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As the number falls, alternatives to the Hagberg–Perten falling number method: A review

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Abstract

This review examines the application, limitations, and potential alternatives to the Hagberg–Perten falling number (FN) method used in the global wheat industry for detecting the risk of poor end-product quality mainly due to starch degradation by the enzyme α-amylase. By viscometry, the FN test indirectly detects the presence of α-amylase, the primary enzyme that digests starch. Elevated α-amylase results in low FN and damages wheat product quality resulting in cakes that fall, and sticky bread and noodles. Low FN can occur from preharvest sprouting (PHS) and late maturity α-amylase (LMA). Moist or rainy conditions before harvest cause PHS on the mother plant. Continuously cool or fluctuating temperatures during the grain filling stage cause LMA. Due to the expression of additional hydrolytic enzymes, PHS has a stronger negative impact than LMA. Wheat grain with low FN/high α-amylase results in serious losses for farmers, traders, millers, and bakers worldwide. Although blending of low FN grain with sound wheat may be used as a means of moving affected grain through the marketplace, care must be taken to avoid grain lots from falling below contract-specified FN. A large amount of sound wheat can be ruined if mixed with a small amount of sprouted wheat. The FN method is widely employed to detect α-amylase after harvest. However, it has several limitations, including sampling variability, high cost, labor intensiveness, the destructive nature of the test, and an inability to differentiate between LMA and PHS. Faster, cheaper, and more accurate alternatives could improve breeding for resistance to PHS and LMA and could preserve the value of wheat grain by avoiding inadvertent mixing of high- and low-FN grain by enabling testing at more stages of the value stream including at harvest, delivery, transport, storage, and milling. Alternatives to the FN method explored here include the Rapid Visco Analyzer, enzyme assays, immunoassays, near-infrared spectroscopy, and hyperspectral imaging.

KEYWORDS
falling number, hyperspectral imaging, immunoassay, near infrared, preharvest sprouting, α-amylase

1 | INTRODUCTION

Starch, proteins, and cell wall polysaccharides are the three major nutrition components of dry wheat. Wheat trading requires high standards for nutrition and end-use quality related to these components (Ross & Bettge, 2009). Grains are discounted when environmental stress causes a failure to meet receival standards, including starch degradation measured in the wheat industry using the Hagberg–Perten falling number (FN) method (He et al., 2019). Starch degradation is considered a major cause of poor end-use quality, because long, intact starch polymers provide the gelatinization and retrogradation needed for an acceptable product (Bettge, 2018; Finnie & Atwell, 2016). Starch goes through a thermodynamic physical change, known as pasting, when heated in the presence of water. As the semi-crystalline granules swell, hydrogen bonds dissociate, and the granules finally burst. Their constituent polymers of amylose and amylopectin can then interact with large quantities of water. This pasting event dramatically increases viscosity. However, the transformation also renders the starch polymers susceptible to cleavage by α-amylase, and starch cleavage decreases the viscosity. Loss of viscosity can result in problems with poor end-products by altering appearance and texture (Steber, 2017). Thus, α-amylase expression as a consequence of preharvest sprouting, the initiation of germination on the mother plant when rain occurs before harvest, can have a strong negative impact on end-product quality. Even at early stages of germination before the seedling emerges from the grain, α-amylase can be produced at high enough levels to reduce the end-use quality of wheat flour.

The FN method is valuable because it detects starch degradation when there is no visible grain germination (Perten, 1964). Although the FN method was originally developed to measure the activity of α-amylase in wheat and other cereal flours, meals, and malts to assess baking performance (Hagberg, 1960, 1961; Perten, 1964), the method was adopted as a means of assessing the degree of sprout damage. The method is straightforward but requires several specialized pieces of equipment including the FN machine itself, a grinder, a shaker, and an oven or other device for measuring moisture content. In preparation for the FN test, the grain is ground, and the moisture content is determined. The sample weight is adjusted to obtain 7 ± 0.05 g, assuming 14% moisture. The presence of higher or lower moisture is accounted for either by changing the gram quantity of meal in the assay (i.e., a lower gram quantity of meal is used if the moisture is <14%) (AACC, 1999) or using a correction equation after the assay is performed (FGIS Directive 9180.38, 2019). Twenty-five milliliters of distilled water are added, and the sample is dispersed by shaking by hand or by using a dedicated mechanical device. A plunger is placed in the tube and a second, duplicate tube is prepared in parallel, whereupon both are placed in the FN boiling water bath. The FN machine agitates the samples by moving the plunger up and down for 60 s. The plungers are released near the top of the tube, and the machine measures the time in seconds for each plunger to fall through the pasting wheat–water mixture under the force of gravity. The test is terminated when the plunger reaches the bottom, and the total time including the 60 s of stirring becomes the FN. Thus, the minimum FN is 60 s. The FN varies with elevation because the boiling point of water is dependent on the atmospheric pressure. Thus, the FN is corrected for barometric pressure (Delwiche, Rausch, et al., 2018).

Other methods that can detect pasting properties of wheat flour or meal include the Amylograph®, the Ottawa Starch Viscometer, and the Rapid Visco Analyzer (Balet et al., 2019; Ross & Bettge, 2009). The Rapid Visco Analyzer (RVA) was developed as a more accurate and informative alternative to the FN method (Ross et al., 1987). As originally designed, the RVA imitated the cooking process in the FN by stirring a ground meal–water mixture with a paddle at 96°C. The instrument reported a "Stirring Number" (SN) derived from the power required to maintain a constant paddle velocity. SN was approximately equal to 1 cP. SN is an Approved Method (22-08) of the AACCI. Like the FN, SN is affected by the action of α-amylase on starch pasting. The SN shows a high positive correlation with FN ($r^2 = .97$) (Ross et al., 1987). The RVA can also detect differences in the ratio of the two component macromolecules of starch, amylopectin, and amylose, thus making it more versatile than the FN instrument (Walker et al., 1988; Zeng et al., 1997). While the RVA has not widely replaced FN in the international wheat trade, it has been adopted for the wheat and barley trade in Australia and starch analysis in other cereal grains, including maize, barley, sorghum, and rice (Balet et al., 2019; Gelin et al., 2007). Although the RVA is more informative and accurate in measuring true viscosity than the FN method, it still requires milling of the sample and costly, specialized equipment.

The FN test is under increased scrutiny because it is costly, labor intensive, time consuming, requires specialized equipment and experienced practitioners, and can produce variable results (Bettge, 2018; Chang et al., 2002; Delwiche et al., 2015). A trained technician can run 100–200 samples per week, starting with the grinding process. Variation in FN data can arise from users' testing methods, sampling, and FN machine bias, as well as environmental and biological causes (Delwiche, Higginbotham, et al., 2018; Delwiche, Rausch, et al., 2020; Delwiche, Tao, et al., 2020; Delwiche & Vinyard, 2017; Delwiche et al., 2015; Risius et al., 2015). Collectively, in the United States, these issues resulted in industry stakeholders requesting that

the USDA Agricultural Marketing Service (AMS) reduce variation in the FN test (Coale, 2019). The USDA FGIS directive was recently updated (May 2020) in an effort to decrease FN variability through improved standardization. The standard method now includes correction for grain moisture content and for barometric pressure during the procedure (Delwiche, Rausch, et al., 2018; Delwiche et al., 2015).

The standardized FN test generates a variation about ± 30 seconds. However, the discount is strictly applied to measurements below 300 despite the questionable accuracy of the measurements. Traders then attempt to blend low FN grain into high FN grain to meet the global contract, but blending low FN wheat into sound grain is not a linear function. Loss of crop value can result from inadvertent inter-mixing at many stages, including during harvest; during transport in trucks, barges, trains, and ships; or during storage in elevators. Since enzymes are catalysts, a large amount of sound (high FN) grain can be ruined by mixing with a small amount of low FN grain. For example, a single sprouted kernel, when mixed with 2600 sound kernels as representative of a wheat lot, can result in the lot failing to meet minimum FN specifications (PNW, n.d.). While marketers are able to blend some lower FN grain with sound grain, a great deal of sound grain is needed, and marketers must take into account the non-linear relationship between α-amylase activity and FN.

1.1 | Differentiation between PHS and LMA

Resistance to low FN is genetically complex because there are multiple causes of low FN including preharvest sprouting, late maturity α-amylase (LMA), and variation in kernel starch and protein (Sjoberg et al., 2020). During germination, α-amylase levels increase, so that starch in the endosperm can be mobilized as a nutrient for the germinating seedling (Fincher, 1989). Starch mobilization is particularly needed when grain is planted deeply to provide energy for growth until the seedling is able to perform photosynthesis upon emerging from the soil (Horgan et al., 2021). Thus, breeders must select for appropriate α-amylase expression rather than for complete absence, which is challenging under both cases of low FN, PHS, and LMA.

The majority of PHS tolerance has been attributed to higher seed dormancy, the inability to germinate until dormancy is broken by after ripening during dry storage or by exposure to cool, wet conditions (Finkelstein et al., 2008; Rodriguez et al., 2015). PHS resistance is usually selected based on reduced visible sprouting/germination during misting. However, the degree of visible sprouting does not always correlate well with lower FN after rain (Barnard, 2001; Martinez et al., 2018). PHS causes α-amylase expression during germination, whereas LMA results from α-amylase expression during the late maturation stage of grain development (Derkx & Mares, 2020). LMA is triggered not by rain, but by continuous cool or fluctuating temperatures during grain maturation (Barrero et al., 2020; Derkx & Mares, 2020; Farrell & Kettlewell, 2008; Liu, Tuttle, et al., 2021). While first identified in UK and Australian wheat, LMA susceptibility has been observed in wheat from China, Germany, Canada, CIMMYT, and multiple U.S. breeding programs (Börner et al., 2018; Farrell & Kettlewell, 2008; Liu, Parveen, et al., 2021; Mares & Mrva, 2008; Neoh, Tao, et al., 2021). It is even more critical to select for resistance to late maturity alpha-amylase because LMA is not associated with sprouting or other visible phenotypes (Farrell & Kettlewell, 2008; Mares & Mrva, 2014).

The expression of α-amylase is not the only factor impacting FN and end-product quality. For an example, mutations in the wheat granule-bound starch synthase (*waxy*) genes result in a reduction in amylose compared to amylopectin, and in altered pasting or gelatinization properties including a higher hot past viscosity (Zeng et al., 1997). When all three homeoloci are non-functional, starch with no amylose is considered "fully waxy" (Graybosch et al., 2000). Sound, fully *waxy* wheat cultivars have a low FN without the expression of α-amylase. Protein content also affects FN. The lower protein levels found in soft wheat can result in a lower FN without α-amylase expression (Ross et al., 2012). Therefore, the FN method may not be the ideal approach for judging end-use quality, as much depends on the wheat market class and the intended end-product.

Although both PHS and LMA cause low FN, they influence end-product differently. Recent evidence suggests that low FN from PHS has a stronger negative impact on bread, cake, noodle, and white sauce quality than from LMA (Kiszonas et al., 2018; Neoh, Dieters, et al., 2021; Newberry et al., 2018; J.-P. Ral et al., 2016; J. P. F. Ral et al., 2018). There is lack of correlation between LMA FN and end-product quality (Neoh et al., 2020). It is questionable if there is a correlation, especially when α-amylase level is not very high (Cannon et al., 2021). Although both PHS and LMA result in elevated high pI α-amylase levels, LMA results in *TaAmy1* mRNA induction, whereas PHS results in the induction of *TaAmy1*, *TaAmy2*, and *TaAmy4* transcripts (Barrero et al., 2013; Mieog et al., 2017). Moreover, PHS is associated with the induction of a wide range of hydrolytic enzymes, including lipases, proteases, and cell wall degrading enzymes (Ali & Elozeiri, 2017). These hydrolytic enzymes may contribute to the negative effect on end-product quality. A method to differentiate PHS from LMA would be useful in plant breeding to determine

which problem led to low FN in a particular field trial so that the value of LMA-affected grain can be preserved.

There are several differences between LMA and PHS that could be exploited to differentiate the two problems. Although both PHS and LMA cause α-amylase expression in the aleurone layer of the grain, the location of α-amylase expression in the aleurone differs. During PHS, α-amylase expression in the aleurone cell layer starts at the embryo-proximal end and spreads towards the distal (brush) end of the grain (Bewley, 1997). As a result, there is much more α-amylase expression at the embryo-proximal end of the grain. During germination, living aleurone cells lyse after hydrolytic enzyme secretion, releasing hydrolytic enzymes for mobilization of stored reserves in the endosperm (Bethke et al., 1999; Fath et al., 2000). In the case of LMA, α-amylase activity is evenly distributed throughout the aleurone layer (Mrva et al., 2006). It is possible to differentiate PHS and LMA by measuring α-amylase activity in half-grains prepared by equatorial slicing (Mrva & Mares, 1996). If the α-amylase levels are significantly higher in the embryo proximal half than in the distal half, the cause is PHS. Less is known about the biochemical mechanisms controlling cell death during LMA and whether they are exactly the same as those observed during germination (Cannon et al., 2021). Studies of LMA-susceptible wheat lines suggested that LMA induction was associated with randomly spaced patches of cell death throughout the distal-to-proximal length of the aleurone layer, suggesting that α-amylase induction during LMA is associated with programmed cell death just as it is during germination (Mrva et al., 2006).

1.2 | α-Amylase assays and immunoassays

Whereas the FN and RVA are examples of autolytic assays that indirectly measure α-amylase activity on starch from the kernel based on reduced pasting capacity, α-amylase enzyme assays measure activity specifically through the release of chromogenic molecules covalently linked to starch substrates (i.e., AACC approved methods 22-02-01 and 22-05-01) (AACC 22-02-01, 2000; AACC22-05-01, 2000). Wheat α-amylase enzymes catalyze the hydrolysis of α-D-1,4-glucosidic bonds in starch and related polysaccharides to yield shorter polysaccharides such as maltodextrins, or the disaccharides maltose and maltodextrin (Ju et al., 2019; Mieog et al., 2017). Commonly used colorimetric α-amylase enzyme assays include the Ceralpha™ and SD™ assays from Megazyme, and the Phadebas™ Amylase Test (Barnes & Blakeney, 1974; Cornaggia et al., 2016; Hsu & Varriano-Marston, 1983; McCleary et al., 2002; McCleary & Sheehan, 1987; McKie & McCleary, 2015). As α-amylase catalyzes α-glycosidic bond cleavage in the labeled starch substrate, a dye is released into solution. The intensity of the color produced during the reaction positively correlates with the α-amylase enzyme activity of the sample (Barnes & Blakeney, 1974; Mares et al., 1994; Mares & Mrva, 2008; McCleary & Sheehan, 1987; McKie & McCleary, 2015). The substrates used in α-amylase enzyme assays are resistant to cleavage by β-amylase, improving specificity for α-amylase. Colorimetric α-amylase enzyme assays are sensitive, quantitative, and have been adapted to work with high-throughput screening platforms such as 96-well assays and a robotic platform for the Megazyme™ SD assay (Kiszonas et al., 2018; McKie & McCleary, 2015). This is an improvement over the FN method which tests two samples at a time. However, care is needed to obtain consistent results because small differences in pH, incubation time, or incubation temperature can cause significant run-to-run variation in these assays (Barnes & Blakeney, 1974). Although α-amylase enzyme assays cannot distinguish between independent isozymes of α-amylase, these assays can be used to distinguish between LMA and PHS as the source of α-amylase expression when performed on half-grain samples, something not possible with the FN test due to larger sample size requirement of 7 g or more of milled material (Barrero et al., 2013; Mieog et al., 2017; Mrva & Mares, 1996).

Unlike enzyme assays, which measure protein levels indirectly through enzyme activity, immunoassays, including lateral flow assays (LFAs) and enzyme linked immunosorbent assays (ELISAs), are highly sensitive assays that measure target protein levels directly (Engvall & Perlmann, 1971). Immunoassays depend on the specific protein–protein interaction between antibody and antigen to detect the presence of the antigen in a protein extract (Aydin, 2015; Sela-Culang et al., 2013). Since their development, immunoassays including LFAs and ELISAs have been used ubiquitously to characterize the regulation of biological processes across diverse living systems, including the characterization of α-amylase in wheat (Aydin, 2015; Skerritt & Heywood, 2000). Immunoassays can be performed rapidly, in as little as 5 min and can be designed in high throughput 96-well formats.

The first antibodies to individual wheat α-amylases were developed to understand α-amylase protein function during grain development and germination and to characterize tissue specificity and enzyme ontology (Daussant & Renard, 1987). Early work using isoelectric focusing gels found that high pI (pI of 5.5 to 7.0) α-amylases are specifically induced during germination or LMA, whereas low pI α-amylases (pI of 3.5 to 5.5) are not (Mares & Mrva, 2014; Verity et al., 1999). The first ELISA developed to identify grain impacted by PHS used antibodies raised to purified, high-pI α-amylases (pI 6.0 to 7.0). This ELISA appears

to detect the α-amylase isoform encoded by the *TaAMY1* gene, which is expressed during PHS or LMA (Barrero et al., 2013). Based on sequences provided in the original patent, the specific epitopes detected by the monoclonal antibodies used in this ELISA are found in both *TaAmy1* and *TaAmy2* (Skerritt, 2010). A 5-min, field-based assay was developed to estimate FN, and two products were commercialized in Australia from these pioneering efforts, the WheatRite lateral flow immunoassay (LFA) and the Readrite immunonoscanner for reading the signal (Skerritt & Heywood, 2000). The benefits of the WheatRite/Readrite technologies were that they had the potential to improve screening accessibility, efficiency, and accuracy through on-farm testing, reduced sample processing time (5 min or less), and reduced sample size requirements, requiring only milligram quantities of milled grain versus gram quantities as in the FN test (Barrero et al., 2013; Skerritt, 2010; Skerritt & Heywood, 2000). Unfortunately, the WheatRite LFA and Readrite immunoscanner were not made widely available to the global grain industry, and neither tool is currently commercially available.

Since the development of the first wheat α-amylase antibodies more than 30 years ago, collective research efforts have continued to clarify and define distinct roles for the four classes of α-amylase isozymes in wheat and identify their functions and impact on end-use quality (Mares & Mrva, 2014; Mieog et al., 2017). The construction of ELISAs using monoclonal antibodies to wheat α-amylases may play an important role in improving end-use screening platforms, as they are capable of addressing many of the shortcomings associated with the FN test, α-amylase enzyme activity assays, and ELISAs that are not specific for wheat α-amylases. Recent efforts evaluating a 96-well LMA-centric ELISA demonstrated a high correlated total α-amylase activity ($R = .95$) (Neoh, Tao, et al., 2021). This result is important and powerful because it links α-amylase activity to a specific α-amylase isozyme, high pI α-amylase, and to the genetic cause, LMA. Unlike the aforementioned tests which are not specific, wheat α-amylase ELISAs will provide a consistent and reliable method that is not only complementary to both the FN test and α-amylase enzyme activity assays, but can be used to (1) monitor specific changes in α-amylase protein abundance in diverse germplasm and across a broad spectrum of sample types, (2) determine differential expression of α-amylase isozymes occurring with LMA or PHS and at specific developmental time points, and (3) evaluate changes in α-amylase protein expression in response to extreme environmental conditions. As a result, α-amylase ELISAs have the potential to become a valuable tool for researchers, breeders, and growers alike.

1.3 | Spectroscopy for falling number

Spectroscopy of either milled or whole kernels is an attractive approach for estimating FNs because it is a non-destructive, higher throughput approach that can be adapted for use on a combine or at a grain elevator and requires little or no sample preparation (Risius et al., 2015). Such an approach could enable faster identification of low FN samples in early generation breeding lines, thereby improving selection against low FN susceptibility. It could also help to preserve value if used to segregate low FN grain in the wheat industry.

To understand how spectroscopy, specifically near-infrared (NIR) transmission or reflection spectroscopy, might be used to measure FN, a look at some basic principles of this technology is required. NIR spectroscopy, as well as the related fields of mid-infrared (mid-IR) and Raman spectroscopy, are considered vibrational spectroscopies. Vibrations between bonded atoms occur when the energy of a photon matches the difference between the energy levels of two sequential quantum levels of a bond. The jump between the ground state ($\upsilon = 0$) and the first level of excitation ($\upsilon = 1$) characterizes the fundamental vibrations that occur as a result of absorptions in the mid-IR region, 4000 to 400 cm^{-1} (2500–25,000 nm). On the other hand, absorptions in the NIR region (10,000–4000 cm^{-1} or 1000–2500 nm) require more energy and are generated from the vibrations of overtones of the fundamental frequencies and combinations of interatomic bonds. Unfortunately, the overtones are not simple multiples of frequencies, thus making qualitative analysis in the NIR region very challenging. However, quantitative analysis of organic compounds is often well suited to the NIR region.

A more complete description of the process of bond vibration in the NIR region considers the quantum nature of the behavior. For example, anharmonicity arises due to the atoms' dimensions and mass imposing physical limits on the separation distance between bonded atoms that prevents them from being too close (overlapping) or too distant (disassociating). Electrical anharmonicity arises from a non-uniform change in dipole moment as a result of a change in the distance between bonded atoms. The presence of anharmonicity can lead to overtone transitions that arise from a change between non-adjacent vibrational quantum levels (e.g., $|\Delta\upsilon| > 1$), combination bands that occur when the energy from one photon produces simultaneous changes in quantum levels of two or more different vibrational modes, and unequal differences between energy levels of the quantum states (Miller, 2001). These occurrences would otherwise be forbidden under a set of

conditions known as selection rules that arise from group theory in quantum mechanics (Wilson et al., 1955).

The significance of these occurrences becomes apparent when we shift away from the fundamental vibrations of the mid-IR region to the overtone and combination vibrations of NIR. To a first approximation, the frequencies of the overtone bands are integer multiples of the corresponding fundamental frequency, with each higher overtone (and shorter wavelength) being weaker than the preceding. Compared to the fundamental absorption bands of the mid-IR region, absorption bands of the NIR region are weak. Therefore, unlike the mid-IR region where dilution of the test sample is often a necessary step before spectral collection, samples in the NIR region can be run in "neat" form, i.e., as a pure substance, such as ground wheat or, even more convenient, as whole grain, the preferred format used in commercial protein content NIR analyzers. In addition, the NIR overtone and combination bands arise overwhelmingly from bonds involving the lightest atom, hydrogen. Typically, these include the bonds C–H, O–H, and N–H, all of which are prevalent in agricultural products such as grain. Hydrogen bonding and neighboring groups will have secondary effects on the frequency and magnitude of vibrational bands. Furthermore, overtone or combination vibrations along the chain, or within a ring structure, of an organic molecule, are not active in the NIR region.

Using this very brief description of NIR spectroscopy principles, we may now consider the direct or indirect measurement of α-amylase in wheat grain. α-Amylase is the primary enzyme that influences starch paste viscosity, and therefore FN. Although low FN is typically attributed to the endogenous form of the enzyme, it is noted that FN in sprouted grain increases with the addition of silver nitrate, an inhibitor of α-amylase activity. This has led some researchers to theorize that most of the enzymatic changes to starch occur during heating and hydrolyzation stages (Olaerts et al., 2016). If true, this theory may explain the historical challenges confronted by researchers who have attempted to develop NIR spectroscopy models for FN.

Nevertheless, the prospect of producing an NIR procedure for measuring FN, α-amylase, or germination-related changes in the wheat seed has been appealing to the wheat research community for nearly 40 years. The first exploration of NIR reflectance for FN measurement used a spectrometer with 19 fixed interference filters and multiple linear regression (MLR) (Starr et al., 1981). The five-filter (1778, 1818, 1982, 1940, and 2100 nm) MLR calibration produced a standard error of 26.8 s for a small calibration set ($n = 45$) and 62.3 s for a separate validation set ($n = 43$). This increase speaks to the importance of external validation in order to realistically describe NIR modeling capability.

With a scanning monochromator (1200–2400 nm wavelength range), the relationship was not confirmed between FN and sodium dodecyl sulfate (SDS) sedimentation volume suggesting the pitfall of pseudo correlation (Osborne, 1984). In pseudocorrelation, a positive correlation between the desired property (FN in this example) and a known NIR-modelable constituent (e.g., protein content) leads to a false conclusion that the NIR process is directly sensitive to the property. Nevertheless, NIR studies on FN have continued for both α-amylase (Xing et al., 2011) and FN (Caporaso et al., 2017) in laboratory conditions and on a combine during harvesting operations for field mapping of FN (Risius et al., 2015).

In recent work, partial least squares (PLS) regression calibrations were established for FN on a genetically diverse set of Washington-grown white wheat, with the premise that improvements in FN precision and NIR hardware and software over the past 30 years may lead to more accurate NIR models (Delwiche, Higginbotham, et al., 2018). However, these FN calibrations had standard errors of performance ranging from 40 to 77 s, which is larger than those observed for the FN test. As an alternative approach, these researchers attempted classification (low/high FN with a cutoff value) using linear discriminant analysis and PLS discriminant analysis (PLSDA) qualitative models. Unfortunately, model accuracy was still low, with the best model correctly identifying 67–71% of the samples in a set of several hundred. Various linear regression (such as PLS) and non-linear (support vector machine and random forest) algorithms were used for to model wheat flour FN using NIR reflectance (800–2700 nm) (Junior et al., 2020). Improvement was attained using the non-linear models; yet, the root mean squared errors (RMSE), ranging from 57 to 68 s, were on par with previously published results. To date, NIR model development for FN has been unfruitful, leaving open the possibility of using NIR to explore α-amylase and seed germination more broadly.

This leaves the following remaining possibilities for a NIR response: (1) the amount of α-amylase is high enough to be measured, with the condition that the molecular structure of α-amylase is spectroscopically distinguishable from that of the proteins in the endosperm, aleurone layer, and embryo; or (2) the NIR spectral response is sensitive to physical changes in the seed that occur during the onset of germination and result in changes to light scatter in the seed (for whole kernel transmission) or externally among the particles produced during grinding (for meal diffuse reflection). With the first possibility, we assume that levels of α-amylase are high enough to be measured directly by NIR. The common isoform of the cereal α-amylase enzyme synthesized during germination, and best studied in barley, consists of 403 amino acid residues folded into three domains, with the largest domain containing 286 residues

in a (ba) 8-barrel formation which houses the active sites for starch hydrolysis (Kadziola et al., 1994). Although collectively sensitive to proteins through N–H bond vibrations, the sensitivity of the NIR response to individual amino acids, let alone their residues, is extremely challenging. Enzymes are even more challenging because of their low abundance, in all likelihood precluding the use of NIR for quantitative analysis (which typically has lower limits of detection in the tenths or hundredths of a percent, w/w).

If the second possibility is true, then we need to understand the morphological changes leading up to α-amylase production and release in the kernel aleurone layer (Fath et al., 2000). Much of our understanding of α-amylase induction and cell death of the aleurone layer in cereals comes from studies of germinating barley grain. The triploid cereal endosperm is composed of a starchy endosperm surrounded by the aleurone cell layer. The starchy endosperm comprises about 70% of the grain volume and dies at the completion of grain filling. In mature wheat kernels, the aleurone is a single cell layer of living cells that controls the mobilization of starch and other nutrients during germination through the synthesis and excretion of hydrolytic enzymes including the starch degrading enzyme α-amylase. Prior to germination, the thick-walled aleurone cells contain many protein storage vacuoles (PSV) that break down during germination, providing the amino acid building blocks needed for rapid protein/enzyme synthesis (Bethke et al., 1998). During germination, the hormone gibberellic acid (GA) triggers a series of morphological changes in the aleurone that conclude with cell death (Bethke et al., 1998; Jones & Jacobsen, 1991). GA triggers the transcription and translation of hydrolytic enzymes, with α-amylase representing as much as 60% of the rapidly translated protein within 3 to 4 h of GA perception. The PSV swell following hydrolysis of storage proteins in the PSV lumen, then fuse to form one large vacuole that increases in size with longer GA treatment. Prior to cell lysis, the aleurone secretes hydrolytic enzymes into the endosperm, and the PSV becomes acidic and fills with enzymes that have low pH optima. Prior to lysis, aleurone cells become highly vacuolate, mobilize most of their stored protein, and use those amino acids to synthesize secreted hydrolases.

The death of aleurone cells is preceded by increased permeability of the plasma membrane, followed by membrane collapse and cell lysis (Bethke et al., 1999). This process should release all remaining hydrolytic enzymes. Cell walls are digested by glucanases and xylanases secreted from the aleurone during germination, likely to provide access to starch contained within endosperm cells. Thus, whether or not some starch digestion occurs prior to FN measurement will depend on where the grains are in the germination process. The study on cellular changes in the aleurone during LMA suggested that the patches of vacuolated cells and of cell death are present following LMA induction (Mrva et al., 2006). If NIR is not detecting starch degradation or α-amylase protein itself, then it is possible that it is detecting the physical and chemical changes in the PSV, vacuolization, and finally cell lysis. Therefore, it may be necessary to use multiple wavelengths and complex modeling to develop an accurate calibration. For this reason, future spectroscopy studies should take care to differentiate between LMA- and PHS-affected grains during calibration development.

1.4 | Hyperspectral imaging

Spectral signals from PHS- and LMA-associated components may be enhanced with hyperspectral imaging (HSI). This technique provides spatial and spectral information about an object of interest, and the spectrum of each pixel contains hundreds of contiguous bands. HSI data are also called a data cube because it has three dimensions of pixels. The first two dimensions are spatial pixels, and each pixel contains a vector of spectral bands that make up the third dimension. To generate HSI data, the push broom method (Femenias et al., 2020; Sendin et al., 2018), also known as the line-scanning method, is most commonly used. In this method, HSI sensors are designed as a two-dimensional chip. The chip has one dimension collecting spatial signals and the other dimension collecting a spectrum. Using the push broom system, only a narrow line of spatial pixels in an object can be scanned at a time. After each scan, the sensor or the object has to be moved by one unit of distance in order to scan a new area (Elmasry et al., 2012; Fong & Wachman, 2008).

In addition to sensors, lighting units and spectrographs are two additional key components needed to acquire HSI data. Lighting units are used to illuminate objects of interest and to generate a reflection, which is a mixture of optical signals. A spectrograph is used to separate different spectral bands from the mixture by isolating a particular wavelength range and transmitting this spectrum to the sensors for scanning. The combination of sensors, spectrographs, and lighting units can limit what range(s) of wavelengths are available to be scanned.

In the wheat industry, HSI approaches are mostly used to improve the assessment of disease and sprout damage. Fusarium Head Blight (FHB), one of the most common fungal diseases in wheat, can reduce kernel density and as a result lead to a loss in yield. Both sound and FHB-damaged kernels can be identified with 96% accuracy by scanning kernels using short-wave infrared (SWIR) HSI and modeling them using an FHB-damage

classifier (Delwiche et al., 2019). HSI data showed that four effective wavelengths, 1100, 1197, 1308, and 1394 nm, can predict FHB with 95% accuracy. The usage of wavelength range between 400 and 1000 nm showed that this method can predict FHB-damaged kernels with 98% accuracy (Zhang et al., 2020). FHB damage can also be quantified by determining the concentration of deoxynivalenol (DON), an FHB-induced secondary metabolite in wheat kernels. HSI was able to identify wheat contaminated with DON and quantify DON in wheat samples with 73.4% accuracy (Femenias et al., 2020).

Since sprouted kernels are less dense than sound kernels, an X-ray imaging approach was used to inspect the interior structure of wheat and could identify sprout damage with 90% accuracy (Neethirajan et al., 2007). However, considering the difficulty and risk of taking X-ray images, thermal images that capture wavelengths over 9000 nm (i.e., long infrared) are generally used as an alternative. Sprouted kernels are actively respiring and have higher temperatures at the surface of the grain. Thermal images that convert invisible radiation to visible imaging signals can classify healthy and sprouted kernels with 90% accuracy (Vadivambal et al., 2010). A simulated PHS event suggested that there are distinct spectral patterns associated with different degrees of sprout damage in the endosperm tissue (Chen et al., 2013). In addition, HSI-driven predictions of α-amylase activity also showed a high coefficient of determination ($r^2 = .82$) when the enzyme activity ranges was between 0 and 78 Sandstedt Kneed Blish (SKB) units (Xing et al., 2009). Furthermore, NIR HSI data have great sensitivity when FN is above 300 s, but this technique is not reliable enough to identify FN when it is lower or sprout levels are severe (Barbedo et al., 2018).

Another study attempted to predict FN with field-collected data. However, the FN prediction accuracy was relatively low ($R^2 = .44$) (Caporaso et al., 2017). Although this study used an average across pixels to develop a predictive model, the pixel-wise prediction demonstrated that the distribution of FN-associated components had patterns specific to the kernel location, including embryo end, brush end, and crease. HSI of wheat kernels with PHS damage identified changes initially at the embryo end that later spread to larger areas of the kernel. This result suggested that HSI can "see" the effects of PHS. The changes in the kernel appeared to follow a path from the embryo end to the brush end. This result suggested that the path of changes follows the vascular bundle in the grain crease and that the vascular bundle is serving as a conduit for an α-amylase-inducing signal from the embryo. This pattern could enhance the prediction of PHS using artificial intelligence, such as convolutional neural networks. Similar to the immunoassays using the embryo and brush ends of a kernel, monitoring the differences between these two ends could enable HSI to differentiate PHS from LMA (Caporaso et al., 2017).

1.5 | Advances of analytical models

Pre-processing techniques are required to remove physical artifacts and improve subsequent analysis from spectral and HSI data (Rinnan et al., 2009). Multiplicative scatter correction (MSC), de-trending, and standard normal variate (SNV) are the most commonly used scatter-correction methods in pre-processing of NIR spectral data in crop studies (Ahmad et al., 2016; Carbas et al., 2020; Lü et al., 2017; Sampaio et al., 2018, 2020; Sorvaniemi et al., 1993; Xing et al., 2011). For an individual sample spectrum, zeroth, first, and second Savitzky-Golay (SG) derivation are commonly used to reduce physical effects such as light scatter and, for first and second derivatives, to accentuate component spectral bands from a broad overall response, consisting of overlapping individual bands. At the same time, the operation results in spectral smoothing. Although defined as a least squares polynomial curve fitting operation, SG operations become mathematical convolutions in which the derivative window size determines the degree of smoothing. Larger windows (e.g., seven or more adjacent wavelengths) result in greater smoothing at the expense of lessening spectral resolution (Delwiche & Reeves III, 2010). Other pre-processing methods such as generalized least square weighting (GLSW) (Ahmad et al., 2016), enhanced multiplicative scatter correction, area normalization, automatic weighted least squares (AWLS) baseline, and automatic Whittaker filter baseline have also been applied in crop studies (Lü et al., 2017; Xing et al., 2011).

Linear regression models, including PLS, and extension models, competitive adaptive reweighted squares, and single linear regression, are commonly used to analyze infrared spectra data. PLS is a statistical method that assumes there is a linear relationship between components and phenotypic features. The PLS method evaluates coefficients by iteratively seeking the lowest value of the root mean squared errors in the prediction. A previous effort (Armstrong et al., 2016) has been put to use PLS analyze spectrum from single-kernel NIR spectroscopy and silicon light-emitting diode sorter. Their results showed that spectral averages from 30 kernels scanned by single-kernel NIR spectrum could increase R^2 to .95 from .78, where the analysis considers one wheat kernel spectrum as a data entry. A combination of MSC and PLS is applied to analyze wheat flour spectra data collected through fusing a Fourier transform near-infrared spectroscopy (FT-NIR), resulting in R^2 value of .55 (Sorvaniemi et al., 1993). Similarly, another study performed de-trending, GLSW, and MSC to preprocess wheat flour spectra collected using fluores-

cence spectroscopy, genetic algorithm to extract significant spectra features and PLS for FN prediction. The result shows an R^2 value of .48 (Ahmad et al., 2016). A study compared PLS with partial least squares discriminant analysis (PLSDA) to predict α-amylase activity, results indicate that R^2 of PLS ranges from .56 to .69, while using PLSDA for limited wavelengths achieved accuracy of 91% of α-amylase activity classification (Symons et al., 2010).

PLS extension models, such as interval PLS, synergy PLS, and moving window PLS, have been used to analyze rice amylose content (Sampaio et al., 2018). Other models, such as artificial neural networks, have been used to analyze NIR spectra of wheat flour (Mutlu et al., 2011). Artificial intelligence algorithms, such as genetic algorithms, have been shown to optimize PLS prediction by selecting the significant spectral blocks from a wheat kernel NIR spectrum (Lü et al., 2017). No difference was found between spectral data collected from a short-wavelength infrared (SWIR) HSI system and data collected from FT-NIR instruments for α-amylase activity prediction (Xing et al., 2011).

2 | PROSPECTS

Developed in the 1960s based on viscosity, the Hagberg–Perten FN method is still the international standard method to access wheat damage due to PHS and LAM across the entire production chain, including breeding, harvesting, storage, transportation, milling, and baking. The method is laboriously slow, vulnerable to grain sampling variation, inconveniently demanding for expensive lab equipment, and unable to differentiate the two major genetic causes that influence end-use quality differently. Low FN due to LMA has less impact on end-use quality than PHS, which also degrades protein in addition to starch. Although none of the FN alternatives has reached the stage to replace the current method, some of them demonstrated great potential or provide complementary solutions. Not only are α-amylase enzyme assays and immunoassays fairly fast at examining starch damage due to α-amylase in typical laboratory settings but also able to differentiate PHS and LMA by measuring α-amylase activity in half-grains. LMA has similar activity between the distal and embryo halves, while PHS has more activities in the embryo half than in the distal half. Spectroscopy, especially HSI, has the advantage of directly screening intact grains without destroying the grain. α-Amylase enzyme assays and immunoassays are preferred over the FN method to develop prediction models, as the FN method is not capable to produce measurements on a single grain. The images on individual grains provide the opportunities to develop machine vision systems for real-time screening to segment grains at different stages. For example, a harvester with such a system would prevent the contamination of sound grain from the damaged gains in the first place. Benjamin Franklin, one of the founding fathers of the United States, famously advised in 1736, "An ounce of prevention is worth a pound of cure." The best solution to the economic pain of PHS and LMA is to breed more resistant (or less susceptible) cultivars. The development of FN alternatives not only promotes the wheat trade but also provides tools in breeding programs. More rapid and accurate screening in breeding programs translates to fewer opportunities for large-scale PHS/LMA events that affect the global wheat industry.

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Yang Hu: Methodology; writing – review & editing. **Amber L. Hauvermale**: Methodology; writing – review & editing. **Craig F. Morris**: Methodology; writing – review & editing. **Ashley E. Cannon**: Methodology; writing – original draft; writing – review & editing. **Camille M. Steber**: Methodology; writing – original draft; writing – review & editing. **Zhiwu Zhang**: Methodology; writing – original draft; writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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