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Hardness Methods for Testing Maize Kernels

GLEN FOX[†,§] AND MARENA MANLEY*[,§]

[†]Department of Primary Industries and Fisheries, Queensland Grain Research Laboratory, P.O. Box 2282,
Toowoomba, Queensland 4350, Australia, and [§]Department of Food Science, Stellenbosch University,
Private Bag X1, Matieland (Stellenbosch) 7602, South Africa

Maize is a highly important crop to many countries around the world, through the sale of the maize crop to domestic processors and subsequent production of maize products and also provides a staple food to subsistance farms in undeveloped countries. In many countries, there have been long-term research efforts to develop a suitable hardness method that could assist the maize industry in improving efficiency in processing as well as possibly providing a quality specification for maize growers, which could attract a premium. This paper focuses specifically on hardness and reviews a number of methodologies as well as important biochemical aspects of maize that contribute to maize hardness used internationally. Numerous foods are produced from maize, and hardness has been described as having an impact on food quality. However, the basis of hardness and measurement of hardness are very general and would apply to any use of maize from any country. From the published literature, it would appear that one of the simpler methods used to measure hardness is a grinding step followed by a sieving step, using multiple sieve sizes. This would allow the range in hardness within a sample as well as average particle size and/or coarse/fine ratio to be calculated. Any of these parameters could easily be used as reference values for the development of near-infrared (NIR) spectroscopy calibrations. The development of precise NIR calibrations will provide an excellent tool for breeders, handlers, and processors to deliver specific cultivars in the case of growers and bulk loads in the case of handlers, thereby ensuring the most efficient use of maize by domestic and international processors. This paper also considers previous research describing the biochemical aspects of maize that have been related to maize hardness. Both starch and protein affect hardness, with most research focusing on the storage proteins (zeins). Both the content and composition of the zein fractions affect hardness. Genotypes and growing environment influence the final protein and starch content and, to a lesser extent, composition. However, hardness is a highly heritable trait and, hence, when a desirable level of hardness is finally agreed upon, the breeders will quickly be able to produce material with the hardness levels required by the industry.

KEYWORDS: Maize; hardness; hardness methods; protein; starch

INTRODUCTION

Maize (*Zea mays* L.) is the largest crop produced internationally, at ca. 700 million tonnes per annum, and is grown in most countries. It was first cultivated in South America, but soon after spread around the globe after it was discovered by 16th century explorers. Maize or corn, as it is also often referred to, is a member of the grass family (Poaceae) and belongs to the Tripsaceae tribe of grasses. It is more related to sorghum and some millet species than the other economically important grass crops such as wheat and barley.

Maize is used for human food as well as animal feed. It has a higher level of starch and provides a source of protein higher in lysine compared to the winter cereals. More recently its high starch content has been of interest in the fuel (ethanol) manufacturing sector, resulting in debate on the use of grain for food versus fuel.

Maize is used as a food source in most countries around the world and grown in most of those. A number of processed foods are produced from maize, including breads, tortillas, corn chips, breakfast cereals, and snack bars. In poorer developed countries, where maize is a staple and produced by subsistance farming practices, it undergoes limited processing and would be used in flat-style breads and doughs and porridges or for alcohol production. For commercial production, maize can undergo either dry or wet milling to produce specific end-products. Maize hardness has been shown to have an influence on the efficiency of production or quality of the final product. Numerous methods have been devised and used to evaluate hardness, and some of these have been used to compare hardness with products produced from maize, irrespective of maize type, that is, flint, dent, or popcorn. This review will explain the relationship

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*Author to whom correspondence should be addressed (telephone: +27 21 808 3511; fax: +27 21 808 3510; e-mail: mman@sun.ac.za).

between a range of hardness methods and the biochemical aspects of maize.

STRUCTURE OF MAIZE

As one of a few C4 plants of economic importance, maize is a summer cereal and produces a low number of tillers or stalks, which develop cobs containing the kernels. Maize kernels are quite large relative to those of other cereals but similar in composition in that the most dominant component is the starchy endosperm. The endosperm accounts for >80% of the physical kernel structure. Within the endosperm, individual compartments (cells) contain the starch as well as storage proteins and lipids. These compartments have walls, which are made up of nonstarch polysaccharides (β-glucan and arabinoxylan), protein, and phenolic acids.

Maize kernels also have a germ, as well as outer layers (pericarp and aleurone) surrounding the starchy endosperm. The tissues in these outer layers are alive, unlike the endosperm, and provide important metabolic systems for the protection of the kernel against pathogens and insects. In addition, these tissues drive the biochemical processes to degrade the endosperm when the kernel is required to germinate. Numerous publications and book chapters describe the structure and functions of these tissues. However, these tissues play no role in the endosperm hardness and texture and will not be discussed in this paper.

The physical shape of maize and the structures of the individual components have been shown to affect maize hardness. Maize can be short and round (popcorn) or long and flat, with an oval, distal end (flint), or short and flat with a dented distal end (dent). In general, flint and dent types of maize are more common, with dent maize being softer than flint maize. However, whereas the physical shape and size of flint or dent maize affect hardness, the interesting characteristic of maize is that there can be both soft and hard endosperm within a single kernel.

The endosperm is the main internal structure and contributes significantly to hardness. As mentioned, starch and protein are the most abundant components, and both of these components affect hardness. However, although protein is much lower in proportion than starch, it plays the major role in final physical hardness. This will be discussed in more detail later.

IMPORTANCE OF HARDNESS IN CEREALS

Hardness is a term usually used by the wheat industry. Hard wheats generally are used in the production of breads, whereas soft wheats are used for cakes and biscuits. Numerous studies have been carried out on wheat hardness and its relationship to quality end-uses (*1*−*18*). Detailed genetic studies have also been carried out [see reviews by Morris (*9*, *19*)]. A limited amount of work has been carried out on the effect of hardness on quality in barley (*20*−*24*), triticale (*25*−*27*), durum (*19*, *28*−*30*), and sorghum (*31*−*34*). However, at this stage wheat is still the main industry that classifies new cultivars on the basis of hardness.

The literature has numerous publications describing physical and biochemical aspects of maize hardness. Maize does not have hardness genes that transcribe into a single protein which is related to hardness, unlike wheat and barley, in which hardness has been linked to a specific gene family with allelic variation related to variability in hardness and quality (*19*, *20*). In terms of maize physical characteristics, kernel size and shape, weight and density, and resistance to milling and compression have all been linked to hardness and subsequent effects on processing. Most of these methods are destructive and provide variable information on the range of hardness from a maize sample. At this stage, there is no published data using a single kernel testing methodology for maize, whereas for wheat and barley, the single kernel characterization system (SKCS) has been shown to be suitable for determining hardness and providing an indication of quality (*21*, *35*).

HARDNESS IN MAIZE AND RELATED BIOCHEMICAL FACTORS

Protein. The storage protein in cereals is named prolamin, after the high *prol*ine and glut*amin*e content found in these proteins. Unlike members of the Triticeae family, maize has a relatively low level of proline and glutamine. The storage protein in each cereal has been given a certain name to easily identify the specific storage protein families, and for maize the prolamin fraction is called zein. However, compared to the prolamins of the Triticeae family, the members of the zein family have quite a different protein structure. In maize and sorghum, the protein exists in a layered ball-like structure or body, instead of forming a matrix, linked by covalent or molecular amino acid bonding as is the case for wheat, barley, rye, durum, and triticale. These protein bodies adhere to the starch granule. Abdelrahram and Hoseney (*36*) proposed chemical bonding rather than physical attachment to the starch granules (as one theory for wheat), similar to second theory for wheat whereby water-soluble material links protein to starch.

With regard to the biochemical contribution to hardness in maize, both protein and starch composition have been associated with maize hardness. Protein in maize comprises around 8−12% of the total composition. Protein content has been correlated to hardness, and the variation in zein classes has been linked to differences in hardness (*32*, *37*−*51*). For most of these traits, the best correlations have been shown in samples gathered from larger sample sets grown over multiple sites and years. When samples have been collected randomly, the protein hardness relationship does not always fit. Dorsey-Redding et al. (*52*) developed a regression equation using protein, with oil and hectoliter weight. This was based on two seasons' samples with over 180 samples per season.

Although the protein content comprises a very low proportion of the total kernel composition, it would appear that it does play a significant role in influencing hardness; however, work by Mestres et al. (*53*) suggests that protein was not linked to hardness, based on a small number of samples from four sites, not grown in a designed trial. It could be considered that the type of hardness test may be influenced by protein, and some tests could be more influenced by the actual endosperm structure, thereby giving a stronger correlation to protein content. At this stage very few studies have carried out multiple hardness tests and link the results to protein content.

The possible effects of individual zein classes on maize hardness are not unique within the context of variation in cereal prolamin content or composition affecting quality. Variation in barley prolamins has an effect on malt quality, and variation in the kafirin profiles protein composition affect the digestibility of sorghum. These prolamin effects on maize are quite different from those of wheat, in which the control of hardness is a different mechanism. Although wheat protein content does have an effect on hardness, wheat hardness is not related to wheat protein composition.

Zeins. Four classes have been identified within the zein storage protein. These classes have been named alpha (α), beta (β), gamma (γ), and delta (δ). The size and composition of these classes differ. In regard to size, the apparent molecular masses of these are α, M_r 16 and 27 kDa; β, M_r 14 kDa; γ, M_r 22 kDa; and δ, M_r 10 kDa (*43*). These proteins are similar to sorghum kafirin proteins, but much smaller than most of the storage proteins in other cereals such as wheat, barley, and rye. Amino acid sequence comparisons between the smallest fraction of the wheat and barley storage protein, gliadin, which is the fraction most similar to maize zein, have been carried out (see more detail below).

When the zein classes were separated on the basis of size and overall charge of the protein using reverse-phase high-performance liquid chromatography (RP-HPLC), two groups where identified, Z1 and Z2. The Z1 group contained the α- and δ-zeins, whereas the Z2 group contained the β and γ groups (*38*, *44*, *48*, *49*). A more recent technology called matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a more rapid technology than HPLC and provided more precise information on actual molecular masses of the zein proteins (*54*, *55*).

The results from a number of studies show some homology between maize zein and other cereal species. β-, γ-, and δ-zeins have shown some homology to the gliadin classes from other cereals, whereas α-zein has shown homology to only Panocoid families (sorghum and some millets). Skerritt and Lew (*56*) demonstrated a low level of homology between zein proteins and prolamins from wheat, barley, rye, oats, and rice using an enzyme-linked immunosorbent assay (ELISA) developed from a wheat gliadin monoclonal antibody. However, studies using RP-HPLC as a protein separation technique (protein separated on size and overall charge) have shown very low levels of homology between maize zein and these cereal species. On the other hand, sorghum kafirin prolamins have been shown to have a close homology to maize. These results would suggest that extraction (*57*) and separation techniques have a strong effect on the final protein extracted and subsequent amino acid composition analysis.

A number of reports have described in some detail the composition and possible role of the individual zeins [reviews by Shewry et al. (*58*−*60*)]. The most important change in the presence of zeins was the development of opaque mutants. These mutants resulted in the decrease or disappearance of the α-zeins with equivalent increases in γ- and δ-zeins. In addition, there were increases in lysine in nonstorage proteins. These mutants resulted in a much softer endosperm with lower protein content. However, the introduction of high-protein, soft endosperm hybrids called Quality Protein Maize presented breeders with the opportunity to select for a range in protein or hardness, which would be targeted at specific end-uses. More is discussed on breeding later in this review.

In most studies, a limited number of cultivars have been studied for the amino acid composition of zeins. There have been obvious varietal effects with the expected agronomic or environmental effects on amino acids and zeins. Maize, like most cereals, is low in the essential amino acids, methionine, tryptophan, and lysine. Targeted breeding has improved nutritional properties with increases in these amino acids, in particular lysine. The sequences of outer zein (γ) could be important in binding zein to starch. Increase in the methionine level, particularly in the γ- and δ-zeins, has been shown to increase hardness. Early work by Phillips et al. (*61*) suggests that through simple inbreeding with suitable parents, it was possible to increase the level of methionine in maize, with a strong increase in the δ-zein fraction. Swarup et al. (*62*) mapped a δ-zein from wild germplasm with high methionine levels as well as high lysine and tryptophan; however, no possible effect on function or impact on texture was postulated.

Lipid Transfer Protein (LTP). In addition to having some homology to gliadins from other species, there is a possibility that zein peptides may also have some homology to a LTP. LTPs are ubiquitous in the plant world. Within plants, they exist in the growing plant tissues, and within the grain they are present in the germ, aleurone, and endosperm tissue. This protein has also been identified as a possible 14-3-3 binding protein (*63*) or protease inhibitor (*64*, *65*) and may be associated with plant defense (*63*, *66*, *67*).

LTPs have been linked to hardness in other species such as barley (*68*, *69*). These proteins are heat stable and survive heating processes such as malting and brewing and, specifically in the case of barley, are linked to beer foaming properties (*70*). They are also associated with foam stability in breadmaking (*71*). The 9 kDa LTP identified in maize has been identified as an allergen to humans (*72*, *73*). LTPs from other species have also been identified as human allergens (*74*, *75*) and are present in many raw and processed foods.

Starch. The starch has two forms, being amylose (25%) and amylopectin (75%), and exists within a spherical granule. Genetic variants can have either 100% amylopectin (waxy) or 100% amylose (high amylose). The high level of starch makes maize suitable for a range of end-uses including human food and animal feed. Starch is the primary source of energy from cereals, although protein and lipids also contribute to the total energy of grains. Starch composition and structure can be influenced by growing conditions with extreme temperatures or severe moisture stress, causing changes in structure through an effect on starch-forming protein such as starch synthases and starch-debranching enzymes (*76*, *77*).

Starch makes up ca. 80% of the maize kernel and comprises two components, amylose and amylopectin (see above). Amylose and amylopectin are relatively simple structures and behave in a more predictable way, being made up entirely from glucose. This is unlike protein, where there are numerous copies of the 20 amino acids that each have unique characteristics and properties. Despite the simple structure of starch, there is some association between maize starch and kernel hardness. An earlier review by Mestres and Matencio (*45*) summarized previous studies showing how variation in amylose content correlated to changes in kernel hardness. Subsequent studies have highlighted the effect of amylose content as well as starch granule size and their relationships to hardness. Although there was variability of amylose content and starch granule size within the grain, generally grains with higher amylose content and larger starch granules in the endosperm were softer (*29*, *32*, *78*−*82*). Genetic variation in starch content and composition, induced by genetic mutations, can increase our understanding of that effects of changes in starch on hardness (see below).

Genetics contribute a significant proportion of the final composition of kernel quality, but the growing environment also plays a role and starch, like protein, is affected by growing conditions. Maturity, grain fill temperatures, and available moisture all affect starch content and granule size and shape—which can in turn affect kernel hardness (*81*, *83*). As in other cereals, temperature during grain fill can affect both starch formation pathways, that is, granule bound starch synthesis for amylopectin and starch synthase for amylose. In addition, genetic mutations of any of the enzymes in either of these pathways could affect amylopectin or amylose production and as a consequence affect hardness.

Some recent research has reported relationships between starch properties and hardness. The most recent technology to be used has been the Rapid ViscoAnalyser (RVA). This technology provides a number of starch-related traits with peak viscosity and peak height most related to hardness. A number of starch mutants, including high amylose (amylose extender gene *ae*) and high amylopectin (waxy gene *wx*) have also been reported and their impact on maize hardness has been examined, with both mutant types producing variation in kernel texture. A comparison of normal, waxy (*wx*), and high amylose (*ae*) lines showed differences in vitreousness, suggesting some role of starch structure on endosperm texture (*84*).

Cell Walls. Within the endosperm, the starch and protein are housed inside block-like structures called endosperm cells. These cells have walls that are composed of nonstarch polysaccharides (NSP), namely, β-glucan and arabinoxylan, as well as a small amount of protein. To date, the impact of the cell wall on hardness has been shown in barley; samples with an increased hardness as measured by testing the resistance to crushing single kernels, using the SKCS, also had higher total β-glucan (*21*), higher molecular mass β-glucan (*85*), or β-glucan that showed decreased solubility (*85*). In wheat, the effect of the outer layers, also containing the same NSP but in differing proportions, also exhibited variation in hardness—which was related to the level of these NSP.

In maize, there is a very low level of NSP in the endosperm cell walls as well as the aleurone cell walls. At this stage, there has been no work demonstrating any effect of maize NSP on hardness. However, it cannot be ruled out that, although the level of these components may be very low, there could still be some contribution to the hardness.

EFFECT OF ENVIRONMENT ON HARDNESS

The growing environment of any agricultural crop has a major impact on final quality. For cereal crops, the soil nutrient profile, available moisture, and environmental conditions prior to and during grain filling can influence starch and protein content and composition. This is also the case for maize kernel quality and, as discussed previously, because both protein and starch can influence maize hardness, this trait was influenced by growing environment. A number of researchers have highlighted the effect of preplanting fertilizer treatments, available water, diurnal temperature effects on grain size, and starch and protein synthesis as well as crop maturity. Nitrogenous fertilizers have a major impact on final protein content. As a consequence, increase in protein can be linked to an increase in vitreousness and hardness (*76*, *83*, *86*−*91*). Robutti and co-workers (*39*, *48*, *49*, *92*, *93*) have provided some of the most detailed genotype by environment (G×E) studies on maize hardness using hybrids, landraces, and wild maize species grown in Argentina with both environmental and genetic effects. The ranges in hardness as measured by a number of methods show the broad range in hardness that could be introduced through the use of landraces or wild types.

In field trials, within-field variation even within a small microenvironment can affect protein content and, as a consequence, kernel hardness (*94*). Kelly et al. (*95*) and Smith et al. (*96*, *97*) have highlighted the benefits of modeling for within and between environmental variation to ensure all variances are accounted for and error from field and any measurements of the samples is minimized, thereby calculating true genetic variance.

Any field environment is subjected to a range of temperatures during the growing season. Excessive high or low temperature can affect grain filling characteristics, which then affect starch and or protein synthesis (*76*, *77*).

A sound understanding of the environmental component contribution to maize hardness would provide important information to industry personnel in developing cultivars and growing commercial crops so as to optimize yield for growers and have the right quality for specific end-use markets. A number of studies have been conducted using maize samples gathered randomly, which then attempted to show variation in hardness; although these may have been successful in showing the possible range in hardness based on a particular methodology, or for a specific end-use, the variation in hardness could not be explained by any environmental effects (*53*, *98*, *99*).

BREEDING

For thousands of years, man has carried out selection of crop seeds with the aim of improving the following years' cropping yield and/or quality (grain size). Over the past 100 years, specific breeding efforts have targeted economically important crops. Maize, like most economically important cereals, was domesticated from a wild grass (*100*). Most countries growing maize have breeding companies developing cultivars that will perform best under those countries' growing environments and meet the end-use requirements of the consumer. In terms of breeding for hardness, it is known there are varietal (genetic) effects. However, more recent breeding efforts have introduced a range of germplasm and cultivars that carry mutations which affect hardness.

Opaque mutants first described in the 1960s (*101*) have been shown to affect maize hardness by producing a softer endosperm, with reduced protein and increased lysine content (*38*, *40*, *46*, *47*, *79*, *102*−*105*). The biochemical effect of these *opaque*-2 mutations resulted in changes in zein content, which affects hardness. The α-zein was not expressed, and there were increased levels of γ- and δ-zein.

The most recent development has been the breeding of cultivars termed QPM, which carry the recessive *o*2 gene and modifier *opaque* genes as well as *floury* genes, which produce a hard vitreous endosperm (*87*, *89*, *102*, *103*, *105*−*107*). A number of studies have explored these modifier genes, including *o*5, *o*7, and *o*11, which produce a harder endosperm (*108*). Whereas a number of mutants resulting in changes in the zein groups result in changes in hardness, changes in amino acid composition have also been shown to result in changes in hardness (or vitreousness) (*105*, *109*). Additional information has been presented showing that the variation in γ- and δ-zein amino acid sequence in various opaque mutants, in particular with increases in methionine, affect these zeins. The increase in this sulfur amino acid affects the disulfide binding, resulting in a stronger protein structure and thereby possibly increasing hardness (*110*, *111*), especially if a change in γ could affect binding. However, some of these studies have measured zeins but not always measured total protein content or hardness. Zhang et al. (*51*) transformed barley with a γ-zein but found no change in hardness or vitreousness, whereas Gutierrez-Rojas et al. (*109*) studied a number of opaque mutants as well as their corresponding zein amino acid sequences. The information was linked to the level of vitreousness. However, the effect on total protein content was not reported. Some additional important information presented was the high level of genetic and phenotypic correlations and heritability. These data suggested both zein content and composition were heritable traits and could be selected for through appropriate crossings. These results supported a previous study in which specific breeding efforts increased hardness as measured by a density test (*112*).

As mentioned above, the results of breeding efforts have provided variation in amino acid sequence of zeins, with high lysine, methionine, or tryptophan. These changes have had an impact on total protein content and hardness. It has been shown in wheat and barley that allelic variation in the hardness genes resulted in variation in protein sequence and hence hardness variation (*9*, *20*, *113*). However, maize is not like wheat and its close relatives in terms of having a hardness locus, but has multiple loci associated with zein protein and other protein families. A number of quantitative trait loci have been linked to a range of hardness measures, including vitreousness. Some QTLs were associated with protein and or zein (*114*−*117*).

A number of studies have used the same hybrid lines, in particular B73, A64, or Mo17 and their respective mutants, either as individuals or in a number of crosses with other mutants or wild types (*77*, *104*, *107*, *109*, *112*, *118*). These studies have shown that these hybrids provide excellent variation in endosperm texture and hardness, as well as associated variation in zein composition.

There have also been a number of other mutant lines tested for variation in starch composition based on a number of single- or double-mutant combinations (*119*). These mutants include amylose extender, sugary and waxy. Whereas the inbred lines and hybrids gave variation in starch composition and content, they were not tested for hardness. It could be assumed that such variation in starch would result in variation in hardness on the basis of previously published literature stating such effects.

A number of studies investigated the effects of *opaque* mutants in a breeding population using diallele crosses and subsequent effects on hardness (*103*). The use of the diallele crosses identified additive effects for hardness characteristics, with possible epistatic effects (*91*, *103*, *120*).

PROCESSING

Processing is influenced by hardness, and depending upon the processing method and the end-product being produced, different hardness types may be required. In many countries, a dry-milling process is used for the production of maize meal and other milled products. This process usually requires a large, hard kernel. The maize is run through a series of mills with various roller gaps, and different products are produced from these various particle sizes. Softer kernels can reduce efficiency in the extraction yield.

Breakage is an important consideration when maize is received and processed. Increased breakage during harvesting and transferring of grain, possibly due to softer kernels, has the potential to reduce storage time and processing efficiency. Softer kernels are more prone to cracking and fracturing. Increased cracked, split, or broken kernels can have the potential to reduce storage conditions, through increased moisture uptake and greater insect and mold presence on those kernels. In addition, softer kernels can reduce milling yields and milling efficiency. Two main tests have been used to measure breakage and its relationship to hardness. The main breakage tester is the Wisconsin Breakage tester, with a number of reports detailing the effects on hardness prior to and after being tested in the breakage tester (*121*−*123*). A number of factors affect breakage including temperature, moisture, genetics, growing environment, and hardness.

As mentioned above, variation in kernel texture can affect the handling ability of the kernels. The condition of the kernels prior to storage as well as storage conditions may also affect the final kernel texture. One study showed through accelerated aged storage changes in endosperm texture when soft, floury endosperm became harder (*124*).

The current literature indicates that hardness has a strong influence on the processing properties of maize. Hardness and a number of other parameters such as moisture, protein, and breakage susceptibility should be known prior to storage and processing.

HARDNESS TESTS

There have been a range of tests used over the past 50 years to determine the hardness of maize. These include measuring resistance to grinding, abrasion, yield of grits, and starch gelatinization properties as well as grinding followed by sieving and the amount of throughs being determined. In terms of the use of near-infrared (NIR) spectroscopy, both reflectance and transmission modes have been used to various levels of success.

The early work by Tran et al. (*125*) showed a number of grinding and pearling methods using a single sample, tempered to different moisture contents. The moisture content had a major impact on the hardness results due to a softening of the endosperm. A number of tests also report on the effect of various moisture levels on hardness. A summary of these tests including ranges for these tests is provided in **Table 1**.

Particle Size Index (PSI). One of the most common methods used to determine maize hardness has been to mill the sample and then fractionate the ground material through sieves. This method is referred to as the PSI method (*29*, *36*, *126*−*128*) and was first used to determine wheat hardness (*129*). There has been little consistency in earlier research in terms of type of grinding mill, mill sieve size, or whether a single or combination of separating sieve sizes was used, for example, 75, 150, 210, or 300 μm (or the equivalent in empirical sizes). The selection of grinder sieves would be dependent upon the type of milling process, that is, hammer versus disk, but more importantly on the size of the holes in the sieve that the grinder passes the particles through, for example, <1, 1, or 2 mm or large holes (*99*, *130*−*133*). One of the simplest procedures was carried out by Abdelrahman et al. (*36*), who used a single sieve (150 μm) and related hardness to chemical treatments. Nonaqueous solvents reduced the hardness, which suggested a different effect of binding of the zein to the starch granules than the protein matrix and wheat (*36*).

The PSI method has a number of benefits, in that if multiple sieves were used, then some information could be gained on the variation of hardness within a sample as well as calculating an average particle size. In addition, the ratio between larger particle sizes and smaller ones can be calculated, thereby giving a coarse/fine ratio, with a higher number indicating harder samples. In addition, the various grain fractions could be used to study endosperm structure and composition, which affect hardness. No other test provides a range of endosperm fractions, as most used a method by which the milled sample is collected as a single sample.

Stenvert. The Stenvert test was introduced by Stenvert for wheat hardness in 1974 (*134*) and then used with a great deal of success by Pomeranz during the 1980s to show variation in maize hardness. This test timed the period for a 17 mL tube, attached to a grinder (sometimes with differing sieves), to fill with ground material, thereby relating hardness to resistance to grinding. A shorter period would suggest the kernels were softer (*122*, *123*, *135*−*137*). Similar to the PSI method, this method could give only an indication of the total hardness of a sample and not provide any information on possible variation within kernels.

Mestres et al. (*130*) proposed classifications of hardness based of the friability of maize using a modified Stenvert test. The modification was based on the addition of different sieves and computer-controlled data capture to report parameters such as energy usage and time of grinding. These authors also suggested a classification system of four grades based on 18 cultivars that were collected from four regions in Africa. Although there was discussion of the diversity of the cultivars used, the study was potentially limited by the low number of cultivars and the localities. A structured G×E study may provide a greater range in the friability values reported and hence alter the classification grades.

Density. An interesting alternative approach was to assess the density of maize kernels (*47*, *79*, *83*, *86*, *90*, *112*, *131*, *136*−*143*). This was carried out by two methods. One method was to count the number of maize kernels that floated in a solution. Different solutions were used, including sodium nitrate, sucrose, kerosene−carbon tetrachloride, and ethanol, all at various specific gravities,

Table 1. Descriptive Summary of Possible Ranges for Hard and Soft Maize Using Various Hardness Techniques[a]

	Stenvert[b]					density[f]				PSI[i]	
	time (s)	height (mm)	TADD[c]	NIR[d], 1680 nm	RVA[e] peak viscosity (RVA units)	floaters (%)	pyc (g/mL)	vit[g] (%)	comp[h]	APS	<150
soft	low (10)	high (200)	high (70)	low	high (700)	high	low (1.25)	low (30)	low	low	high
hard	high (20)	low (150)	low (20)	high	low (400)	low	high (1.40)	high (100)	high	high	low

[a] A list of most methods used to provide an indication of maize hardness with a descriptive low or high to indicate where soft or hard maize would fit, as well as some indicative values. [b] Stenvert—based on time to mill a fixed amount of maize and the height of the flour in the collection tube. [c] Tangential abrassive dehulling device. [d] Near-infrared reflectance at 1680 nm. [e] Rapid ViscoAnalyzer peak viscosity—only one of a number of RVA traits that could be used. [f] Denisty—two methods, the first being a method measuring the number of floating kernels (this method varies according to the solution used) and the second using a pycnometer to measure the volume of liquid displaced by the kernels. [g] Vitreousness—percent of kernel considered to be nontransparent. [h] Compression tests—where a kernel is crushed and the resistance to crushing to determined. [i] Particle size index—a number of variations to this method have been used, mostly depending upon the sieve size used. For this we have shown average particle size and % flour <150 m sieve as two possible hardness indicators.

although a solution of sodium nitrate at a specific gravity around 1.250 g/mL was most common. A higher number of floating kernels indicated the samples have a greater number of softer kernels.

An alternative method was to measure the density based on the mass of kernels in a defined volume. This method used a pycnometer, and lower densities were considered to be softer. This method is dependent upon the moisture content of the kernels, and all samples should be at a similar moisture content prior to the test.

Tangential Abrasion Dehulling Device (TADD). The TADD entails a process where kernels are abraded for a defined period of time. The amount of material removed from the kernel is calculated, with higher values indicating softer kernels. This method has been used in a few studies and compared to other milling tests as well as the maize starch pasting properties (*131*, *137*, *143*). Similar to all previously discussed methods, the TADD process uses a bulk sample and provides no data on the variation within a sample.

Rapid ViscoAnalyser (RVA). The RVA is the only method that relates biochemical components to hardness. The RVA is a technique that uses ground material, mixed with water and constantly stirred while the sample is heated to 100 °C. The method provides information on starch properties, including paste viscosity, gelatinization temperature, and time. This information has been related to maize hardness (*80*, *81*, *131*, *144*) as the RVA can measure variation in amylose content and starch granule characteristics that have been shown to contribute to maize hardness (see above). This technique, like the PSI and Stenvert methods, requires a grinding step. It also takes >10 min for the RVA to complete the pasting of the samples. There is a large amount of data generated, which would require a trained operator to interpret. This method does provide good qualitative data on maize starch (and, to a lesser extent, protein) properties but would not be suitable as a routine method for determining hardness.

Roff Milling Index. In South Africa, a novel process is used to ascertain the level of maize hardness. A method referred to as the Roff milling index or milling index (MI) is used (*145*). With this method the MI is calculated from the meal and bran fractions obtained from milling a sample through a roller mill system. A maize sample (preconditioned to 14% moisture) is milled through three rollers with gaps of 0.3, 0.38, and 0.08 mm. The method provides results adequately robust to distinguish clearly between maize cultivars of different hardnesses and to identify cultivar and environmental effects of maize hardness. The MI method has then been used to develop calibrations for a whole grain near-infrared transmission instrument (Foss Infratec 1251) operating in the 800−1100 nm wavelength range. No data are available to show if this study investigated any G×E or protein effects on the hardness values.

Compression Tests. Another method to measure hardness and one of the few that has the potential to measure single kernels (similar to for the SKCS for wheat) is the compression test. This test relies on a resistance measure of single kernels when a rod is pressed into the kernel. This has been used in maize and shown to have relationship to hardness (*29*, *146*, *147*).

Indirect Measurements of Hardness: Near-Infrared (NIR) Spectroscopy. NIR in both reflectance and transmission modes has been used for over 20 years to estimate maize hardness. The applications of NIR spectroscopy have mainly been to demonstrate efficiencies in breeding selection or for ascertaining the hardness of samples prior to milling (*39*, *92*, *21*, *119*, *135*, *142*−*144*, *148*−*151*). The accuracy and precision of the reference method are critical to the development of any useful NIR calibration. To date, calibrations have been developed using the TADD (*143*, *144*), Stenvert mill (*135*, *144*), floaters method (*39*), kernel density (*142*), and coarse/fine ratio (*92*) as reference methods. In addition, some of these calibrations have used samples corrected to a common moisture content (*119*, *135*, *144*), whereas the remaining have used maize samples on an as-is basis (*39*, *92*, *142*, *143*). The use of a single wavelength (860 or 1680 nm) (*92*, *144*) as well as the maximum absorbance/reflectance between 620 and 680 nm has also been used to relate to hardness (*92*). The use of a single wavelength (1680 nm) is based on the early instrumentation in which filters were used, as opposed to current monochromator or band splitting technology. The wavelength was first correlated to wheat hardness, on the basis of a PSI method. This NIR wavelength was subsequently used as the basis for an AACC method (*152*). A number of recent reports have used scanning NIR instruments but still used only the 1680 nm wavelength to provide an indication on hardness. Of interest, because maize hardness is correlated to protein, the 1680 nm wavelength was not associated as a protein wavelength as this wavelength is more associated with particle size.

From the current literature the most common technology has been the NIR system operating in the spectral range from ca. 840 to 1080 nm in transmission mode. Most have used whole grain, which provides speed in analysis and also avoids a milling/grinding step. Only one reference provided data on the accuracy and precision of the calibration with better results for a milling method (Stenvert) than the TADD method (*143*). In addition, the calibrations applied have varied from fewer than 100 samples in the data set to over 1000 samples.

The value in any NIR calibration is for the range of the reference data to cover the values of any future unknown samples to be tested. If the unknowns will always fall into the calibration range, then little work will be required to update any calibration. However, and this is generally the case for breeding material, there will be location and harvest year effects that sometimes result in samples that fall outside the calibration range. Hence, yearly updates are then required.

As mentioned by Mestres et al. (*130*) at this stage, single kernel testing for maize hardness is somewhat impractical. However, very recent advances in hyperspectral imaging suggest that individual kernel hardness can be identified. There were a number of wavelengths (starch and protein) associated with hard or soft endosperm, although 1680 nm was not one of those identified by Williams (*153*). However, as there are no single kernel tests available for maize, there was no correlation to any hardness values.

The additional benefit of NIR is simultaneous estimation of protein and moisture, which both have major impacts on the processing of maize.

Vitreousness. One of the earliest methods to provide an indication of endosperm texture is vitreousness. Although not a direct measure of hardness, vitreousness provides a measure of the endosperm that is translucent. Studies on those areas of the endosperm that are translucent against the nontranslucent areas show differences in protein content and composition (zein fractions) (*29*, *40*, *41*, *45*, *51*, *88*, *98*, *99*, *103*, *109*, *115*, *120*, *132*, *146*, *154−180*). A number of studies have correlated other hardness measures with vitreousness with the more vitreous samples being harder than the less vitreous samples. In addition, studies have correlated the level of vitreousness to end-use quality (*88*, *132*, *158*, *166*, *168*, *169*, *175*, *176*, *179*). The measurement of vitreousness requires an image analysis system, with software that can precisely calculate the percentage of vitreousness in each kernel. This system has been shown to be useful for a number of other hulless cereals including durum (*155*) and wheat (*159*, *170*).

CONCLUSION

Earlier research into maize has uncovered important biochemical aspects in the relationship of protein content and composition to hardness. The presence of particular zein fractions affects hardness, with the absence of α-zein resulting in softer endosperm. Hardness has also been shown to be influenced by cultivar and environment, with both of these factors impacting hardness through affects on protein and/or starch. *Opaque*-2 mutants along with other mutations can affect hardness from variation in gene expression of particular protein or starch components. Breeders are aware of the genetic and environmental affects and can select for high-yield soft or hard types. However, testing for maize hardness has not been standardized, and as a result there is a considerable range in methodologies, some of which would be useful in breeding programs, although most do not reflect the actual commercial milling process. NIR spectroscopy has been shown to be an excellent surrogate for estimating hardness and will continue to play an important role in testing either in breeding programs or at receival. The current level of information on hardness as well as communication with breeding programs will assist in the development of cultivars that will ensure a strong maize industry in South Africa, supporting the food, feed, and fuel sectors.

There is an association between protein and hardness, although few studies have gone to the trouble of analyzing samples for protein content. In the future, measurement of protein content would continue to provide useful information on this relationship in normal hybrids and mutants as well as wild types. Conversely, when analysis on protein composition and amino acid composition has been carried out, few studies compared these with hardness, although it was not the objective of those studies. However, for future studies, a single hardness test combined with protein composition or amino acid sequence could provide useful information on the relationship between these important traits.

In addition, when hardness tests are performed, it would be helpful to select maize types with known differences, that is, opaque versus normal, with expected differences in hardness and from structured trials grown in multiple sites. This becomes more complex when commercial samples are tested, although the protein content and hardness need to be considered together even for samples used for commercial processing. Testing individual cultivars provides background information on underlying influences on hardness, so hectoliter weight, protein, and moisture would be useful data in any future maize hardness analysis. This also applies to breeding trials, wherein field variation as well as laboratory variation and processing order has been shown to affect final data. Most studies used only limited number of samples, sometimes with an expected hardness variation to demonstrate a new method, correlations between existing methods, or correlations to individual or multiple grain traits. In some cases a single sample was conditioned to differing moisture contents to demonstrate the effect of moisture on hardness, decreased breakage, or other physical measures.

To date, there is no single international standard method for maize hardness. A recent collaborative study suggested within- and between-laboratory variance was high for TADD and time-to-grind for the Stenvert, whereas there were maize hybrid and laboratory effects for dry and wet milling (*137*). These results suggest that there is still work to be carried out to standardize a method for maize hardness, with no one physical test more suited to providing hardness level than another.

ABBREVIATIONS USED

E, environmental; G, genetic; LTP, lipid transfer protein; MALDI-TOF MS, matrix-assisted laser desorption ionization time- of-flight mass spectrometry; MI, milling index; NIR, near-infrared; NSP, nonstarch polysaccharides; QPM, Quality Protein Maize; RP-HPLC, reverse-phase high performance liquid chromatography; PSI, particle size index; RVA, Rapid ViscoAnalyser; SKCS, Single Kernel Characterization System; TADD, Tangential Abrasion Dehulling Device.

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