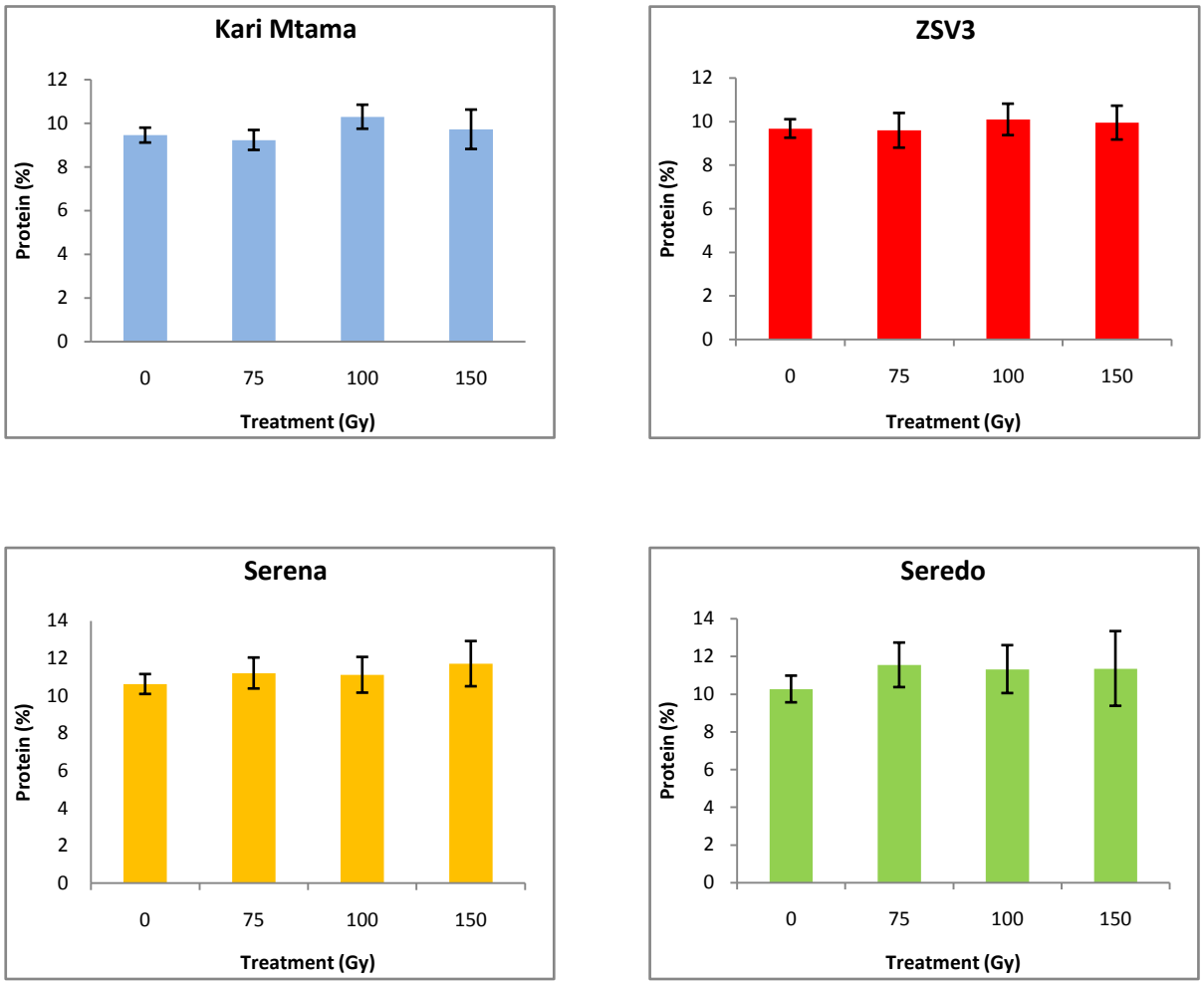

Figure 2.10: Mean crude protein levels and standard deviation of 10 treated and untreated control sorghum heads from 4 varieties following 2 generations of selfing (M_2 plants yielding M_3 seed).

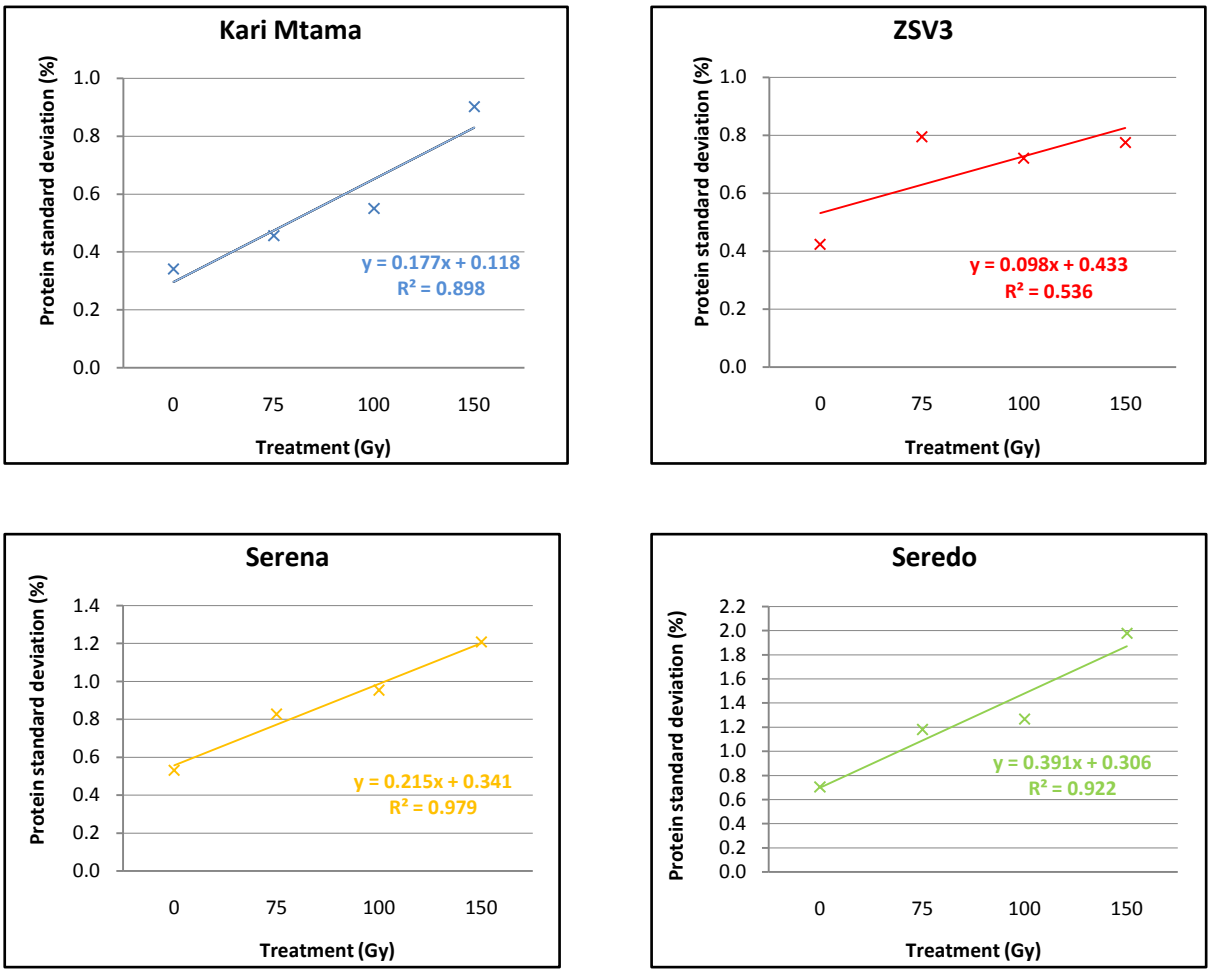

Figure 2.11: Regression analysis of crude protein standard deviation of 10 sorghum heads versus mutagenic dose rate (Gy) following 2 generations of selfing (M_2 plants yielding M_3 seed).

2.4 DISCUSSION

It is widely accepted in the literature that mutagenic treatment of plant populations should release genetic variability and that as the dose rate increases, there should be a quantifiable correlation at a molecular, cellular and phenotypic level (Goud et al., 1970; Singh, 2003; Dong et al., 2008; Zhang et al., 2008). In the use of low-LET mutagenesis methods of the past, the quantification of treatment success was measured by visual deleterious effects of treatment such as decreases in germination, survival, plant injury, visible chlorophyll mutations, etc. (Medina et al., 2005). In this study, the results of such measurements are reported and clearly showed that cyclotron treatments had a definitive effect which varied between varieties. However, no consistent trends were observed between treatment doses and plant damage.

An optimal mutagenic dose rate is typically selected as that which results in the most genetic change without debilitating the plant from producing viable seed for further breeding (Singh, 2003). Dose rate selection criteria have been suggested to be near or at the LD_{50} treatment, but this has assumed that treatments have large negative effects on plant viability (Medina et al., 2005). Possibly the largest motivation behind the use of ion-beams to induce mutations is a reduction in non-viable mutations and plant damage (Yu, 2006). The lack of change in germination and survival observed in two sorghum varieties in this study, even at the highest dose rate, has been observed in rice treatments (Song et al., 2007). Song et al.(2007) observed no significant change in germination or survival in rice variety Wanjian 2090 yet, 98.66 % of treated plants showed morphological changes in the M_2 generation.

Only two of the seven varieties treated displayed typical responses in that 02MN5453 exhibited lower germination rates at 75 and 150 Gy and the surviving fraction of ICSV3 and 02MN5453 were significantly damaged by treatments of 150 Gy. These data suggest that higher dose rates need to be applied and that in future, dose trials should start in the region of 150 Gy. In a recent study published after the commencement of this work, Dong et al. (2008) ascertained that the median lethal dose (LD50) to treat sweet sorghum seeds was 120 Gy using 100 MeV u^{-1} carbon ions. This was the lowest treatment in the range from 120 to 240 Gy at 40 Gy intervals and a dramatic decrease in

germination and survival was observed as a result of 120 Gy treatment when compared to untreated controls (approximately 50 %). The data in this study, however, is inadequate to determine LD_{50} as the levels of plant damage were so low.

The apparent increase in germination of four varieties following treatment was contrary to expectation and prior research, and also suggests a need for higher dose rates. As there was no difference in seed between the untreated control and treated seed the source for experimental error was relatively low. Nevertheless this could be explained by some uncontrolled environmental factor during storage of control seeds while the others were sent to $RARF^2$. If accurate, however, a linear response across the treated samples should still have been observed. Chemical mutagenic treatment of sorghum has shown an increase in emergence of the first leaf and germination speed with an increase in treatment, although this was strongly negatively correlated to seed fertility, and less but still negatively correlated to total germination, survival, seedling height, and coleoptile and primary root length (Sree Ramulu, 1972).

It is possible that unconsidered effects of treatment could account for these unexpected trends in total germination. One such possibility could be a sterilizing effect of ion beam treatment potentially eliminating endogenous or parasitic organisms from the seed and reducing competition for nutrient resources as the seed was germinating. Sorghum is prone to colonization from a number of fungal and mould species in the field and during improper storage (Waniska, 2000). These include: *Fusarium thapsinum* (Klittich et al., 1997); *Claviceps africana* (Isakeit et al., 1998); *Curvularia lunata*; other *Fusarium* spp.; *Alternaria* spp.; *Phoma sorghina*; and *Dreschlera* spp. (Castor and Frederiksen, 1980) amongst others. Ion beam research using $^{12}C^{6+}$ at $RARF^2$ on the model reporter organism *Mesorhizobium loti* reported that high dose rate high-LET irradiations causes cell inactivation “attributable to the formation of lethal and complex DNA damage” (Ichida et al., 2008). Dose rates used in this experimentation were from 2.5 – 25 Gy, which is much lower than the treatments used in this study. Other work on the effect of heavy ions on bacteria shows that bacterial mutations increase up to a maximum, above which cellular non-viability is induced (Horneck et al., 1994). Irradiation is widely used in sterilization of health and food products, although the required dose rates to meet ISO

standards are orders of magnitude higher: 15 or 25 kGy for health products (International Organization for Standardization, 2006). Further experimentation by culturing microbial isolates from sorghum seeds treated with different radiation levels would be required in order to explore these possibilities further.

Varietal differences in response to radiation have been well documented from some of the earliest work on sorghum mutagenesis (Sree Ramulu, 1970), where treatment responses from the physical mutagens (X-rays and γ-rays), as well as those from four different chemical mutagens, varied significantly between three different varieties of sorghum. A dose dependant relationship of a linear nature with a saturation point was observed in only one of the three varieties tested (Sree Ramulu, 1970). Sorghum seeds displayed a wide degree of variability, both between varieties and within caryopses of the same varieties (Waniska, 2000). A combination of inherent and environmental factors could account for varietal differences in response to treatment. Differences in pericarp thickness, and endosperm and germ size, vary strongly between varieties (Waniska, 2000). In particular, variation in germ size, and thus nuclear volume, would account for some variation in radiosensitivity (Singh, 2003). Seed moisture is the most important environmental factor in determining radiation effects and small changes between varieties can result in the production of varying levels of reactive oxygen species (Singh, 2003). It is important to mention that these factors play a secondary role to dose level. Varietal differences should have been less observable had the dose been high enough. The results of this study suggest that treatment dose level needs to be optimised for specific sorghum varieties.

The definitive effects of radiation in reducing seedling growth, inducing visible abnormalities such as chlorophyll deficiencies in the M_1 and M_2 generations, and increasing crude protein variation linearly to treatment dose, suggest a need for alternative methods of quantification of induced genetic change. Zhang et al. (2008) utilized assays for reactive oxygen species and free radical quenching enzymes to indicate the optimum dose effect in wheat seedlings. Dong et al. (2008) used molecular techniques such as random amplification of polymorphic DNA (RAPD) analysis to investigate induced genetic change. Due to the less deleterious nature of ion beam

mutagenesis over previously employed approaches, biochemical and molecular tracking tools may be more informative than those employed in this study.

The morphological abnormalities and reduced growth observed in treated seedlings could be explained by oxidative stress (Singh, 2003). The increased presence of chlorophyll mutants in the M_2 generation is concordant with previous mutagenesis in sorghum and genetic theory which infer that most mutations occur as recessive genes that require amplification to the homologous state through selfing (Sree Ramulu, 1972; Singh, 2003). Induction of these chlorophyll mutations at the lowest dose of 75 Gy strongly suggests that the applied treatment indeed had effects despite the fact that this was not clearly reflected in the data presented in this study. Similarly, slight differences in field performances indicated induced polygenic mutations in the mechanisms which control flowering, albeit with a low frequency.

It may be important to consider the treatment facility as a source of error because mutagenesis was performed by RARF[2] and not done in-house. Previous treatment of 20 sorghum varieties at RARF[2] was undertaken in 2005 by the ACCI[1] at their recommended rate of 75 Gy (Laing, pers. comm.[12]). The visible effects of this treatment, however, were far greater than any treatments used in this study that were supposed to have been higher. This raises the possibility of dose level error, either in this body of work, or in that previously performed.

2.5 CONCLUSION

The increasing popularity of heavy ion-beams as a mutagenic method due to high-LET interactions was supported by the discrepancy between a lack in clearly visible plant damage from the highest dose rates used in this study, and the evident genetic change in protein variation and morphology. Indeed one of the greatest limitations of mutagenic methodologies used in the past was excessive plant damage and "unusable" genetic change. While the results of this study call for higher ion-beam dose rates or possibly the use of different atoms in generating the ion beams (such as neon or argon), it would

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be of benefit to use more informative molecular tracking methods more sensitive to high-LET generated mutations.

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CHAPTER THREE

NIR CALIBRATION DEVELOPMENT AND CHEMOMETRICS ON SORGHUM GRAIN

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ABSTRACT

In breeding of grain sorghum, the development of cheap, rapid, non-destructive assays for a range of parameters is needed to avoid costly, slow "wet chemistry" methods. These parameters include moisture, crude protein, phytic acid, fat, apparent metabolisable energy (AME) and the amino acid quantities. In addition, visible near-infrared instruments have been shown to be able to perform the function of standard colorimeters and aid in grain classification using CIELab L*a*b* coordinates. In theory, Near Infrared Analysis (NIRA) provides a technology to measure a broad range of agronomic parameters accurately, cheaply and speedily in a single scan. Calibration development on milled flour and whole grain sorghum was undertaken by partial least squares and test-set validation using a FOSS NIRSystems model 6500. These calibrations were independently tested to render prediction error statistics (SEP). Additional preliminary calibrations were developed on 20-40 milled grain samples for AME, fat and amino acid quantities using PLS and cross-validation. In addition to quantitative calibrations, partial least squares discriminant analysis (PLS-DA) models for colour classification were developed. Moisture levels were estimated using whole grain (n=396, R^2= 0.897, RMSEP=0.351, SEP=0.6 %) but these were inferior to estimates from milled samples (n= 420, R^2= 0.971, RMSEP=0.267, SEP=0.302 %). Similarly, % absolute crude protein was accurately and reliably predicted in milled grain samples (n=364, R^2=0.997, RMSEP= 0.031, SEP=0.177 %), but less so in whole

grain (n=382; R^2=0.955, RMSEP= 0.661, SEP=0.766 %). Phytic acid calibration was shown to be more predictable in whole grain (n=120, R^2=0.780, RMSEP= 0.383 mg g^{-1}) than milled flour (n=160, R^2= 0.94, RMSEP=0.425 mg g^{-1}) depite a significantly lower coefficicient of determination. Colour coordinates were successfully calibrated on 200 whole grain samples using the visible region of the collected spectrum. L* and gloss values were the most predictable (R^2=0.959 and 0.951, RMSEP= 3.085 and 2.547, SEP= 1.84 and 1.612 respectively) whilst a* and b* still yielded good regressions (R^2=0.92 and 0.887, RMSEP= 1.486 and 2.358, SEP= 1.28 and 1.578 respectively). The biological significance of PLS calibrations was investigated through comparing spectral B-coefficients to analyte absorbencies identified in previous calibrations. Preliminary amino acid calibrations yielded R^2 values from 0.915 to 0.996, and ratios of performance deviation between 2.32 and 8.43, for 16 amino acids. Principal component analysis (PCA) clearly distinguished milled from whole grain spectra, as well as white grain from other grain colours. PLS-DA models were able to predict white sorghum grain, but unable to predict any of the other colour classes reliably. In addition, a linear relationship between absolute crude protein calibration accuracy and the degree of milling (ie. particle size) was shown. The potential of NIR in grain sorghum analysis was clearly highlighted and robust and accurate calibrations were developed and rigorously tested. Further research needs to be done to develop reliable discriminant models for grain colour in sorghum.

3.1 INTRODUCTION

The increasing demand for product quality and production rationalization in the food and agriculture industries has led to the development of more rapid and environmentally friendly analytical methods (Kawano et al., 2002). Near-infrared spectroscopy (NIRS) has emerged as an extremely powerful tool for non-destructive, high-throughput analysis in quality control and process monitoring (Kawano et al., 2002). Classical methods, although well established, are limiting due to their expense, time and expertise requirements, destructive nature and inability to analyse more than one parameter at a time (Givens et al., 1997). Near-infrared analysis (NIRA) is highly dependent upon sample-specific calibration and an accurate reference method (Ciurczak, 2001) but can dramatically increase access to analysis following completion of calibration processes

(Osborne et al., 1993, Ciurzack et al., 2001). Due to the time, expense and expertise required to calibrate near-infrared (NIR) instrumentation, these calibrations are valuable commercial entities and not widely accessible to research communities (Borello[13], pers. comm.; Holman[14], pers. comm.; Oosthuizen[15], pers. comm.).

Calibrations are not easy transferrable between NIRS instruments due to instrumental responses that vary greatly between different manufacturers and models, and even between identical instruments of the same make and model, although to a lesser degree (Givens et al., 1997; Feudale et al., 2002; Bouveresse and Campbell, 2007). Advances in multivariate chemometrics standardization methods such as The PDS method, Shenk-Westerhaus method and neural networks, as well as newly developed software packages such as TransStar™ (Unity Scientific Asia Pacific[16]) have addressed transfer issues but are patent-protected and therefore relatively inaccessible (Shenk and Westerhaus, 1989; Kowalski et al., 1995; Despagne et al., 1998). There is thus a need to investigate the potential of NIRA in crops that have been relatively unexplored, such as sorghum, and to develop "in house" calibrations.

The development of NIRS calibrations would benefit the breeding, production, commercial and industrial use of grain sorghum (Pomeranz, 1986; Rodriguez et al., 2005). Efficient and informative data generation is critical to both conventional and biotechnology-based plant breeding programs (Singh, 2003). Classical analytical methods are a widely acknowledged bottleneck to plant breeders (Mayo, 1987; Bos and Caligari, 1995; Givens et al., 1997). The wide diversity of commercial and industrial uses of sorghum, especially trends to cultivate grain sorghum for liquid biofuels production, suggest an ongoing requirement for low input, high throughput analytical methods (National Research Coucil, 1996; Vermerris et al., 2007). In particular, the biofuels industry will need to screen existing varieties for their suitability towards this

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end and would benefit from advanced analytical approaches (Vermerris et al., 2007; Carpita and McCann, 2008).

The potential for NIR calibrations in sorghum is supported by success in other grain crops such as maize, wheat and barley and some research in sorghum grain (Correa et al., 2002; Fontaine et al., 2002; Munck et al., 2004; Cocchi et al, 2006). Most applications in sorghum have been for analysis of stover for animal feed and biofuels production (Budiongo et al., 1996; Bruno-Soares et al., 1998; Vermerris et al., 2007). Rodriguez et al. (2005) reported that no models had been developed for sorghum grain prior to their calibration attempts for density, moisture, crude protein, neutral detergent fibre (NDF) and tannins. However, Hicks et al. (2002) had already reported accurate predictions of crude protein in sorghum grain. Similarly, Fontaine et al. (2002) reported that highly informative and accurate predictions could be obtained for amino acids and absolute crude protein in milled sorghum grain. Good calibrations were developed for crude protein in sorghum on milled flour and whole grain, whilst weaker but acceptable calibrations were shown for lipid content, endosperm texture and hardness (de Alencar Figueiredo et al., 2006). More comprehensive work on sorghum grain for monitoring breeding programs has been undertaken by Texas A&M University. However, this work has not been extensively published (McDonough and Rooney, 2003; Rooney[17], pers. comm.).

NIR calibrations have not been reported on sorghum for several parameters that have been reported for other crops. Phytic acid is an antinutritional compound found at varying levels in most cereals that binds mineral cations, thus reducing their bioavailability (Zhou and Erdman, 1995). The classical method of analysing for this compound is destructive to the seed and involves the use of 2,2'-bipyridine, a toxic chemical which is hazardous to work with and requires specialized disposal measures (Young, 1936; Haug et al., 1983). NIR applications have been successfully developed for phytate in oilseed rape (Möllers et al., 1999), rice grain (Frenzel, 2003) and in plant feedstuffs including four sorghum samples (de Boever et al., 1994). Colour characteristics of sorghum grain and flour are typically recorded to assess quality, insect

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and mould infestation and track changes in breeding programs (de Milliano[18], pers. comm..). Spectrometers which include the visible range of the spectrum have been calibrated to predict CIE L*a*b* colour coordinate values (Hunter 1975; ASTM 1996). Calibrations that predict grain and flour colour from an instrument capable of NIR spectroscopy would eliminate the requirement for an additional colorimetry step in data collection. This has been shown to be of value in wheat, barley and red lentils (Black and Panozzo, 2004).

The broad aims of this study were to further investigate the potential of NIR analysis of sorghum grain and flour, and its reliability and suitability for use in sorghum breeding. This was to be accomplished through developing in-house calibrations for several parameters on a significant scale, and testing their functionality through validating their prediction error in routine analysis. In addition preliminary calibrations were to be developed for parameters such as phytate, amino acids and colour coordinates where previous research was lacking or funds were prohibiting large-scale calibration processes (such as expensive amino acid analysis). The degree of sample preparation required was also to be investigated to establish whether NIR analysis could function as a non-destructive technique. Lastly, discriminant classifications were to be explored to determine whether NIRS could assist in classifying sorghum grain colour from spectral information, thus reducing the time required for field data collection.

3.2 MATERIALS AND METHODS

3.2.1 Seed Collection

Approximately 420 Sorghum grains from various origins were initially collected from UKZN[19] ACCI[20] nurseries and seed stores (Table 3.1). Approximately 260 additional samples were collected during routine analysis and screening over the following 2 years for use in independent validation. Diversity was introduced in the sample set through collecting grain cultivated under a variety of conditions over a period of 6 years including: Year-round nurseries; pot, tunnel and field trials; irrigation and nutrition trials; and inbred and out-crossed lines. Additional variation was introduced to the

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calibration set by including samples from different individual plants of 19 varieties exposed to heavy-ion beam mutagenesis, and self-pollinated for 2 generations.

3.2.2 Sampling and sample preparation

Grain samples of approximately 50 g were collected from either seed bags stored at 4° C in a seed storage facility, or from freshly harvested heads displayed upon threshing tables. Where grain was collected from seed bags, the spoon method (ISTA, 1996) was used to generate the sample of at least 10 composite sub-samples, after decanting the bag into a flat tray. Where grain was collected from individual heads, and the heads were larger than the required sample size, composites of 20 random, hand-pinched sub-samples were made. Samples showing visible insect damage were either excluded or labelled as such. All samples were shaken over a 2 mm mesh for 1 min to remove excess dust and chaff. Any remaining awns were removed by hand. Significantly weathered samples were discarded.

Table 3.1: Diversity of ACCI sorghum seed used in NIR calibration

Origin	No.
South African landraces	32
Elite Kenyan materials	28
SADCC/ICRISAT Zimbabwe	21
Purdue B-lines	39
Kansas State lines	10
Texas A&M Lines	21
Mexican R-lines	5
Ethiopia	3
USA Sweetstems	11
Mozambique	5
Uganda	3
Botswana	4
India	7
Malawi	4

3.2.3 Milling

All samples were milled using a Kenwood CG100 coffee grinder (Kenwood[21]). Crude milled fractions were simply milled for 30 seconds to pass through a 2 mm mesh. Fine milled flour fractions were milled to pass through a 0.5 mm mesh.

3.2.4 Near-infrared spectroscopy

3.2.4.1 Instrumentation

A NIRSystems Composite Monochromator 6500 with static sample window and reflectance detector with an autogain function was used (FOSS NIRSystems Inc.[22]).

3.2.4.2 Sample measurement

The reflectance spectra of samples were recorded over a spectral range of 400 – 2498 nm at 2 nm increments. All spectra were recorded as log (1/*R*) where *R* was the relative reflectance. All samples were equilibrated to 24° C for 48 hrs in the laboratory prior to scanning. Each sample was scanned 32 times and averaged by the instrument. Two repeated scans were performed on each sample following 90° rotation and these were also averaged. A ceramic tile reference was scanned before and between samples within the static module attached to the machine. All milled and grain samples were packed in a static cup cell with quartz glass plate and placed over the scanning window.

3.2.4.3 Calibration and chemometric software

Instrument operation and initial spectral visualization were performed by Vision software (Version 2.22, FOSS NIRSystems Inc.[10]). Preliminary principal components analysis (PCA) and partial least squares (PLS) models were completed using the same Vision software. Spectra were exported from Vision using NSAS or ASCII formats. Thorough PCA analysis, data pre-processing, PLS1, PLS2 and/or PLS-DA were performed using The Unscrambler^{R} software (Version 9.2, CAMO PROCESS AS[23]) at the Institute for Wine Biotechnology[24]. Further data pre-processing, modified PLS and

[21] Kenwood, Highway Business Park, Park St (south), Centurion, 0061, South Africa
[22] FOSS NIRSystems Inc., 7703 Montpelier Rd, Laurel, MD, USA
[23] CAMO PROCESS AS, Nedre Vollgate 8, N-0158, Oslo, Norway
[24] Institute for Wine Biotechnology, University of Stellenbosch, Private Bag X1, Matieland, 7602, SA

cross-validation was performed by WinISI III (Version 1.50e, Infrasoft International[25]). All calibration procedures were performed in compliance to AACC method 39-00 (AACC, 2009). Calibration statistics included the following parameters: number of samples (N); coefficient of multiple determination (R^2 for both calibration and validation); regression slope; 1-variance ratio (1-VR); i.e., R^2 in cross-validation; standard error of cross-validation; root mean square error of calibration (RMSEC); root mean square error of prediction (RMSEP); standard error of independent prediction (SEP); ratio of performance deviation (RPD). RPD was calculated in accordance with the AACC method 39-00.01 (2009) by dividing the standard deviation of the sample set by the error of cross-validation. The standard error of the reference method (SEL) was also calculated and reported. It is important to note that neither the RMSEP or SEP reported here were corrected for bias. RMSEP reports the error on the validation set, whilst SEP reports the error on the independant test set.

Initial calibration was performed on the first 420 samples collected. Independent validation was performed over the following 2 years on the totally independent set of 260 samples collected during that period.

3.2.5 Reference methods

3.2.5.1 Moisture

Moisture analyses were performed immediately after NIRS to ensure minimum errors between sample NIRA and its reference chemistry. Moisture was determined by drying samples in a ventilated oven. Whole grain samples were dried for 72 hrs at 103° C (ISTA, 1996). Milled samples were dried for 4 hrs at 90° C (ISTA, 1996). All samples were weighed on a Precisa Instruments Fine Balance Model 125A (Switzerland). Moisture values were determined in duplicate due to the relatively small sample size and the mean of the two values was calculated and used.

All other wet chemistry procedures were performed on a dry matter basis and the values back calculated to an "as is" basis upon which calibration was performed.

[25] Infrasoft International, Silver Springs, MD, USA

3.2.5.2 Crude protein quantity

The nitrogen content of the samples was determined on fine milled samples by the Dumas method according to Official Method 990.03 of the AOAC International (2005) using a LECO FP2000 nitrogen analyser **(LECO** Corporation[26]). Nitrogen values were multiplied by the constant 6.25 to give % absolute crude protein. Analyses were performed in duplicate due to budget limitations and the mean of the two values calculated. Several samples were analysed 10 times and the mean and standard deviation calculated to establish the precision of the instrument and method used.

3.2.5.3 Phytic acid

Phytate phosphorous was determined on fine milled samples according to a spectrophotometric method described by Young (1936) and modified by Haug et al (1983). Separation following extraction was performed using a Beckman-Coulter^{R} Avanti^{R} Centrifuge Model J-26 XPI (USA). Further modifications were made to increase throughput and reduce error: The reaction step described by Haug et al. (1983) was performed in triplicate in 1.5 ml Eppendorf tubes (Merck Chemicals (Pty) Ltd.[27]) and the visualization step was performed in triplicate in 300 µl wells. Heated reactions at 100° C for 30 min were performed using an Accublock^{TM} D1200 (Labnet International Inc.[28]). Spectroscopy was performed at 519 nm on a 96 well Versamax Tunable microplate reader (Molecular Devices[29]).

3.2.5.4 Colorimetry

CIE L*a*b* values were obtained using a BYK-Gardner portable colorimeter (BYK-Gardner GmbH, Geretsreid, Germany). This instrument used a pulsed Xenon lamp with geometry of 45°/0° ie. The light path angle from the lamp is 45° and circumferential, whilst the viewing angle is 0° with the specular component included (Black and Panozzo 2004). They are filtered to match the CIE Standard Observer Response (Commission Internationale de l'Eclairage, ASTM 1996). The measuring area is 11mm

[26] LECO Corporation, 3000 Lakeview Ave, St. Joseph, Michigan, USA
[27] Merck Chemicals (Pty) Ltd., 259 Davidson Road, Halfway House 1685, Wadeville, Germiston, 1428, SA
[28] Labnet International Inc., PO BOX 841, Woodbridge, NJ, USA
[29] Molecular Devices, 1311 Orleans Dr, Sunnyvale, CA, USA

which is more suited to fine particles thus whole grain measurements required numerous repetitions. The device was referenced on three supplied ceramic tiles (black, white and green) between samples. Milled samples were measured in replicates of three and whole grain samples were measured in replicates of nine, repacking and levelling the sample between measurements. CIE L*a*b* values were averaged and standard deviation calculated. These measurements were performed on the same identical samples scanned with the NIR instrumentation. The software supplied with the device (Easy-Link v.3.20, BYK-Gardner GmbH, Geretsreid, Germany) was used to record data and export into Microsoft Excel format for further chemometric analysis.

3.2.5.5 Amino acid quantiy

Amino acid analysis was performed on fine milled samples according to the Official Method 994.12 of the AOAC International (2000) using a Dionex Corporation ISO System and detector (Dionex Corporation[30]) and a Pickering Post-Column derivitization instrument (Pickering Laboratories Inc.[31]).

3.2.5.6 Fat quantity

Fat was analysed according to AOAC method 920.39 (1990) at the Department of Animal and Poultry Science[32], UKZN.

3.2.5.7 Apparent metabolisable energy

Gross energy was calculated by calorimetry using a DDS isothermal CP500 bomb calorimeter (Digital Data Systems (Pty) Ltd.[33]) according to the method described by AOAC (2000).

[30] Dionex Corporation, 1228 Titan Way, Sunnyvale, CA, USA
[31] Pickering Laboratories Inc., 1280 Space Park Way, Mountain View, CA, USA
[32] Department of Animal and Poultry Science, University of KwaZulu-Natal, Private bag X01, Pietermaritburg, 3209, KZN, SA
[33] Digital Data Systems (Pty) Ltd., 22 Arbeid Ave, Strijdompark, Randburg, South Africa

3.3 RESULTS

3.3.1 Spectral differences between whole grain and milled flour

In both the visible and NIR regions of raw and pre-processed spectra clearly visible differences can be seen that differentiate whole grain from milled flour samples (Fig. 3.1). Larger baseline offsets, scatter effects and general noise are clearly visible in whole grain spectra, despite having similar profile curves to those of milled flour (Fig. 3.1). Following pre-processing, milled flour spectra showed more distinct peaks and valleys with clearer separation than those of whole grain samples.

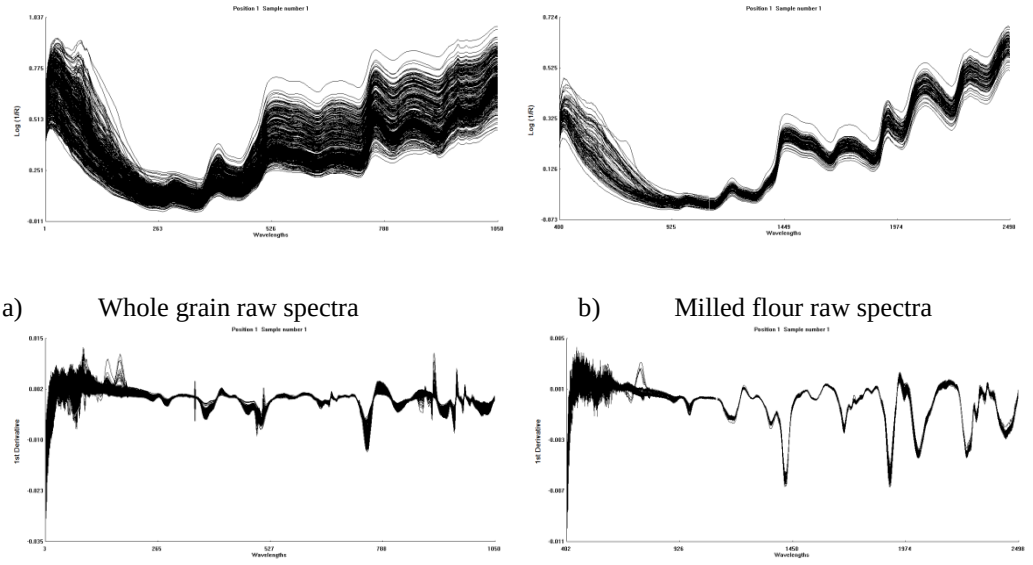
Principal component analysis (PCA) score plots and biplots of whole grain and milled flour NIR spectra (1000-2500 nm) are shown in Figure 3.2. Score plots of the 1st and 2nd principal components (PC) show clear distinctions between whole grain and milled flour samples. The 1st PC describes 90 % of the spectral variation in the dataset. However, whole grain and milled flour samples cannot be distinguished along this PC alone because there is some overlapping of whole grain samples on the negative axis of the 1st PC. The second PC, describing a further 9 % of the spectral variation, was thus required to clearly separate the two sample groups. It is important to note that milled flour samples showed tighter clustering than whole grain samples on the both PC's on the score plot, thus showing less variation between samples. Plots of explained and residual sample calibration variance of the second PC further highlight the differences in variation between samples of milled flour and whole grain samples. Most of the variance in PC 2 in milled flour samples was almost completely explained (near 100 %) whilst whole grain samples showed a much lower level of explanation which ranged from as low as 24 % to near 100 %. Similar trends were observed in plots showing variance between sample residuals and the regression line fitted by the PCA on the 2nd PC. Although milled flour samples showed levels of residual sample calibration variance, these were all below 0.01 whilst those of whole grain samples ranged up to 0.034 and were clearly distinguishable on comparative plots (Fig. 3.2). The second PC thus appears to be modelling variation introduced by whole grain samples.

3.3.2 Effect of pre-processing on spectra and calibrations

Raw spectra of whole grain samples were particularly noisy, showing large baseline offsets, scatter effects and non-linear relationships despite similar profiles when

compared to milled grain spectra (Fig. 3.2). Pre-processing of whole grain spectra with a standard normal variate (SNV) reduced some of the multiplicative interferences of scatter and particle size (Fig. 3.3b) when compared to raw spectra (Fig. 3.3a). Detrending reduced apparent non-linear relationships (Fig. 3.3c). Combinations of SNV and detrend produced significantly less noisy spectra with reduced baseline offset and good linearity (Fig. 3.3d). Further pre-processing was necessary however to reduce the remaining noise further. Generation of the first derivatives of spectra using the Savitsky-Golay algorithm (Savitsky and Golay, 1964), after SNV and detrend, successfully maintained peak integrity and facilitated good spectral separation at peaks and valleys (Fig. 3.3f). In addition, two types of multiplicative scatter correction (MSC) pre-processing methods were tried: weighted and inverse (Fig. 3.3g and 2.3h respectively). The former compared the most favourably to SNV and detrend but did not result in as clear separation of spectra at absorption peaks (Fig. 3g). In addition a 4 nm smoothing was usually applied to further optimise the signal-noise ratio.

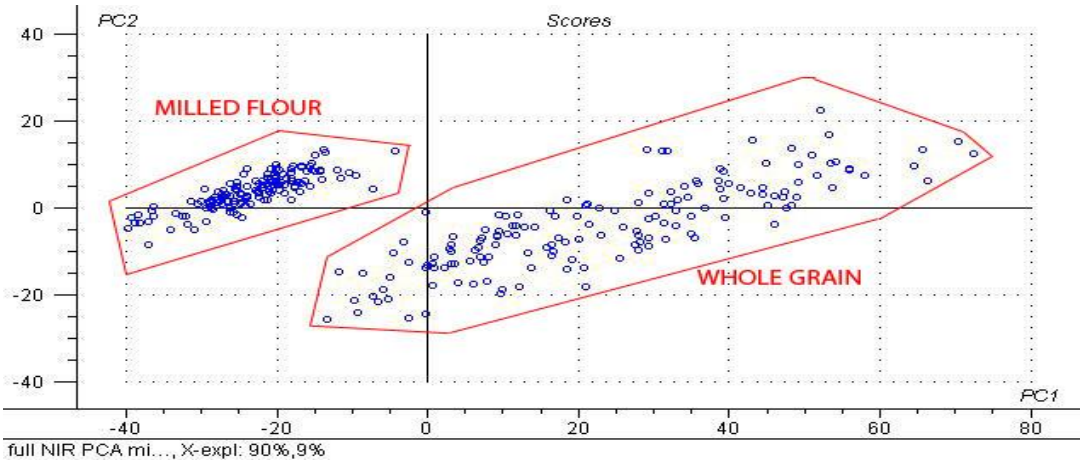
Selection of suitable pre-processing methods requires more than visualization of spectra, so comparative models were developed using partial least squares (PLS) and cross-validation and/or independent test set validation on the same data sets for the same parameter to confirm the choice of pre-processing method. Comparisons of suitable pre-processing methods fell outside the scope of this study. The pre-processing method providing the best calibration statistics was selected for all models for all parameters. Differences were observed between calibration statistics of different pre-processed spectra (Appendix 1) which showed little visible difference (Fig. 3.3).



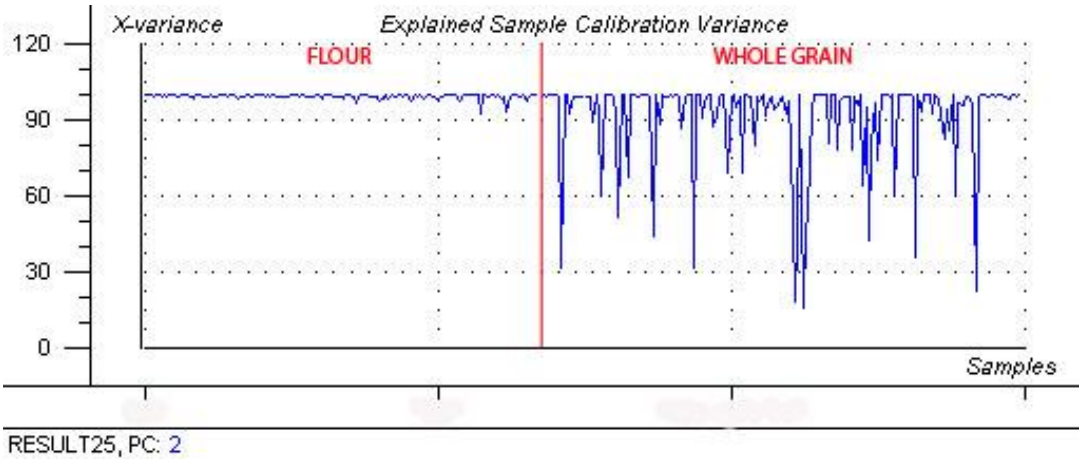
a) Whole grain raw spectra b) Milled flour raw spectra

c) Whole grain pre-processed spectra d) Milled flour pre-processed spectra

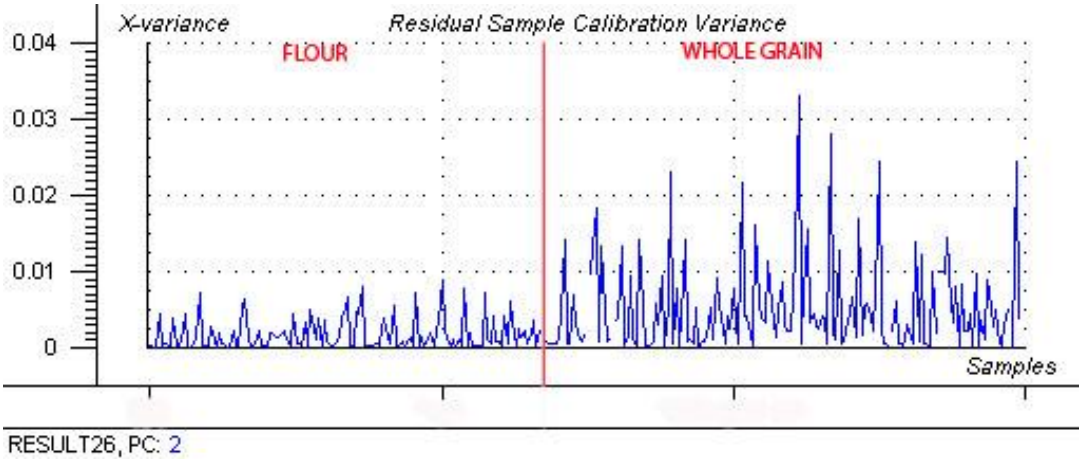
Figure 3.1:Near-infrared reflectance spectra from 400-2500nm (expressed as Log1/R) collected on a FOSS NirSystems 6500 monochromator on "as is" basis (a) whole grain sorghum and (b) milled sorghum flour before (a;b) and after pre-processing (multiplicative scatter correction, Stavitsky-Golay 1st order derivation with 4nm smooth) (c;d).



a



b



c

Figure 3.2: Principal components analysis (PCA) of milled and whole grain NIR spectra (1000-2500 nm) on "as is" basis: a) score plot showing groupings on the 1st and 2nd principal component; b) explained sample variance; c) residual plot showing the difference in spectral variance on the 2nd principal component.

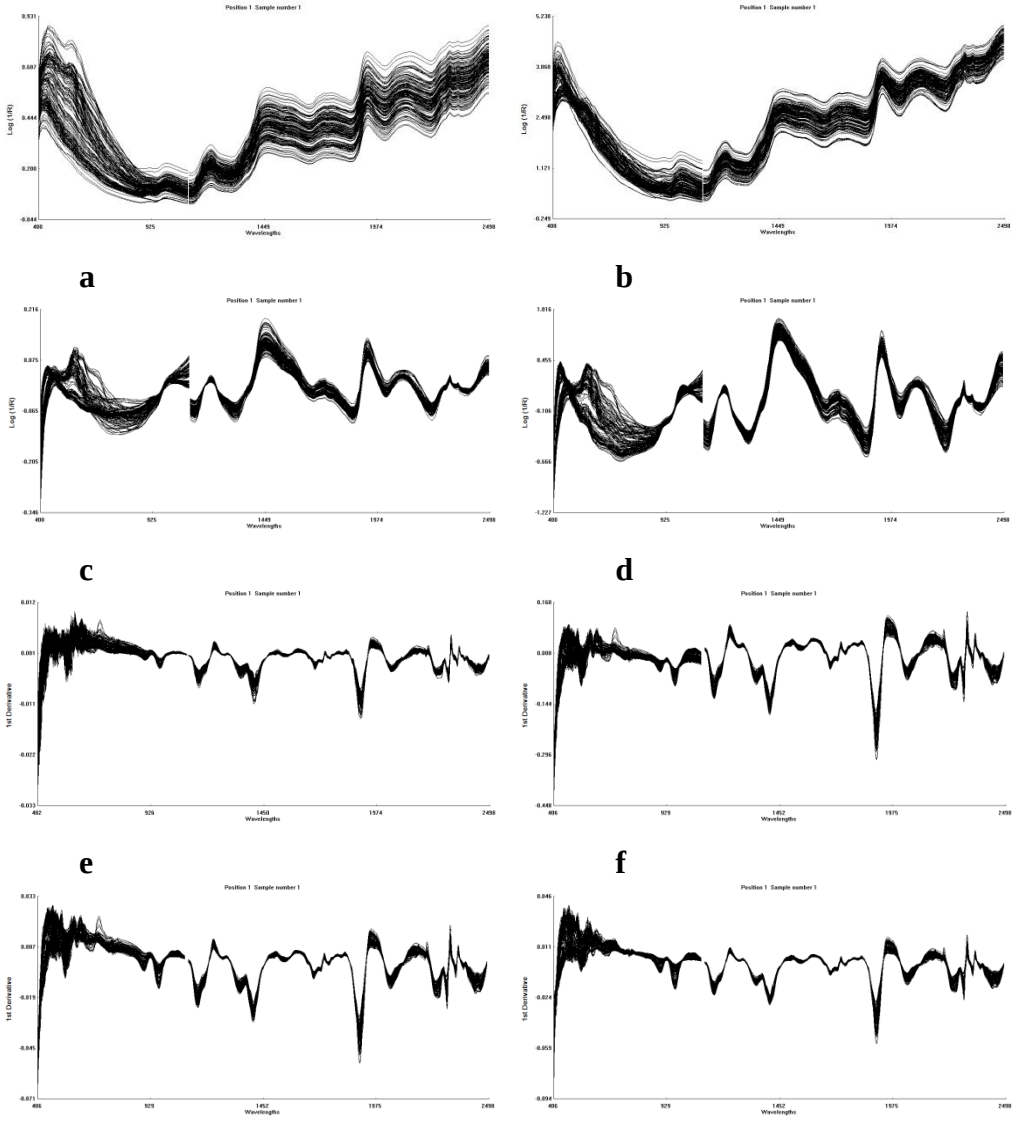


Figure 3.3: Whole grain near-infrared reflectance spectra from 400-2500nm (expressed as Log1/R) collected on a FOSS NirSystems 6500 monochromator on "as is" basis after several pre-processing methods performed in WinISI III: (a) raw data, (b) standard normal variate (SNV), (c) detrend, (d) SNV and detrend, (e) 1st derivative, (f) 1st derivative, SNV and detrend, (g) 1st derivative and inverse MSC, (h) 1st derivative and weighted MSC.

3.3.3 Calibration

3.3.3.1 General differences between milled and whole grain models

Calibrations developed on spectra of milled flour samples were consistently better than those based on whole grain spectra for moisture and absolute protein (Table 3.2). Moisture and protein models based on milled flour spectra contained fewer PLS factors (PC's) than whole grain models. The converse was observed for phytic acid models, in which whole grain models appeared more accurate and predictive.

3.3.3.2 Moisture

Both moisture models performed best when the region from 400-900 nm was excluded from PLS regressions. The moisture calibration based on milled flour samples had lower SEP (0.302 %) and higher coefficients of determination (0.971) than whole grain (0.6 % and 0.897 respectively) (Table 3.2). A notably lower error in the reference method (SEL) for moisture determination in milled flour was reported (0.119 vs 0.340 %). Independent validation reported that milled flour calibrations display almost half the error of whole grain calibrations when applied to practical routine analysis (0.6 vs 0.302 %). Nevertheless, error and R^2 values for models on both sample types agreed well and were acceptably close.

Despite fewer smooth distinguishable peaks in whole grain moisture models, several similarities were observed in the B-coefficients selected (Fig. 3.4). Five absolute B-coefficient peaks were located on or near the same wavelength at approximately: 1102-114 nm, 1300-1306 nm, 1662-1668 nm, 1842-1843 nm, and 2020-2040 nm. Following variable selection and model optimization, whole grain models had two major absolute coefficients below 1102 nm and none between 1102-1300 nm in which the milled flour model contained four peaks. Both models contained a further four significant absolute coefficients between 1300-1306 nm and 1662-1668 nm. The milled flour model used more information from the region above 2000 nm with three relatively large absolute coefficients at 2242, 2362 and 2453 nm.

3.3.3.3 Absolute crude protein

Crude protein PLS models for both milled and whole grain were built on the region from 900-2500 nm (Fig. 3.4). More clearly defined loading peaks were observed in milled flour calibrations, whereas whole grain loadings appeared noisier and less clear to interpret. Calibrations on both milled flour and whole grain underwent extensive variable selection and reduction in number of wavelengths, whilst avoiding over-fitting. The accuracy of milled flour models (0.177 %) far exceeded that of whole grain (0.766 %) and displayed a near perfect coefficient of determination (0.997). Error and R^2 values for calibration and validation of both models compared well. The milled flour calibration was based upon two PC's and displayed extremely low RMSEC and RMSEP values of 0.026 and 0.031 %, respectively. Independent validation of this model yielded a more realistic SEP of 0.177 % when compared to the SEL of 0.109.

The largest absolute milled flour crude protein model B-coefficients were at and between 1050, 1463-1570, 1664-1780, and 1200 nm amongst others (Fig. 3.4). Several of the largest absolute whole grain calibration B-coefficients correlated with these at 1018, 1570, 1200, and between 1640 and 1780 nm. The whole grain model, however, contained far fewer coefficients between 1463 and 1570 nm than milled models. Both crude protein models also contained similar smaller absolute B-coefficients at 1270, 1850, 2100 and 2200 nm.

3.3.3.4 Phytic acid quantity

Phytic acid coefficient of determination significantly improved (0.780 vs. 0.940) when based upon milled flour spectra. However, the whole grain calibration for this parameter was more predictive (RMSEP of 0.371 vs. 0.425 $mg.g^{-1}$) and yielded a higher RPD of 2.61 (Table 3.2). Time and repetition were required to reduce the error of this reference method. As a result, independent validation of these calibrations fell outside the scope and scale of this study.

Regression B-coefficients differed quite significantly in the final models of both sample types (Fig. 3.4). Milled flour models had two distinct characterising absolute coefficients at 1664 and 1726 nm. Similarly, absolute coefficients were observed in whole grain models at 1665 and 1719 nm, but were less heavily weighted in the model. The main absolute B-coefficients in the whole grain model were at 1393, 1415, 1898, 2208, 2254 and 2420 nm. The milled flour model used several wavelengths on the edge of the visible light region at 786, 840 and 996 nm whilst the whole grain model contained no values below 1132 nm. The milled flour model also had two lower order, but clearly significant, absolute B-coefficients at 1204 and 2042 nm.

3.3.3.5 Colorimetry

Calibration of the FOSS NIR instrument to CIE Lab colour values of whole grain gave good results despite high differences between RMSEC and RMSEP, and the error of the reference method. Better calibrations were developed using the visible region of the spectrum (400-1000 nm) than the NIR region (1000-2500 nm) or full spectrum (400-2500 nm) (Appendix 1). The highest calibration correlations were observed for L* and gloss, which displayed the highest RMSEP values of 3.085 and 2.547 units respectively. These values correlate in that L* and gloss had the highest SEP and reference method error. Lower R^2 values were observed for a* and b*. B* had the lowest R^2 of all colour coordinates (0.887). The lowest SEP was observed for a* models (1.28 units). The order of increasing RMSEP values correlated well with the SEP values for all colour models, although RMSEP values were significantly higher and over-estimated the error shown by independent validation (SEP). Calibration error was, however, significantly higher (3-4 times) than colorimeter derived reference values.

Several similarities and differences were observed in the weighted loading plots and coefficient plots (Fig. 3.4) for all four colour coordinates. The highest absolute coefficient values for all colour coordinates were as follows: a* at 400, 410, 540, 780 and 950 nm; b* at 408, 582 and 870 nm; L* at 556, 878 and 1000 nm; and gloss at 556, 876 and 1000 nm. L* and gloss models possessed absolute B-coefficient peaks at identical spectra with similar values. These coefficients, however, appeared to be reciprocals of one another, with inverse weighting at the same wavelengths.

3.3.3.6 Preliminary calibrations

3.3.3.6.1 Fat quantity

The preliminary NIR calibration for fat quantity was based on a sample set showing a flat distribution across the range of values (Appendix 2). The model was based upon a small number of samples and it is important to note that cross-validation statistics are typically optimistic. Nevertheless the calibration statistics reported for this parameter are acceptable and clearly depict a strong correlation between spectra and the reference values (Table 3.3). A large difference between R^2 and 1-VR, and SEC and SECV was observed. Thus the accuracy of this calibration was limited and relatively poor.

3.3.3.6.2 Apparent Metabolisable Energy

AME preliminary calibration was also based on a sample set with relatively flat distribution across the range of values (Appendix 2). Despite differences between R^2 and 1-VR values, these values were relatively high and depict a strong coefficient of determination (Table 3.3). The difference between SEC (0.1858 %) and SECV (0.2939 %) was quite large, yet in light of the scale of this calibration, there was fairly good agreement between these values and the models clearly did not overfit. These results suggest that AME is indeed predictable by NIR in sorghum flour.

3.3.3.6.3 Amino acid quantity

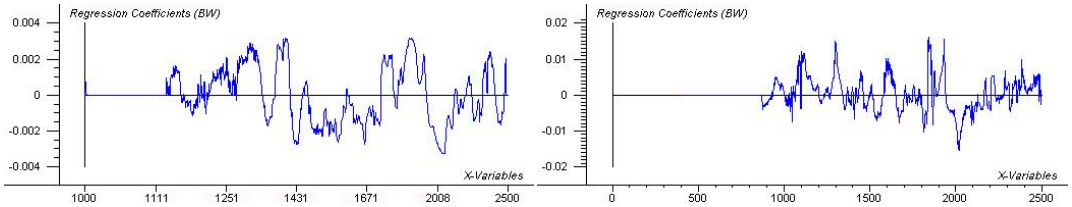
Preliminary amino acid calibrations using PLS with cross-validation were performed upon sample sets manually selected to show flat distributions across the widest possible value range, to optimise results on such small sample sets (Appendix 2). The sample size in this study was limited by the cost of amino acid analysis. Once again, it is important to note that cross-validation results overestimate the potential of calibrations. Nevertheless, good results were shown for almost all parameters tested (Table 3.3). Regression analysis showed strong correlations between absolute crude protein and all but three amino acids (Table 3.4). PCA plots of 16 amino acids and crude protein show correlations between specific amino acids and crude protein (Fig. 3.5). Crude protein results were similar to more comprehensive calibration attempts shown in Tables 3.2 and 3.4. All 16 amino acids showed good R^2 and 1-VR values. Aspartic acid displayed

the lowest 1-VR (0.7763). The best calibration and validation correlations above 0.95 were observed in decreasing order for: glutamic acid, leucine, alanine, phenylalanine, tyrosine and glycine. Glutamic acid, leucine, alanine and glycine yielded

Table 3.2: Summary of optimised model statistics using test-set validation including independent data set prediction, ratio of performance deviation and reference method variance

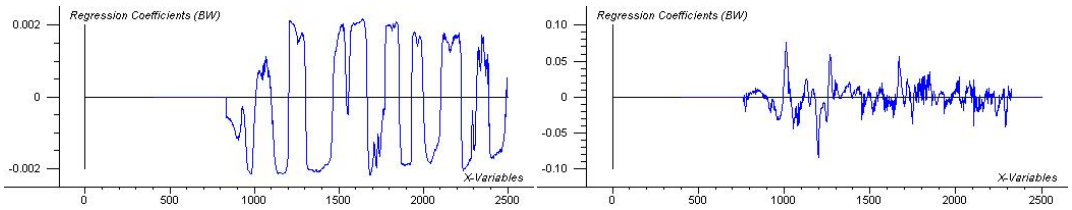
Parameter	Sample[d]	Statistics				Calibration				Validation			Independent validation			Reference method
		N	max	min	mean	R^2	slope	PC's	RMSEC	R^2	slope	RMSEP	n	SEP (%)	RPD	SEL (%)
Moisture[a]	WG	396	18.18	7.14	11.31	0.897	0.828	3	0.343	0.875	0.804	0.351	157	0.600	1.74	0.345
	F	420	12.12	3.97	8.797	0.971	0.950	2	0.203	0.950	0.941	0.267	150	0.302	2.55	0.119
Protein[a]	WG	382	19.21	7.35	13.09	0.955	0.913	6	0.562	0.930	0.897	0.661	260	0.766	2.72	0.106
	F	364	19.21	8.29	13.10	0.997	0.994	2	0.026	0.995	0.974	0.031	230	0.177	11.92	0.106
Phytic acid[b]	WG	120	4.73	1.25	2.68	0.780	0.601	5	0.299	0.654	0.515	0.371	-	-	2.61	0.109
	F	160	4.80	1.11	2.79	0.940	0.884	7	0.239	0.883	0.815	0.425	-	-	2.35	0.109
L*[c]	WG	200	63.46	20.36	46.56	0.959	0.910	3	2.857	0.934	0.896	3.085	60	1.840	5.32	0.787
a*[c]	WG	200	17.53	4.34	9.92	0.920	0.843	4	1.292	0.893	0.824	1.486	60	1.28	2.92	0.377
b*[c]	WG	200	29.53	8.04	22.84	0.887	0.786	4	2.153	0.855	0.769	2.358	60	1.578	3.28	0.504
Gloss[c]	WG	200	75.34	41.50	54.48	0.951	0.903	3	2.438	0.947	0.891	2.547	60	1.612	5.09	0.619

[a] Values expressed in percent (%)
[b] Values expressed in $mg.g^{-1}$
[c] Values expressed in CIE Lab values
[d] WG = whole grain; F = flour



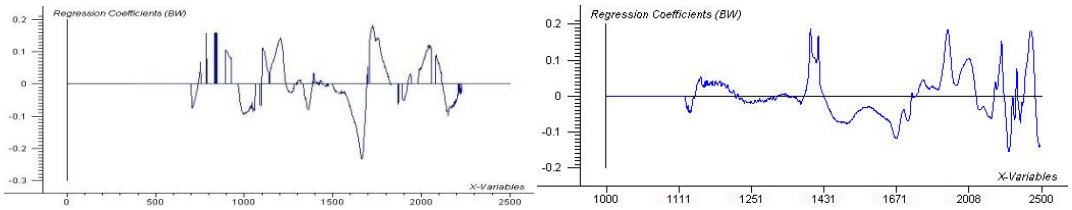
a) Milled flour moisture

b) Whole grain moisture



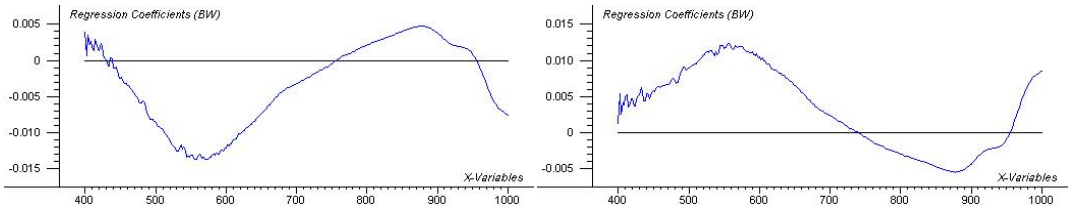
c) Milled flour crude protein

d) Whole grain crude protein



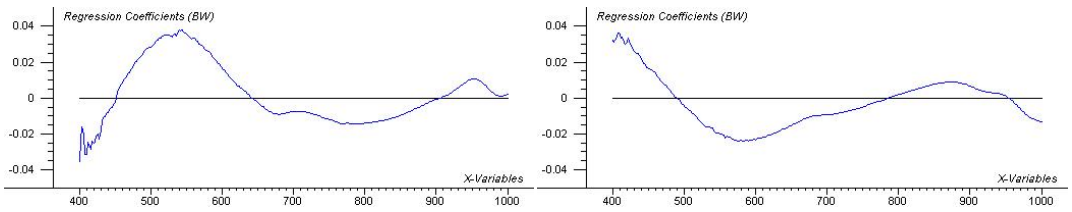
e) Milled phytic acid

f) Whole grain phytic acid



g) L* whole grain

h) Gloss whole grain



i) A* whole grain

j) B* whole grain

Figure 3.4: Weighted Regression Coefficient plots for calibrations.

Table 3.3: Preliminary calibration statistics performed on small sample sets using cross-validation in WinISI III on "as is" spectra after 1st order derivative, 2nd order polynomial, standard normal variate, detrend and 8nm smooth pre-processing

Parameter	n	Calibration set			Calibration		Cross-validation		
		Mean	Min	Max	SEC	R^2	SECV	1-VR	RPD
Crude protein	39	13.575	8.673	16.582	0.2902	0.9924	0.3160	0.9853	10.52
Fat	20	3.154	2.644	3.783	0.0882	0.9357	0.1388	0.8349	2.51
AME	20	13.97	12.700	15.283	0.1858	0.9157	0.2939	0.8001	2.18
Aspartic	33	0.771	0.509	1.315	0.0395	0.9618	0.0954	0.7763	2.32
Threonine	35	0.329	0.211	0.528	0.0097	0.9849	0.0195	0.9380	4.05
Serine	35	0.417	0.284	0.624	0.0140	0.9824	0.0265	0.9356	3.98
Glutamic	35	2.245	1.423	3.396	0.0403	0.9956	0.0721	0.9856	8.43
Proline	35	0.761	0.522	1.016	0.0173	0.9826	0.0308	0.9448	4.25
Glycine	32	0.235	0.107	0.356	0.0097	0.9779	0.0140	0.9536	5.11
Alanine	35	0.987	0.579	1.549	0.0251	0.9934	0.0420	0.9809	7.33
Valine	34	0.546	0.347	0.766	0.0144	0.9833	0.0249	0.9496	4.53
Methionine	35	0.181	0.087	0.312	0.0103	0.9761	0.0166	0.9371	4.01
Isoleucine	35	0.418	0.303	0.585	0.0127	0.9788	0.0207	0.9429	4.20
Leucine	35	1.494	0.881	2.391	0.0349	0.9947	0.0641	0.9817	7.48
Tyrosine	34	0.349	0.195	0.607	0.0147	0.9873	0.0269	0.9571	4.83
Phenylalanine	35	0.559	0.358	0.857	0.0152	0.9904	0.0291	0.9638	5.30
Histidine	35	0.238	0.127	0.354	0.0132	0.9432	0.0216	0.8510	2.56
Lysine	35	0.239	0.121	0.313	0.0130	0.9340	0.0210	0.8313	2.42
Arginine	33	0.410	0.293	0.567	0.0129	0.9736	0.0241	0.9065	3.23

Table 3.4: Regression analysis of 16 amino acids against crude protein quantity on dry matter basis

Variable	*Coefficients*	*Standard Error*	*t Stat*	*P-value*
Intercept	14.942	0.175	85.583	2.9251E-29
Aspartic Acid	3.853	0.822	4.686	0.000113
Threonine	18.002	8.383	2.147	0.043043
Serine	9.890	5.408	1.829	0.081055
Glutamic	23.376	3.160	7.398	2.10807E-07
Proline	-39.990	3.290	-12.155	3.10574E-11
Glycine	-25.083	8.897	-2.819	0.009992
Alanine	57.452	5.734	10.020	1.16442E-09
Valine	-63.533	8.861	-7.170	3.46385E-07
Methionine	-85.446	6.814	-12.539	1.70117E-11
Isoleucine	-26.001	15.129	-1.719	0.099724
Leucine	-63.132	4.825	-13.085	7.41545E-12
Tyrosine	12.191	9.162	1.331	0.196943
Phenylalanine	52.006	8.230	6.319	2.33001E-06
Histidine	208.860	13.504	15.466	2.64999E-13
Lysine	-51.362	8.087	-6.351	2.16464E-06
Arginine	-13.753	4.748	-2.896	0.008374

Table 3.5: Effect of milling on protein quantity models on "as is" basis

Sample prep	n	R^2	Slope	RMSEC	R^2	Slope	RMSEP
Whole grain	196	0.8965	0.8824	0.7042	0.8767	0.8620	0.7233
2mm milled	196	0.9572	0.9430	0.1780	0.9362	0.9354	0.2020
0.5mm milled	196	0.9944	0.9840	0.0846	0.9899	0.9756	0.1033

the best ratios of performance deviation above 5. The lysine calibration showed an acceptable coefficient of determination and low SEC and SECV values. However, it was based on a relatively small range of values and therefore displayed an RPD below 2.5, along with aspartic acid.

3.3.4 Effect of milling on crude protein calibrations

Cross-validated calibrations based upon the same 196 samples yielded similar results to those shown in Table 3.2 in that significant improvement of calibrations were observed when based upon milled flour spectra when compared to whole grain spectra (Table 3.5). Furthermore, these data suggest a linear relationship between the degree of milling and the RMSEC and RMSEP of resulting calibrations. Lower calibration and prediction error values were observed for calibrations on spectra of samples milled to pass through 0.5 mm mesh (0.1033 %) than those on samples milled to pass through 2 mm mesh (0.202 %). The improvement of these calibration statistics, however, was far more significant between whole grain (0.7233 %) and milled samples (between 0.1033 and 0.202 %) than 0.5 mm and 2 mm milled samples. It is important to note that these calibrations were not optimised through extensive variable selection in order to allow for fair comparisons between the degree of sample preparation. Particle size effects, therefore, may not have been excluded to the degree they could have with extensive model optimization. Nevertheless, similarities between RMSEP's presented in Tables 3.2 and 3.5 suggest that optimization would not have improved prediction errors much more.

3.3.5 Colour classification analysis

PCA analysis of CIE Lab reference values and visual classification colour rankings decomposed 96 % of the data variation onto the first 2 PC's (Fig. 3.6). PC 1 explained 83 % of the sample set variation. A score plot of PC 1 verses PC 2 depicted clear separation between samples visually classified as white and those classified as all other colour classes. This depiction occurred along the first PC axis with white samples located in the positive half of the axis and all other samples in the negative half. Corresponding biplots overlaying the positions of CIE Lab coordinate variables L*, a*, b* and gloss indicate a strong correlation between the 1st PC and L* and gloss values. Samples classified as white are strongly correlated with the L* variable along the first PC whilst all other samples are more strongly correlated with gloss. Gloss and L* were negatively correlated along the first PC, which correlated to data presented in Figure 3.4. Similarly there were positive correlations between b* and L*, and a* and gloss on the first PC but negative correlations between the two correlated groupings. A repeated

PCA in which gloss was excluded from the analysis, however, still yielded similar results. L* still displayed a positive correlation to b* yet both were negatively correlated to a* along the first PC. Along the second PC a* and b* were positively correlated with one another and negatively correlated to L*. No consistent groupings with respect to each other or CIE Lab values were observed for the other colour groupings red, brown, yellow or brown-red.

Discriminant calibrations were developed using the UNSCRAMBLER^{R} software (Version 9.2, CAMO PROCESS AS[10]) which were able to predict white from other colour groupings with a high degree of accuracy. Calibrations to predict other colour groupings were inaccurate and incorrectly classified colour groupings with a high frequency.

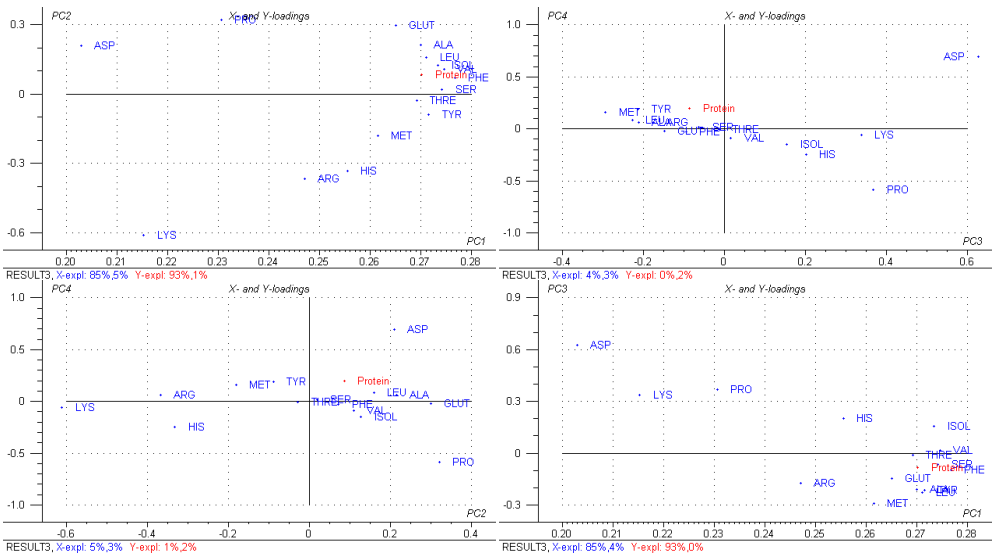
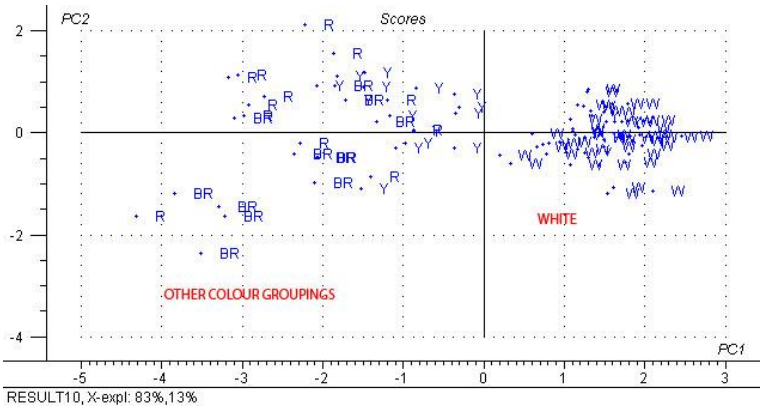
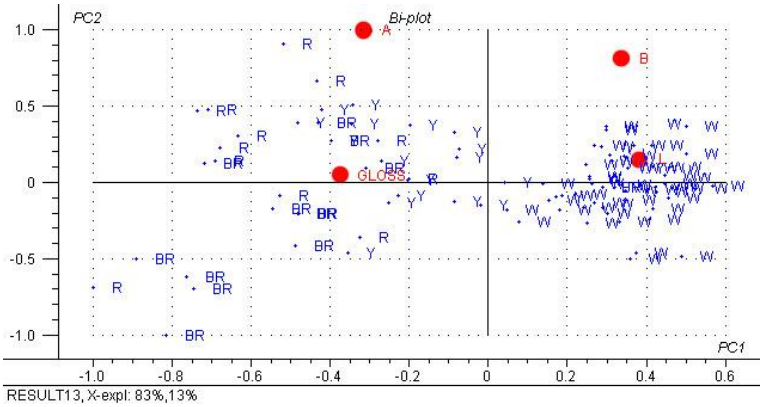


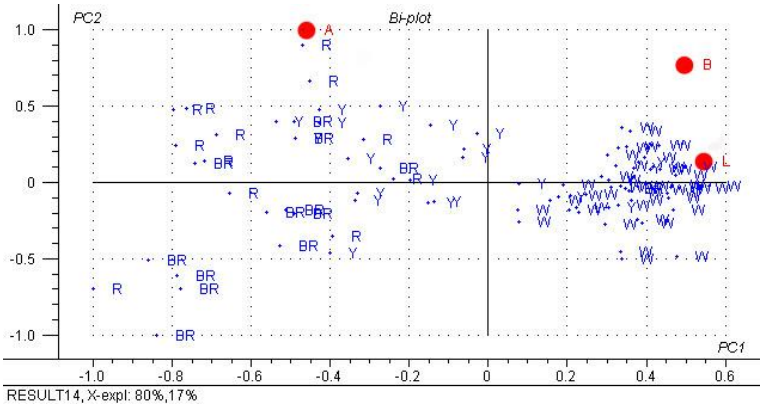
Figure 3.5: X and Y loading plots from principal components analysis of crude protein against 16 amino acids on dry matter basis.



a



b



c

Figure 3.6: Principal component analysis (PCA) of "as is" colour discrimination based on CIE* Lab values and visual classification rankings shown on the 1st and 2nd principal components including: (a) score plot, (b) biplot of L* a* b* and gloss, and (c) biplot of L* a* and b*.

3.4 DISCUSSION

This study has served to highlight the potential of NIR calibration development on grain sorghum and identified some of its limitations. The successful development and implementation of calibrations confirm the reliability of NIR methods and advocate their suitability as a tool in sorghum breeding, classification and analysis. The need for accurate reference data for calibration development appears critical, as is the need to identify the application for which calibrations are being developed.

3.4.1 Whole grain verses milled spectra

It is widely accepted in the literature that there are large spectral differences between whole grain and flour samples due to particle size, and that there is a strong positive correlation between fine particle size and strong calibration predictability (Blanco and Peguero, 2008). These particle size effects were clearly visible in raw and pre-processed spectra in this study. In previous work they have been shown to account for up to 90 % of the NIR spectral variance and to persist through derivitization of spectra (Blanco and Peguero, 2008).

It is important to consider the purpose and functional role of calibrations when deciding on the degree of milling prior to NIR calibration development (Shenk et al., 2001). For the purposes of rapid screening in plant breeding programs, for example, the error level of 0.766 % crude protein dry matter may be acceptable where breeders are screening for significant outlying samples and the volume to be screened is high. The same error, however, may not be acceptable to quality control of baking flour where such a large error could seriously impact the suitability of the final product. Thus the selection of calibrations needs to be suited to purpose.

3.4.2 Data pre-processing

The advantages and applicability of different data pre-processing methods has been widely discussed in the literature and comprehensively reviewed by Beebe et al. (1998). It is generally accepted that different pre-processing treatments are suited to specific constituents and the matrices in which they are bound. For example: first derivatives are

usually applied to spectra for moisture calibrations, and second derivatives are applied to spectra for starch and other carbohydrate related calibrations (Shenk et al., 2001). Pre-processing serves to reduce noise and reduce size effects, allowing trends in the spectral data to become "more visible" to the regression method (Esbensen, 2006). In the past, a several different spectral pre-processing methods have been used for calibrations in sorghum. These include: mean centre (Hicks et al., 2002; Rodriguez et al., 2005); mean centre, second order derivative, standard normal variate and detrend (de Alencar Figueiredo et al., 2006); mean centre and Savitsky-Golay second order derivative (Savitsky and Golay, 1964; McDonough and Rooney, 2003). The calibrations of Hicks et al. (2002) and Rodriguez et al. (2005) reported lower accuracy and prediction for absolute crude protein than those using second order derivatives (McDonough and Rooney, 2003; de Alencar Figueiredo et al., 2006). In addition, the Stavitsky-Golay treatments have been shown to be particularly promising for noise suppression (Tsai and Philpot, 1998). The nature and complexity of multivariate modelling often means that models for the same parameters are built differently yet remain as effective (Esbensen, 2006). As the nature of these models are not empirical and do not obey laws such as Beer-Lambert's, the true test of their robustness and predictability is when they are practically applied and the predictions are confirmed (Esbensen, 2006).

3.4.3 Calibrations

3.4.3.1 Moisture

Some of the earliest applications of NIR were for measuring moisture in grains and seeds (Norris and Hart, 1965). Moisture calibrations are reported as being some of the more reliable NIR models, particularly due to NIR sensitivity to atoms bonded to hydrogen ions and the water molecule containing two such bonds to oxygen (Shenk et al., 2001; Büning-Pfaue, 2003). Fontaine et al. (2002) reported moisture calibrations with R^2 of 0.97 and SECV of 0.257 % in 167 sorghum flour samples with a range of 6.3-14.1 %. Rodriguez et al. (2005) reported moisture calibrations on milled flour samples with R^2 of 0.96 and SEP of 3.72 % in 212 milled flour samples with a range of 7.2-16.9 % moisture. The range of milled flour moisture values in this study were lower, ranging from 3.97-12.12 %, yet yielded a similar R^2 and SEP of 0.302 %. The

results of both studies and the work presented here reflect those of Fontaine et al. (2002). This work was based upon cross-validation, which is accepted to be over-optimistic with respect to calibration predictive ability, thus would generally yield higher R^2 values and lower SECV (Esbensen, 2006). The moisture calibrations developed in this study on milled flour samples are probably more robust than those of Fontaine et al. (2002) as they were developed on a larger sample size, although would not be as predictive in the range of moisture values from 12.12-14.1 %. Similarly, the calibrations of Rodriguez et al. (2005) are more predictive in the range from 12.12-16.9 % moisture and are more easily described, as they were based upon three individual wavelengths using Multiple Linear Regression (MLR), where as the calibrations reported in this study were developed on the full NIR spectrum.

There are not many reports of whole grain moisture calibrations for sorghum in the literature. Hicks et al. (2002) oven dried their samples to approximately 10 % moisture. de Alancar Figueiredo et al. (2006) equilibrated their whole grain samples to 11.5 % prior to NIR scanning. The approaches used by Hicks et al. (2002) and de Alancar Figueiredo et al. (2006) removed the need to create a calibration for moisture. McDonough and Rooney (2003), however, reported moisture calibration attempts on 95 (190 duplicated) whole grain sorghum samples. They obtained an R^2 of 0.824 and SEVC of 0.235. These calibrations were developed on spectra between 955 and 1650 nm using cross-validation after Savitsky-Golay 2^{nd} derivation pre-processing. Brown and black kernel samples had to be removed from these calibrations due to significant contribution to unexplained variability in the spectral range used.

Possible limitations in NIR moisture calibrations might relate to the accuracy of the reference methodology. Heat based moisture analysis has been reported to remove phenolic content of the grain and thus skew apparent moisture values (Shenk et al., 2001). This study used a relatively small sample minimum of 10g for moisture analysis. Increasing this, or using a longer time at a lower temperature to reduce the risk of phenolic losses, could increase the accuracy of moisture data. The use of lower temperatures has however been shown to leave some moisture behind in the sample (Shenk et al., 2001). It is commonly assumed that moisture calibrations are among the

simplest to develop as the instruments are particularly sensitive to O-H bonds, yet it has been suggested that the factors affecting reliable moisture calibrations are indeed more complex (Büning-Pfaue, 2003). Further strategies to improve the calibrations in this study could also include expanding the range of moisture values present in the calibration data set through harvesting sorghum grain earlier in the season to ensure higher water content. Reliable and accurate moisture calibrations are essential to the further development of "as is" calibrations for other parameters as most reference methods are dry matter based (Haug et al., 1983; AOAC, 2000). Thus inaccuracies in moisture predictions would enhance inaccuracies in predictions of other parameters, unless the samples all contained very similar moisture contents (Shenk et al., 2001). Approaches to down-weigh the contribution of moisture sensitive spectral regions to calibrations have been investigated, such as the use and inclusion of repeatability files (Baker et al., 1994). In some cases, such as predicting the absolute protein content of wheat, the use of partial least squares has been shown to remove the effects of moisture amongst other confounding factors (Dardenne et al., 1995).

3.4.3.2 Crude protein

The literature contains many reports where NIRS has been used to reliably predict absolute crude protein in various forages and feeds including sorghum (Givens et al., 1997), yet there are few reports of calibrations for crude protein in whole and milled sorghum grain. Reliable and accurate models for crude protein in sorghum have been obtained on milled samples (Fontaine et al., 2002; Hicks et al., 2002; Rodriguez et al., 2005; de Alencar Figueiredo et al., 2006), and good but less accurate models have been developed for crude protein on whole grains (Hicks et al., 2002, McDonough and Rooney, 2003; de Alencar Figueiredo et al., 2006). There is strong concurrence between the results presented here and those in the literature that calibrations based on milled samples are more accurate than those based on whole grain samples (Williams and Sobering, 1993; Flinn et al., 1998; Hicks et al., 2002; de Alencar Figueiredo et al., 2006). On milled flour samples Fontaine et al. (2002) showed R^2 and SECV values of 0.99 and 0.214 % respectively. The results presented in this study reflect the same R^2 of 0.99 yet show a lower RMSEP and SEP of 0.031 and 0.177 % respectively. More samples (197) were used in this study than that of Fontaine et al. (2002). Additionally,

the calibrations reported here covered the 14.6-19.21 % protein range, but lacked the low protein samples with values from 5.71-8.29 % present in the calibration of Fontaine et al. (2002). The use of independent test set validation in this research implies that the reported accuracy is more reliable than that of cross-validated models (Esbensen, 2006). Prior to independent validation, the RMSEP of milled flour calibration reported was a third of the accuracy of the reference method. Although the SEP increased significantly, whilst remaining acceptably low, it is important to note possible over-fitting in this model. Rodriguez et al. (2005) applied multiple linear regression (MLR) to spectra of milled samples collected on a fixed filter instrument to yield an R^2 of 0.93 and SEP of 0.186 %. These results suggest that PLS models based upon the entire spectrum may be unnecessary and could include excess noise, despite resulting in potentially more predictive calibrations. A core collection of 257 sorghum samples by de Alencar Figueiredo et al. (2006) was used to develop calibrations for absolute crude protein in whole and milled grain using identical instrumentation to that used in this study. Calibration performance in their study was particularly good and reflected similar values to those presented in this study. R^2 values and SECV percentages for milled grain and whole grain were 0.98 and 0.35, and 0.95 and 0.62 respectively (de Alencar Figueiredo et al., 2006). McDonough and Rooney (2003) reported whole grain calibrations for crude protein on 200 samples with R^2 and SECV of 0.984 and 0.246 respectively. These calibrations used nine PC's and excluded dark grains, yet reported more accurate predictions than this body of work, possibly advocating the development of separate calibrations for different grain types where improved accuracy is required. Both the calibrations presented in this study as well as those of de Alencar Figueiredo et al. (2006) and McDonough and Rooney (2003) report far more predictive and accurate calibrations than those of Hicks et al. (2002). Hicks et al. (2002) also showed better crude protein calibrations based on milled flour than on whole grain. However, the reported R^2 and SECV percentage values for milled flour and whole grain were 0.82 and 0.62, and 0.64 and 0.88 respectively. Possible explanations for this could lie in the lack of first or second order derivative pre-processing by Hicks et al. (2002).

In other cereal crops, good absolute crude protein models have also been obtained. These include wheat, barley, corn, triticale, cassava, oats and rice (Gerlach, 1990;

Osborne et al., 1993; Givens et al., 1997; Fontaine et al., 2002). Crude protein was calculated by multiplying total nitrogen values by the constant 6.25 and used to indicate a measure of total protein. Due to the low levels of non-protein nitrogen in sorghum grain, crude protein calibrations are relatively good approximations of actual protein levels in this cereal (Dogett, 1988). This approach, however, is limited in that NIRS cannot directly measure nitrogen as it does not have a "vibrational response" in NIR, but NIR can indirectly measure N-H molecular vibrations (Shenk et al., 2001). Comprehensive reviews of the range of spectra used in crude protein calibrations have been compiled and show large variation in wavelength selection (Shenk et al., 2001). The characterising protein wavelength in non-derivatised spectra is typically 2180 nm (Shenk et al., 2001). As crude protein is thus an expanded measure of nitrogen content, these calibrations typically use less information from the C-H stretch/C=O stretch combination and focus more on the primary stretch of the carbonyl amide and the N-H bend first and second overtone regions (Shenk et al., 2001). The reported B-coefficients for milled flour correlated with the N-H second stretch overtone at 1050 nm, N-H stretch first overtones between 1463 and 1570 nm, C-H first stretch overtones between 1664 and 1780 nm, and C-H second stretch overtones at 1200 nm. The largest whole grain calibration B-coefficients also correlated with the second stretch N-H overtone at 1018 nm, N-H first stretch overtone at 1570 nm, second stretch C-H overtone at 1200 nm and first stretch C-H overtones between 1640 and 1780 nm. Whole grain and milled flour models also contained similar unidentified high values at 1270 and 1850 nm. Combination N-H bond vibrations between 1900 and 2500 nm were less heavily weighted in models of both sample types.

Extensive variable selection and reduction in number of wavelengths were used to exclude those displaying non-linearities, large noise fractions and irrelevant information. In turn this reduced the number of PC's upon which the PLS model was based and reduced the RMSEP. Interestingly, further reduction of spectra to less than eight wavelengths, based on selecting those with the highest B-coefficient values, resulted in only marginally reduced calibration statistics for milled flour models and yet drastically reduced the accuracy of whole grain models. This is possibly because B-

vectors can either indicate an important variable (as with milled flour models) or a variable with small absolute values but significant relative differences (Esbensen, 2006).

3.4.3.3 Phytic acid

Phytic acid has been reliably predicted previously by NIR in a range of vegetable and cereal feedstuffs (de Boever at al., 1994) with a relatively high degree of accuracy. It is important to note, however, that phosphorus itself has no spectral signature, and thus NIR calibrations detecting phytic acid are indeed detecting the matix in which the phosphorous is bound (de Boever et al., 1994). Similarly to moisture, calibrations for this parameter were hampered by relatively inaccurate reference methods which reportedly yield 10 % error at best (Raboy[34], pers. comm.). The reported range of phytic acid values for the four sorghum samples included in the study by de Boever et al. (1994) was 1.7-2.2 $mg.g^{-1}$ which compare favourably with the values reflected in this study, although the values determined herein reflect a wider range from 1.11-4.8 $mg.g^{-1}$. Similarly, phytic acid values of 3.73 $mg.g^{-1}$, for red sorghum grains, and 5.03 $mg.g^{-1}$, for white sorghum grains, were reported by Mitchikpe et al. (2007). Lestienne et al. (2005) analysed a variety of sorghum with 8.44-10.06 $mg.g^{-1}$ phytic acid. Maghoub and Elhag (1997) 2.48-3.0 $mg.g^{-1}$. 8.86 $mg.g^{-1}$ reported by Marfo et al. (1990). 3.01-3.66 $mg.g^{-1}$ (Osman 2004). 10.12 $mg.g^{-1}$ (Garcia-Estapa et al., 1999). It is thus evident that the range of phytic acid values upon which the calibrations in this study were based could be expanded.

The standard errors of phytic acid calibrations from 0.38 to 0.43 mg g^{-1} reported in this study were significantly higher than error of the reference method (0.1 mg g^{-1}). These values do, however, correlate with the reported SEP's of between 0.4 and 1.3 mg g^{-1} by de Boever et al. (1994), which were developed on 19 different feedstuffs. Frenzel (2003) obtained NIR calibrations for phytic acid in brown rice and compared these to spectra of pure sodium phytate. Clear peaks at approximately 1650 and 1720 nm in the sodium phytate spectrum point to the biological significance of the calibrations developed in this study. In addition, the model of Frenzel (2003) had strong B-

[34] Dr. V. Raboy, United States Department of Agriculture, ARS, Pacific West Area, 1691 S 2700, W. Aberdeen, Idaho, USA

coefficeints at approximately 2000 and 2220 nm where the whole grain model reported here had similar coefficients at 2008 and 2254 nm, possibly explaining its improved accuracy over the milled flour model which lacked coefficients in the 2200 nm region.
It is suggested that the inclusion of greater sample numbers would greatly contribute to the robustness and accuracy of these calibrations and the reduction of the number of PC's upon which they are based. NIR calibrations previously reported for phytic acid used between eight and ten PC's where the calibrations reported in this study use between five and seven PC's (de Boever et al., 1994). Prior conclusions drawn in this study, as well as prior research on the relationship between calibration accuracy and particle size suggest that whole grain calibrations should yield higher errors in prediction and lower correlation coefficients (Blanco and Peguero et al., 2008). It is apparent from the RMSEP's reflected in this study, however, that phytic acid is more accurately predictable in whole grain than in milled flour despite higher coefficients of determination in milled flour calibrations. Expansion of the data sets used in these calibrations would be necessary, however, before any such claims could be made authoritatively.

The calibrations reflected here appear to be an improvement on those previously developed by de Boever et al. (1994) because they reflect lower errors and are based upon fewer PC's, and therefore they should be more robust. This is almost certainly because these calibrations were developed exclusively on sorghum samples. It is suggested that further research be performed to refine these calibrations before use, although the potential for their successful development is strongly evident. These calibrations may, nevertheless, be sufficient to identify outliers in a high throughput screening process.

3.4.3.4 Colorimetry

Calibrations to predict the CIELab colour values, contrary to all other calibration parameters tested in this study, performed best without pre-processing. This result is expected as CIELab values are based upon light reflectance at fixed wavelengths selected to correspond to the peak values for red, green and blue light (ASTM, 1996). These values are in turn used to determine the chromatic values such as CIE L*,a*, b*,

and gloss (ASTM, 1996). Previous attempts to calibrate a visible NIR instrument to predict CIELab values in 69 food samples by McCaig (2002), and in barley grain and lentils by Black and Panozzo (2004), concluded that the most effective manner in which to achieve this aim was not to use a PLS regression, but to formulaically calculate the CIELab values from fixed data points in the visible spectrum through applying the standard observer curves (E308) of the American Standards of Testing Materials (ASTM) (ASTM, 1996). Contrary to this work, Black and Panozzo (2004) used second derivative pre-processing to develop calibrations for CIELab values. Inaccuracies using PLS regression methods were attributed to its secondary nature and thus dependency upon accurate sampling, calibration development methodologies, expertise in calibration software packages and analytical accuracy (Williams and Cordeiro, 1985; Shenk and Westerhaus 1993; Black and Panozzo et al., 2004). Approaches to predict CIE L*a*b* values with a lower degree of error than the methods employed in this study would be of great importance to discriminant classification models based upon CIE L*a*b* values as error in prediction would greatly increase error in classification. Sorghum grain contains a high degree of colour variation even on grains from the same plant head (McDonough and Rooney, 2003). The results of this study suggest that highly reliable calibrations would be able to be developed for sorghum flour due to the homogenous nature of its colour. Where accuracy is important, future applications of visible NIR instruments should thus be calculated through the methods described by ASTM standard E308-99 in a spreadsheet program and preferably not through classical NIR calibration software packages. Unfortunately this approach does not facilitate easy and rapid analysis of multiple parameters through one statistical package, which is possible when PLS calibrations are developed to predict CIE L*a*b* values in the same packages which facilitate calibration development for other parameters.

3.4.3.5 Preliminary calibrations

3.4.1 Fat and AME

The calibration statistics for fat and AME strongly motivate scope for further research and calibration development. The small, albeit representative, size of the calibration set and the use of cross-validation in PLS strongly call for further investigation into the predictability of these parameters. The RPD values suggest that fat quantity calibrations

may be usable in plant breeding or feed screening; however, that is assuming that the calibration statistics remain constant following expansion (AACC, 2009). Sorghum has a reportedly low range of fat of between 2.5 and 3.5 % (FAO, 1995), thus the applicability of good calibration may indeed be limited. NIR calibrations on the composition of sorghum fats (in other words fat quality) would be of value to sorghum breeders focussing on nutrition, as significant variation has been reported within different sorghum varieties (Rooney, 1978).

3.4.2 Amino acids

Previous research has shown that NIR can provide "highly informative and predictive" predictions in sorghum with respect to amino acid content (Fontaine et al., 2002). The results of preliminary calibration attempts reported here strongly support these prior. Typically, such accurate results from sample sets of small size should be met with scepticism, especially due to the employment of cross-validation. Cross-validation methods were necessary in this situation due to the small sample size, which was limited by the cost of full amino acid analyses involved. A further reason to place merit in these findings can be found in the careful selection of representative samples across the range of values in sorghum. The samples used in this study were selected at equidistant points across the range of variable values, and the analyses were performed with multiple repetitions to ensure the highest degree of accuracy possible. In effect a method of variable selection was used to force the model to fit across the range and focus on reliable regions of the spectra (Nørgaard and Bro, 1999). These data motivate further research and "in house" calibration expansion.

Fontaine et al. (2002) developed cross-validated calibrations on 167 sorghum flour samples for similar parameters. The R^2 and SECV values for the amino acids (methionine, cystine, lysine, threonine, arginine, isoleucine, leucine and valine) ranged from 0.86-0.98 and 0.009-0.0046 respectively. The R^2 and SECV values in this study ranged from 0.93-0.99 and 0.016-0.249. Higher correlations and cross-validation errors were thus observed in these calibrations than those of Fontaine et al. (2002). This is most likely because the calibrations in this study were based on smaller sample sizes, resulting in less robust models with reduced predictive prowess (Esbensen, 2006). In

addition, good calibrations for amino acids have also been shown in wheat, barley, corn, triticale, rice, soy, rapeseed meal, sunflower meal, peas, fishmeal, meat and poultry meal products (Fontaine et al., 2001 and 2002). This study reflects the results of prior research with respect to the potential and ease with which calibrations for amino acids can be developed.

3.4.4 Calibration applicability

The purpose of calibrations can be judged by their RPD values (AACC, 2009). These have been considered adequate for quality control when ranging from 5-10, and suitable for screening in breeding programs when ranging from 2.5-4.9 (Williams and Sobering, 1993). The exceptional statistics and RPD value of 11.92 for crude protein calibrations in milled flour strongly support their use in routine analysis because the proven accuracy is close to the reference method. It has been recommended that the error of NIR calibrations should be approximately twice that of the reference method to avoid over-fitting (Fontaine et al., 2001; Freznel 2003). Absolute crude protein calibration on milled flour meets that requirement adequately. Several of the calibrations developed here do not meet the required accuracy for analytical and quality control applications but are suitable for the screening of breeding programs. These include: crude protein, phytate, a* and b* calibrations on whole grain, and the moisture calibration of milled flour. These whole grain calibrations appear quite adequate for non-destructive screening in plant breeding programs, and would greatly reduce the time and preparation required for analysis and facilitate the reuse of the seed for further breeding trials. In addition, L* and gloss calibrations on whole grain showed high enough RPD's to be used for quality control (AACC, 2009).

3.4.5 Colour classification

The CIELab system describes a colour in terms of three colour coordinates. L*describes lightness and was the most accurately predictable colour value in PLS calibration (Hunter, 1975). Similarly, discriminant classification models were able to clearly distinguish between white sorghum grain and all non-whites, which are effectively described along the L* axis. Sorghum grain is not very uniform in colour, as can be seen by the high standard deviations shown in characterising colour using a standard

colorimeter. It also has a wide range of colours characteristic with its wide genetic diversity (Dogett, 1988). Sorghum additionally has a wide range of awn colours, which are not always completely removed from the grain and can be quite different in colour to that of the grain (Dogget, 1988). White is the lightest of the visual classification colours, and red, brown and yellow grains are darker. These are easily distinguishable along the L* axis. Some lighter yellows were misclassified as white by the model. As these classifications are visually determined they are quite subjective and it is quite possible that human error could contribute to the inaccuracies of these models. a* and b*describe chromacity, where a* describes a red/green axis and b* describes a yellow/blue axis (Hunter, 1975). It was expected that yellow grains would correlate to a higher degree with the b* axis, and red grains would correlate to the a* axis. It is possible that inaccuracies in visual classification masked trends in the data upon which the models were based. Possible future solutions would include increasing the sample size significantly and using experts to visually classify the grain. It is quite possible that the grain classifications do not strongly correlate to real CIELab colours.

In other crops, such as durum wheat, researchers have been able to use the NIR spectra alone to discriminate between varietal differences (Marini et al., 2008). These classifications were, however, built using artificial neural networks (ANN) and not PLS-DA as was done in this study. Thus different chemometric classification approaches to this type of modelling, such as SIMCA, may also yield improved results (Bro, 1997; Esbensen, 2006). These may also facilitate the further investigation of the relationship between colour parameters and the presence of polyphenolic compounds in sorghum caryopses (Waniska, 2000). Such approaches have been used in wine classification models (Melendez et al., 2001).

3.5 CONCLUSION

This body of work shows that highly accurate and informative predictions can be obtained through NIRS for grain sorghum. NIRS is a complex science which is application specific. However, for the purposes of plant breeding it offers huge potential to reduce the costs and delays of getting analyses of each generation of breeding material. Plant breeders require many analyses of multiple parameters for each sample.

NIRA offers a relatively low technology solution, once calibrated, which operates at low cost. Extreme accuracy is not necessarily required as the function of analysis is to make a rapid comparison and selection between breeding materials.

This research has highlighted the widely accepted need for extremely accurate reference chemistry and large representative samples in calibration development. NIR analysis of amino acids and phytic acid would greatly reduce costs and enhance breeders' access to useful and important information in breeding for enhanced nutrition. Further work developing a wide range of sorghum analytical models is required. However, their potential has been highlighted. There is also scope for NIR calibration development on a range of additional sorghum parameters.

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CHAPTER FOUR

THESIS OVERVIEW

4.1 INTRODUCTION

The research completed in this dissertation was centred on the improvement of the important crop species *Sorghum bicolour* (L.) Moench. This was accomplished through investigating a new method of inducing genetic diversity through heavy-ion beam mutagenesis (Chapter Two) and developing non-destructive screening applications using near-infrared spectroscopy (Chapter Three). The review of the literature in Chapter One highlighted the importance of grain sorghum in developing countries and its current and potential contribution towards food security. Attention was additionally drawn to some of the constraints facing sorghum's use as a crop both in terms of its inherent nutritive composition, challenges in cultivation and breeding. Attempts were made to address these constraints in this body of work.

4.2 SUMMARY OF CONCLUSIONS IN THIS STUDY

The potential of near-infrared spectroscopy as a screening tool for sorghum was shown in Chapter Two. Development of near-infrared (NIR) calibrations on grain sorghum was successful for several grain constituents including moisture, crude protein and phytic acid, although moisture was only reliably predictable on milled flour. Additionally, the potential for reliable calibration for detection of several amino acids, fat and apparent metabolisable energy (AME) was shown through initial calibration development on smaller sample sets. Unfortunately, due to the cost of analysis and timescale available for the completion of this work, these calibrations could not be developed further. The possibility of using NIR instruments able to detect the visible region of the light spectrum to determine colour coordinates was also investigated, but not shown to be particularly reliable.

4.3 IMPLICATIONS OF RESEARCH PERFORMED

4.3.1 Near-infrared spectroscopy

Mutagenesis of sorghum seed by heavy-ion beams generated by an AVF cyclotron was shown in Chapter Two. Typical relationships between dose level, germination and plant survival were not observed as clearly as previously recorded in the literature, suggesting the need for higher treatment levels. However, visible chlorophyll mutations, abnormalities in flowering patterns and changes in protein variation within the sorghum varieties treated showed that treatment had a mutagenic effect. The apparent reduction in plant injury shown Chapter Three, combined with evident genetic change, confirmed the potential of this method of mutagenic treatment that was highlighted in the literature review in Chapter One.

The potential shown in Chapter Three for NIR calibration development on grain sorghum motivates further calibration development. Existing calibrations developed during the course of this research can be expanded with new samples from multiple locations over different generations to increase robustness and reliability. Grain sorghum samples can currently be scanned to generate data rapidly, and tracking of breeding material for those populations is now possible. Additional parameters can be added and further calibrations can be developed.

There is a need to repeat moisture analysis on whole grains with larger sample sets and more replications as originally advised by Norris and Hart (1965), and supported by AOAC (2005), in order to make moisture calibrations more feasible. Strong motivation has been provided to perform more analyses on sorghum for amino acids in order to develop calibrations assisting the selection of quality protein material at a dramatically reduced cost. The potential for discriminant modelling of colour groups has also been highlighted and there is scope for further work in this area. More thorough focus on colour classification methodology and principles is called for.

4.3.2 Heavy-ion beam mutagenesis

Due to the limiting timescale over which this research was completed, further tracking of mutant populations was unfortunately not possible. However, following two generations of self-pollination bulking of germplasm, much material has been generated though this work which could potentially contain beneficial mutations. Continued self-pollination of this material should render segregation resulting from any induced mutations evident (Singh, 2003). There is thus a need to continue screening this material for any beneficial change. The NIR calibrations developed in Chapter Three could potentially be of benefit in this regard.

The work presented in Chapter Two recommends further heavy-ion beam treatment of sorghum seed to be done at higher levels than those used. The differences observed in response to treatment between different varieties, however, also suggest that it may be necessary to treat all future sorghum seed with a range of treatment levels unless an optimal dose level has already been established.

4.3.3 Sorghum grain selections

Over the course of this study, several sorghum selections were made both visually in the field and as a result of analysis by NIR. In particular, heads of M_2 plants showing high levels of crude protein or low levels of phytic acid following routine NIR scanning were selected and marked. These selections of M_3 seed require planting out and further analysis and selection in order to select homozygous lines displaying the desired characteristics.

In addition, screening for resistance to the sulfonylurea group of herbicides was performed in this study. No sorghum seedlings survived the herbicide treatment, thus no tolerant plants were isolated. It is, however, important to mention that further screening for a herbicide resistant trait is advised following more generations of self-pollinations. Continual self-pollination for several generations may still render a recessive homozygous trait which confers resistance (Medina et al., 2005).

4.5 LIMITATIONS

The timeframe available for this study limited the scope and scale of both NIR calibration development and successful mutant isolation. Whilst seed stored in the ACCI seed cold store facility over many seasons was included in the sample sets used for calibrations, their physiological viability could not be tested by "growouts" as has been recommended (Medina et al., 2005). Similarly, mutagenesis studies are recommended over a three to seven seasons to thoroughly establish the rate of mutation induction, especially using a high LET inducer such as heavy-ion beams (Medina et al., 2005).

The methods used to track mutant induction in this study were all secondary methods (Singh, 2003). To comprehensively answer questions regarding treatment levels a molecular method would be far superior, because it will give a primary indication of actual genetic change and could be quantified in a manner similar to that performed by Dong et al. (2008).

4.6 CONCLUSIONS

The research presented in this dissertation has highlighted the potential of heavy-ion beam mutagenesis and near-infrared spectroscopy in improving grain sorghum. Whilst further work is required to continue tracking mutant populations generated in this study, and to refine and further expand some of the NIR calibrations developed, there is sufficient motivation in this body of work to continue the use of both of these tools towards the improvement of sorghum.

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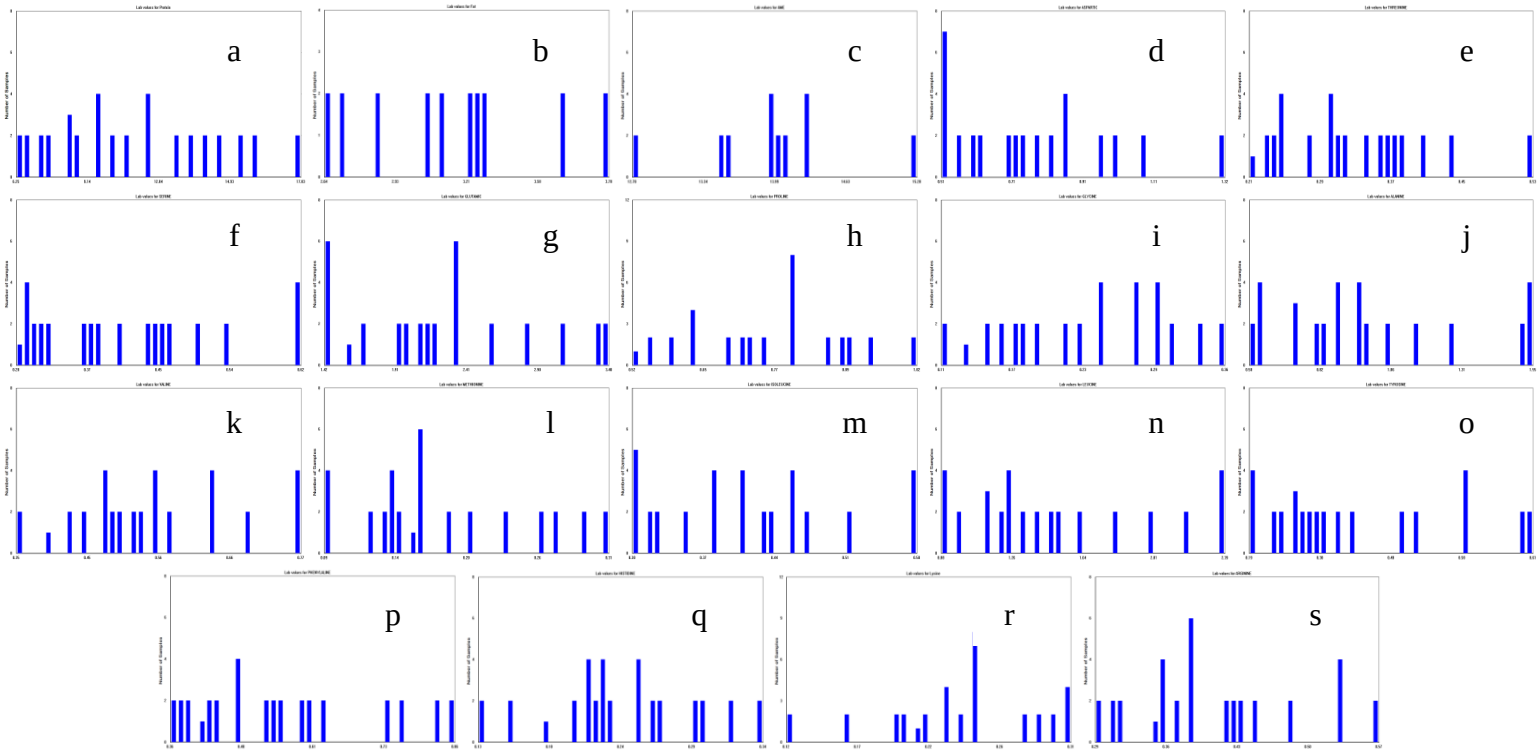
APPENDIX

Appendix 1: Calibration statistics for L*a*b* and gloss using PLS 1 and 2 on a variety of pre-processing methods

Constituent	Software	Regression type	Validation	Spectra	Pretreatment	Derivitive	Smooth	Gap	n	Outliers	No.	Calibration		Validation	
										Removed	PC's	R-squared	RMSEC	1-VR	SECV
L*	Unscrambler	PLS2	TS	400-1000	none	0	0	1	115	12	5	0.8701	4.1931	0.9134	3.9485
A*	Unscrambler	PLS2	TS	400-1000	none	0	0	1	115	12	5	0.9223	1.3310	0.8415	1.6318
B*	Unscrambler	PLS2	TS	400-1000	none	0	0	1	115	12	5	0.7410	2.7253	0.8507	2.1502
Unk*	Unscrambler	PLS2	TS	400-1000	none	0	0	1	115	12	5	0.8851	3.2431	0.9099	3.1183
L*	Unscrambler	PLS2	CV	400-1000	none	0	0	1	115	12	4	0.8902	4.0026	0.8806	4.1647
A*	Unscrambler	PLS2	CV	400-1000	none	0	0	1	115	12	4	0.8911	1.4724	0.8774	1.5570
B*	Unscrambler	PLS2	CV	400-1000	none	0	0	1	115	12	4	0.7797	2.5367	0.7583	2.6433
Unk*	Unscrambler	PLS2	CV	400-1000	none	0	0	1	115	12	4	0.8977	3.1147	0.8874	3.2599
L*	Unscrambler	PLS1	TS	400-1000	none	0	0	1	115	12	4	0.9455	3.0630	0.8958	4.1941
A*	Unscrambler	PLS1	TS	400-1000	none	0	0	1	115	12	4	0.8534	1.6678	0.8598	1.6341
B*	Unscrambler	PLS1	TS	400-1000	none	0	0	1	115	12	4	0.8867	2.1978	0.8346	2.7372
Unk*	Unscrambler	PLS1	TS	400-1000	none	0	0	1	115	12	4	0.9505	2.5335	0.9466	2.4003
L*	WINISI	PLS1	CV	400-2500	none	0	0	1	135	3	5	0.9193	3.0002	0.9103	3.1507
A*	WINISI	PLS1	CV	400-2500	none	0	0	1	137	1	5	0.854	1.2321	0.8156	1.3851
B*	WINISI	PLS1	CV	400-2500	none	0	0	1	134	4	5	0.8432	1.9409	0.8141	2.1059
Unk*	WINISI	PLS1	CV	400-2500	none	0	0	1	133	5	5	0.9275	2.2622	0.9227	2.3279
L*	WINISI	PLS1	CV	400-1098	none	0	0	1	134	4	7	0.9464	2.4434	0.9336	2.7107
A*	WINISI	PLS1	CV	400-1098	none	0	0	1	136	2	7	0.867	1.2041	0.833	1.3501
B*	WINISI	PLS1	CV	400-1098	none	0	0	1	132	6	7	0.8574	1.7756	0.8179	2.0017
Unk*	WINISI	PLS1	CV	400-1098	none	0	0	1	132	6	7	0.9324	2.1417	0.9147	2.3975
L*	WINISI	PLS1	CV	400-1098	none	0	1	1	134	4	3	0.9359	2.5334	0.9344	2.5619
A*	WINISI	PLS1	CV	400-1098	none	0	1	1	135	3	3	0.8659	1.157	0.8613	1.1778
B*	WINISI	PLS1	CV	400-1098	none	0	1	1	133	5	3	0.8946	1.4793	0.8678	1.6547
Unk*	WINISI	PLS1	CV	400-1098	none	0	1	1	135	3	3	0.91	2.3902	0.911	2.3769
L*	WINISI	PLS1	CV	400-1098	none	1	4	4	136	2	6	0.8967	3.3953	0.852	4.0515
A*	WINISI	PLS1	CV	400-1098	none	1	4	4	137	1	6	0.8948	1.0554	0.8335	1.3268
B*	WINISI	PLS1	CV	400-1098	none	1	4	4	135	3	6	0.7898	2.2236	0.7701	2.3181
Unk*	WINISI	PLS1	CV	400-1098	none	1	4	4	137	1	6	0.8829	2.8672	0.8379	3.3645
L*	WINISI	PLS1	CV	400-1098	none	0	4	4	134	3	3	0.9273	2.6908	0.9268	2.7
A*	WINISI	PLS1	CV	400-1098	none	0	4	4	137	1	3	0.8716	1.1287	0.8539	1.203
B*	WINISI	PLS1	CV	400-1098	none	0	4	4	133	5	3	0.8789	1.5863	0.8486	1.7708
Unk*	WINISI	PLS1	CV	400-1098	none	0	4	4	134	4	3	0.9122	2.3647	0.9134	2.3488
L*	WINISI	PLS1	CV	400-1098	snv; Detrend	0	1	1	134	4	8	0.9419	2.4137	0.9295	2.6564
A*	WINISI	PLS1	CV	400-1098	snv; Detrend	0	1	1	135	3	8	0.8448	1.2438	0.8343	1.2871
B*	WINISI	PLS1	CV	400-1098	snv; Detrend	0	1	1	134	4	8	0.8729	1.7098	0.8477	1.8709
Unk*	WINISI	PLS1	CV	400-1098	snv; Detrend	0	1	1	135	3	8	0.9101	2.3903	0.8987	2.535
L*	WINISI	PLS1	CV	400-1098	snv; Detrend	1	4	4	136	2	6	0.895	3.2183	0.87	3.5797
A*	WINISI	PLS1	CV	400-1098	snv; Detrend	1	4	4	136	2	6	0.8684	1.1517	0.8331	1.297
B*	WINISI	PLS1	CV	400-1098	snv; Detrend	1	4	4	134	4	6	0.8047	2.0669	0.7969	2.1046
Unk*	WINISI	PLS1	CV	400-1098	snv; Detrend	1	4	4	137	1	6	0.8655	2.9133	0.8406	3.1695
L*	WINISI	PLS1	CV	400-1098	msc	0	1	1	133	5	6	0.9191	2.8461	0.9145	2.9245
A*	WINISI	PLS1	CV	400-1098	msc	0	1	1	137	1	6	0.8543	1.2191	0.8122	1.3835
B*	WINISI	PLS1	CV	400-1098	msc	0	1	1	137	1	6	0.868	1.6943	0.8531	1.7867
Unk*	WINISI	PLS1	CV	400-1098	msc	0	1	1	135	3	6	0.8814	2.7391	0.8788	2.7685



Appendix 2: Histograms showing distribution of parameter quantities for preliminary NIR calibrations: a) crude protein; b) fat; c) AME; d) aspartatic acid; e) threonine; f) serine; g) glutamic acid; h) proline; i) glycine; j) alanine; k) valine; l) methionine; m) isoleucine; n) leucine; o) tyrosine; p) phenylalanine; q) histidine; r) lysine; s) arginine.