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Review

Limitations and current applications of Near Infrared Spectroscopy for single seed analysis

Lidia Esteve Agelet*, Charles R. Hurburgh Jr.

Department of Agriculture and Biosystems Engineering, Iowa State University, USA

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ABSTRACT

Near Infrared Spectroscopy (NIRS) analysis at the single seed level is a useful tool for breeders, farmers, feeding facilities, and food companies according to current researches. As a non-destructive technique, NIRS allows for the selection and classification of seeds according to specific traits and attributes without alteration of their properties. Critical aspects in using NIRS for single seed analysis such as reference method, sample morphology, and spectrometer suitability are discussed in this review. A summary of current applications of NIRS technologies at single seed level is also presented.

Contents

1. Introduction

Plant breeding facilities are constantly looking to improve current varieties and to obtain new seeds with special traits. This is achieved with a careful selection of the best individual traits. Use of bulk samples in the selection process results in a larger seed production with only a fraction with the desired trait, since the heritability of a desired characteristic may be low. Analyzing individual seeds allows researchers to understand the future plant characteristics and the characteristics of its next generation [1], while retaining competitive agronomic performance, or obtaining high yields or resistance characteristics. Common Near Infrared Spectroscopy (NIRS) bulk sample analyzers provide measurements of samples of about 250 g of kernels on average. Single seed differences cannot be identified and no discrimination is possible.

Besides seed producers, farmers, feed processors, animal producers, food companies, and other seed-related industries can benefit from on-site single seed screening as well. The so-called 'dilution effect' of current analytical technologies allows low fractions of unwanted seeds to be mixed with the majority without the chance of identifying the impurity fraction, decreasing the overall batch value. For this reason seed inspection is very relevant for pricing commercial grains, as the undesired fraction of seeds is visually determined at single seed level. For processing and quality improvement purposes, NIRS analysis at the single seed level

* Correspondence to: Food Science Building, Iowa State University, Ames, IA 50014, USA. Tel.: +1 515 294 8319.
E-mail address: esteve.lidia@gmail.com (L. Esteve Agelet).

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followed by a sorting mechanism could help in increasing sample uniformity and purity. Whether the target is to segregate sound seeds from defective/damaged, to keep seeds with a specific concentration of a compound of interest, or to discriminate mixtures of varieties, the quality and economic value of a seed batch could increase considerably when the unwanted fraction of seeds is removed and the batch is uniform in its attributes [2].

This review gathers relevant aspects of seed and grain analysis by NIRS as a tool to quantify, segregate, and discriminate seeds on a fast and non-destructive manner. Current and promising applications are exposed together with limitations and challenges faced by the technology. Although NIRS is already well-known for successfully analyzing bulk grain and bean samples, Single Kernel NIRS (SKNIRS) still has to reach its full potential for industrial applications. SKNIRS needs to have recognized protocols and methods as bulk NIR analyses currently have.

2. Overview on near infrared spectroscopy

It has been over 60 years since the first practical application of NIRS as an analytical method. Karl Norris, pioneer of NIRS, developed the first applications of NIRS on grains and seeds in the 1960s [3,4]. Since then, instrumentation, statistical methods, and software have been improving and the number of applications have exponentially grown. NIRS is now a mature analytical method for grains and seeds, recognized by the American Association of Cereal Chemists (AACC 39-00) and the American Oil Chemist Association (AOCS am 1–9).

Near Infrared spectroscopy (NIRS) technologies have a performance comparable to other wet chemistry analytical methods, but with some important advantages such as short analysis time, small sample preparation, and non-destructiveness. The radiation from the near infrared (NIR) electromagnetic region (700–2500 nm) is absorbed by water and organic compounds such as carbohydrates, protein, oil or alcohols. The apparent absorbed energy by a sample, calculated from either transmitted or diffusively reflected radiation, can be related to the content of the compound. Shorter wavelengths, close to the visible region, are weakly absorbed compared to the longer wavelengths closer to the infrared region. For this reason, shorter wavelengths can penetrate deeper through samples that are not excessively thick and opaque. Fraser et al. [5] showed that, for apples, wavelengths up to 900 nm could penetrate up to 25 mm, while from 1400 to 1600 nm the penetration decreased to 1 mm.

Bulk sample NIR instruments working by transmittance mode mainly work on the region from 700 to 1100 nm. Those instruments measure the transmitted radiation through a fixed pathlength of a bulk sample of grains or beans, assuming that the decrease of the initial radiation in traveling through the sample is due to absorption. The pathlength is optimized according to the commodity being measured and the instrument setup, being common a pathlength around 15 mm for corn and soybeans. Instruments based on reflectance mode, on the other hand, measure the diffusely reflected radiation from the sample. The diffuse reflected signal is a fraction of the initial radiation source which after penetrating the sample few mm, has been interacting with the sample molecules, scattered in several directions, and traveled back to the surface. Only the diffuse fraction of the reflected radiation has interacted with the compound of interest. Other reflected fractions (such as specular) may only have interacted with the sample surface and thus does not contain chemical information related to the sample composition.

In order to correlate the sample absorbance to the concentration of a specific compound, the accurate amount of the compound under analysis must be known. For this reason NIR technologies are initially dependent on other chemical methods (also known as reference methods) to develop a calibration model and validate it properly. After some time, the calibration may need future updates because new sources of variability are most likely to appear when dealing with grains and seeds (variability due to fields, varieties, environmental changes etc.). More samples will have to be added in the model and more reference analyses will be needed. Therefore the selection of an appropriate primary chemical reference method and laboratory a crucial step when developing any NIRS application. Precision and accuracy of NIRS calibrations will be determined by the quality of the reference laboratory data. Combining reference data from different laboratories, even if the method is the same, is highly discouraged because errors from different laboratories differ.

There are many calibration algorithms, but most share the same principles as those that are widely known to perform well on quantitative analysis: multiple linear regression (MLR), principal component regression (PCR), and partial least squares (PLS) [6]. PLS and PCR often lead to very similar results, and MLR performs better when working with a short range of uncorrelated wavelengths or data points. PLS and PCR can be easily adapted for discrimination (i.e. PLS-DA) and are in fact derived from principal component analysis, a popular algorithm in pattern recognition and discrimination.

Once a calibration model is developed, it must be properly validated. True validation is done predicting independent samples, not related with samples included in the calibration set. Bagging and cross-validation are other validation alternatives when sample availability is a limitation. However, validation statistics from cross-validation may be overoptimistic, especially for models developed with few samples or not including all possible sources of variability. The use of suitable validation statistics is extremely important in order to report the calibration performance and determine its future use (screening, quality control etc.). The most widely utilized statistics for quantitative models are well summarized by William [7] and Fearn [8], and include among others those to quantify expected random errors (i.e. standard error of prediction (SEP) or cross validation (SECV)) and systematic error (bias). Other statistics such as the coefficient of correlation (R) – or its squared, the determination coefficient (R^2) and the ratio of the standard deviation of references over the SEP (RPD) give an idea of the overall calibration performance.

3. Impact of seed size and morphology

Single seeds are variable in shape and size. The variability of seed thickness translates in variability of the distance from the sample to the collecting sensor (focal length) and hence the sample distance (pathlength) that radiation travels in transmittance mode. Eq. (1) shows the relationship of the radiation reaching the sensor (F) with the focal length (f) and the irradiated sample diameter (D) [9]. According to that equation, large kernels reflect more radiation than small kernels as the focal length is larger. Once the reflectance is transformed to apparent absorbance, larger kernels will have lower optical density or absorbance offset compared to small kernels.

$$F = f/D \tag{1}$$

Because individual seeds can be small (i.e. 3 mm length for wheat kernels), the irradiated diameter (D) of commercial instruments working in reflectance mode is often larger than the seed diameter. In that case, the collected radiation includes scattering from kernel edges in detriment of relevant biochemical signal. For transmittance measurements, the irradiated diameter should not exceed the seed diameter because any radiation leakage through

the edges may lead to detector saturation. For this reason, measurements by transmittance are more complicated for small seeds. The transmittance measurement of big seeds, on the other hand, may lead to seed edges badly illuminated and cause non-uniform illumination of the sample [10,11].

Seed curvature and shape are among the main sources of spectral variance within seeds of a same variety. Malena et al. [12] found that the principal source of variance contained the information related to the kernel curvature. There are spectral differences between sides (crease and back) in heterogeneous kernels such as wheat or corn as well. In reflectance mode, there will be more light scattering when the crease side face the illumination source. For measurements taken in transmittance mode, the radiation also experiences different scattering and travel path patterns depending on which side the kernel is placed for analysis. Orman et al. [13] showed that corn kernels with the embryo facing the light source (collecting the radiation from the back of the kernel) gave lower errors when quantifying oil compared with calibrations developed with the embryo facing the detector (SECV = 1.2% vs. 1.5%). Cogdill et al. [14], however, showed the opposite when predicting oil content in corn kernels with chemical imaging by transmittance.

Even when analyzed from the same side, the small changes on orientation and positioning of the seed respect to the measurement fiber impact the final spectra and calibration performance: there are appreciable changes in spectra offset and shape. Delwiche [10] found that the repeatability of protein predictions by transmittance was worse for smaller wheat kernels, mostly due to the difficulty of keeping the same alignment in the sampling clamp – more degrees of freedom when being replaced –. Dowell et al. [15] and Weinstock et al. [16] also reported the impact of seed positioning and replacement on corn kernel reflectance spectra when predicting oil.

Some mathematical transformations of the spectral signal have been useful to reduce the impact of kernel morphological characteristics, positioning, and orientation. Preprocessing methods that reduce light scattering effects such as multiplicative scatter correction (MSC) or standard normal variate (SNV) have been utilized either alone [17–20] or combined with methods that reduce peak overlap while smoothing the signal, such as derivatives [10,18,19]. The optimal preprocessing depends not only on the instrument configuration, but also the seed characteristics and compound to be measured. Measuring properties related to seed size or morphological-rheological characteristics involves measuring the indirect scattering behavior (i.e. differences in offset). In that case, developing models with absorbance is better than preprocessing the spectra with other mathematical treatments. This is the case of measuring hardness, vitreousness, and density in wheat kernels [18].

4. Reference methods and detection limit

NIRS is not an analytical method for direct measurement of trace elements – compounds found at part per million (mg/kg) or part per billion in the seed. Given the small size of seeds, the detection limit is a limitation of NIRS on single seed analysis. Dowell and Maghirang [21] suggested that compounds that represent in weight less than 0.1% of the seed cannot be accurately measured. For instance, Patrick and Jolliff [22] observed that predicting meadow foam seeds with oil content below 5 mg were consistently overpredicted so the detection limit was reached. But on the other hand, Janni et al. [23] obtained large errors when predicting oil in corn kernels when including kernels with oil content above 8% on dry mass. Seeds with abnormally high concentrations of any compound (specific hybrids or genetically modified seeds) are a problem for conventional NIR calibrations as those new seeds may present additional genetical or morphological changes that make them different from the rest and add an additional source of variability to be modeled and which may not always be properly done by linear methods like PLS.

The low detection limit of NIR may be even higher than 0.1% depending on the initial size of the seeds and the characteristics of the compound to be measured. The impact of kernel size on calibration accuracy was described by Tajuddin et al. [24] when developing PLS oil calibrations for large (>6 mm) and small (<6 mm) soybean seeds. Larger seeds lead to better calibrations than smaller seeds (SEP = 0.09% and 0.14%, respectively). On the other hand, attributes such as moisture often lead to much better accuracies than compounds such as protein or oil (Table 1), due to the strong absorption of water in the NIR region. This also means that the detection limit for moisture is going to be lower compared to other compounds.

There are feasibility studies in discriminating kernels with mycotoxin contamination which have been controversial as mycotoxins are found in trace concentrations. Dowell et al. [15] showed good accuracies segregating corn kernels above 100 ppm of total fumonisin and below 10 ppm, and Pearson et al. [25] had also success with corn kernels with aflatoxin above 100 ppb and below 10 ppb. Nevertheless, the measurement of those compounds in such low concentration may be due to the indirect measurement of characteristics which are correlated to the concentration or presence of compound of interest. It was suggested that some changes in the endosperm may be what drove the classification of aflatoxin contaminated corn kernels [25]. The measurement of wheat vitreousness is another example of indirect measurement of other compounds. Dowell [26] concluded that protein or starch concentrations together with light scattering effects could be what NIRS was measuring when analyzing kernel hardness. The agreement of his NIRS predictions with graders were very high (99%) for easily classifiable kernels. For vitreousness, similar to what was observed with hardness, Wang et al. [27] and Manley et al. [12] concluded that light scattering, kernel color, hardness, starch content, water binding and protein concentration were relevant components for classification of vitreousness of wheat and corn kernels with NIRS.

Secondary (pleitrofic) effects allowed the segregation of roundup ready (RR) soybeans from conventional with NIRS [28–30]. Therefore, NIRS does not detect the RR gene or modified DNA, but the effect that the gene causes on the fiber of soybean seed hulls [30]. Some papers have reported the determination of minor compounds such as fatty acids [16,31–41] or amino acids [20,42], although the correlation to total oil or protein content, respectively, should be determined in order to find out if what is being indirectly measured is the majoritary compound. This concept is explained by Kovalenko et al. [43] for NIR measurement of amino acids in bulk soybean samples.

The size of single kernels is also a limitation from the reference laboratory point of view. Choosing reference methods and laboratories that can provide accurate results is crucial because the error of the reference method will be added to the NIR predictions. For instance, Cogdill et al. [14] found that the reference chemistry method for oil in corn kernels accounted for 50% of the prediction error of their chemical imaging calibrations.

However, when working with single seeds determining and accounting for the laboratory error is not an easy task as most of current reference methods of seed analysis are designed and optimized for bulk analysis. Even if a single seed is enough sample for certain chemical analysis, the error coming from the laboratory will be proportionally larger relative to the mass of a single kernel compared to bulk analysis. For destructive reference methods (wet chemistry), the kernel is destroyed and given the small sample size most likely there is no chance to determine the standard error of the laboratory or measurement repeatability. In consequence, it is not possible to average measurements to reduce the random

Table 1
Major commodities and compounds quantitatively analyzed by NIRS at single seed level.

Seed	Attribute	SEP	R^2 (%)	Best use	Technology[a]	Citation
Wheat	Moisture	0.3–1.0%	81–98	Quality control	DAR	[53,63]
	Protein	0.4–1.37%	84–99	Quality control	MR, PGT, DAR, CIR	[10,17,18,49,63–68]
	Hardness	7.6–15 h.u.	71–91	Usable	MT, DAR	[50,66,69]
	Oil	0.1%	69	Rough screening	CIR	[68]
	Mass	2.4–2.9 mg	37–79	Screening	DAR	[53]
	DON and Vomitoxin	40–60.8 ppm	64–87	Screening	DAR	[55]
	Ergosterol	100 ppm	64	Rough screening	DAR	[55]
	Amylose	0.4–0.9%	90	Usable	DAR	[70]
Corn	Oil (%), Oil (mg)	0.6–1.4%, 2.7 mg	55–95, 85	Usable, Screening	DAR, CIT, MT, CIR	[13,14,16,19,20,23,71]
	Fatty acids:	(1) 2.2%[b]/3.9 mg	(1) 38/77	(1) Rough Screening	DAR, CIR	[16,41]
	(1) Palmitic	(2) 4.2%[b]/9.6 mg	(2) 39/89	(2) Screening		
	(2) Oleic	(3) 4.2%[b]/13.3 mg	(3) 42/74	(3) Rough screening		
	(3) Linoleic					
	Protein (%)	0.3–1.7%	75–95	Usable	DAR	[19,20,41,71]
	Protein (mg)	2.3–3.8 mg	77–89	Screening		
	Moisture	0.76–1.2%	87–95	Quality control	DAR, CIT, PGT	[14,48,59]
	Starch (%)	0.7%–11.5% 17.8–	66–88	Screening	DAR	[19,41,71]
	Starch (mg)	18.2 mg	85	Screening		
	Mass	27.6–30.0 mg	87	Screening	DAR	[19,20,41]
	Energy	183 cal/g–93.9 cal	n.a.	Rough screening	DAR	[41]
	Fungal infection	6.3–9.6%	80	Usable	GMR	[72]
	Ergosterol	1.74 mg/kg	81	Quality control	GMR	[72]
	Fumonisin	1.33	78	Usable	GMR	[72]
Soybeans	Protein	0.3–1.57%	87–98	Quality control	DAR, GMR, GMT, FTR, FTT	[24,44,46,59]
	Moisture	0.32–0.88%	98–99	Quality control	DAR, FTR, DBT	[45,46,59,64,]
	Oil	0.2–1.44%	96–98	Quality control	DAR, FTR, FTT, GMR	[24,44,46]
	Mass	10–16 mg	77.5–91	Quality control	DAR, GMR, FTT	[44]
	Isoflavones	0.017%	99.7	Quality control	FTR	[73]
Soybean pods	Sucrose	0.37%	75	Screening	GMT	[42]
	Free aminoacids	0.21%	69	Screening	GMT	[42]
Rapeseeds	Protein	0.74–0.77%	94–96	Quality control	MGR	[38,74]
	Oil	1.14%	74–97	Quality control	MGR	[33,38]
	Mass	0.51 mg	85	Usable	MGR	[33]
	Fatty acids:	(1) 2.7–8.9%[b]	(1) 85–97	(1) Quality control	MGR, DAR	[32,33,37,40]
	(1) Oleic	(2) 1.53–4.2%[b]	(2) 53–85	(2) Screening		
	(2) Linoleic	(3)1.13%[b]	(3) 76–85	(3) Screening		
	(3) Linolenic	(4) 0.42%[b]	(4) 72	(4) Screening		
	(4) Palmitic	(5) 3.87%[b]	(5) 14	(5) Rough screening		
	(5) Stearic	(6) 3.24–6.4%[b]	(6) 88–93	(6) Quality control		
	(6) Eucosenoic					
	(7) Erucic					
	Glucosinates	10.3 μmol/g	86	Screening	GMR	[38]
	Indole	1.4 μmol/g	86	Screening	GMR	[38]
Sunflower seeds	Fatty acids:	(1) 27.7 g/kg	(1) 52	(1) Rough screening	GMR	[31,34,35]
	(1) Palmitic	(2) 46.0 g/kg	(2) 80	(2) Screening		
	(2) Stearic	(3) 84.3 g/kg	(3) 89	(3) Screening		
	(3) Oleic	(4) 62.1 g/kg	(4) 91	(4) Usable		
	(4) Linoleic					
Rice	Mass	1.09–1.30 mg	67–71	Screening	GMR	[75]
	Moisture	0.24–0.29%	98	Quality control	MGR	[76]
	Protein	0.39–0.52%	93–94	Usable	MGR	[76,77,78]
	Amylose	2.3–3.6%	67–85	Screening	MGR,MGT	[75,79]
Meadow-foam seed	Oil	3.0%	95	Usable	MGT	[22]
Common beans	Protein	1.6%	82	Quality control	DAR	[80]
	Starch	4.9%	56	Rough screening	DAR	[80]
	Mass	41.2 mg	74	Screening	DAR	[80]
Barley	Protein	0.8%	84	Usable	GMR	[81]
Sesame	Moisture	0.4%	87	Usable	GMR	[36]
	Protein	1.0%	78	Screening	GMR	[36]
	Oil	1.5%	82	Screening	GMR	[36]
	Fatty acids:	(1) 0.8%[b]	(1) 0	(1) Unusable	GMR	[36]
	(1) Palmitic	(2) 0.4%[b]	(2) 52	(2) Rough screening		
	(2) Stearic	(3) 1.3%[b]	(3) 52	(3) Rough screening		
	(3) Oleic	(4) 1.5%[b]	(4) 63	(4) Rough screening		
	(4) Linoleic					

Table 1 (*continued*)

Seed	Attribute	SEP	R^2 (%)	Best use	Technology[a]	Citation
Pine seeds	Moisture	1.9–2.7%	86	Usable	GMR, GMT	[82,83]
Sugar beet seed	Moisture	0.27%	99	Quality control	GMR	[84]
	Ratio: True seed weight/total fruit weight	1.4	81	Screening	GMR	[84]

[a] Abbreviations: Diode array reflectance (DAR), Fourier-Transform reflectance and transmittance (FTR and FTT, respectively), Gratting Monochromator reflectance and transmittance (GMR and GMT, respectively), chemical imaging reflectance and transmittance (CIR and CIT, respectively), prism gratting transmittance (PGT), Dual beam transmittance (DBT).

[b] % of the individual fatty acid over total fatty acid content per kernel.

error of the laboratory. Non-destructive reference methods such as Nuclear Magnetic Resonance (NMR) for oil quantification (i.e. AOCS method Ak 4–95 (09) and ISO 10565:1998) outperform other destructive methods such as supercritical fluid extraction (SFE) for oil quantification [14]. For protein, Kjeldahl digestion and Biuret colorimetry are known methods for bulk samples but single kernels are too small to be analyzed. By combustion (i.e. AOCS method Ba 4e-93 (09)), single kernels can be combusted to obtain %N which is correlated to protein content. The analysis can be fast because without previous grinding of the seeds calibrations are still good [44]. NMR can also be used for protein and moisture determination in seeds, although it has not been as popular when developing NIR calibrations for single seeds as combustion and oven drying, respectively. Moisture determination methods by difference of the sample mass before and after oven drying (i.e. method ASAE S352.2 for soybean) are optimized for bulk samples, although they may also been used in single seeds [45]. One may, however, take in account that those methods may show inconsistencies due to the different forms in which water can be found in seeds and which oven drying can not quantify [46,47]. Karl Fischer titration, another common method to analyze moisture, has been never applied to single seeds – most likely because its use in bulk seed samples was reported being not excessively successful [46].

Visual reference methods involving manual inspection by trained personnel are popular for grain inspection and can be reference methods for qualitative or discriminative applications. Because of the subjectivity between operators and variability within readings of a same operator these reference data should be taken with caution when developing NIR applications. Most of the calibration algorithms are not robust to outliers and if not properly identified can lead to a calibration with low performance. On the other hand, the criteria taken by human operators may not be comparable with the information that can be obtained from the NIR spectra. Wheat vitreousness, for instance, is determined by a Board of Appeals and Review in US. When Dowell [26] developed calibrations for vitreousness he found that inspectors only agreed with NIR only 75% of the times when kernels were difficult to classify.

The development of single seed calibrations with bulk reference data or averaging single kernel spectra to simulate bulk data are alternatives that has been tested by several authors. Finney and Norris [48] averaged single kernel spectra to predict the composition of bulk samples. Developing wheat protein calibrations with bulk reference data and the average of the spectra from several kernels (from 10 to 100) gave calibrations that could be used for screening single seeds, with SECVs from 0.35 to 0.16 % [49]. Thirty to fifty kernels represented well the entire sample for hardness and protein in wheat, and as more kernels were scanned and averaged, the calibration approached the performance of calibrations for bulk samples [18,50]. Working by averaging the spectra of 30 corn kernels per sample and using bulk reference data allowed Tallada et al. [20] to develop screening calibrations to measure crude protein, and potentially predict tryptophan, lysine and oil. Armstrong et al. [51] and Armstrong and Tallada [52] developed calibrations averaging single corn and soybean seeds (30 and 50, respectively) and used bulk reference data, either from wet chemistry or predictions from bulk NIR instruments. They could use those calibrations to predict single corn kernel protein and density at rough screening level (RPDs around 2), although bias correction was necessary.

The units of the reference data are relevant and influence calibration performance. Any mathematical transformation of the reference data may benefit or negatively impact its correlation with the absorbance spectra. The composition of bulk samples is often expressed as mass percentage of any compound over the total sample mass (relative units). In the United States, the total mass can be expressed as dry mass, current mass or as is moisture, or as mass with standard moisture basis (i.e. 13% moisture content for soybeans and 15% for corn). Dry and standard moisture basis are the ones utilized the most, as make two samples with different moisture content more comparable. While predicting the relative content (weight %) of compounds in bulk samples give accurate results and it is widely used by all the bulk instrument calibrations, some researchers pointed out that in single kernel the most accurate results are achieved by working by absolute units (i.e. in mg) [53]. Peiris et al. [53] found that smaller wheat kernels had a higher protein content in % basis compared to large kernel, but Bramble et al. [54] observed a positive correlation between kernel mass and protein content. Hence, the correlation of absolute content of the compound and its % is variable. Using % units may, for this reason, leave to calibrations with higher prediction error, lower spectral variance expressed by the model, and subtle non-linearities. Delwiche [10] and Bramble et al. [54] applied mass corrections when developing protein calibrations for wheat kernels, obtaining better calibration performances. For Baye et al. [41] absolute units gave acceptable models for corn kernel constituents such as protein or starch. Relative unit models (%) only accounted for 50% of the data variability, while absolute unit models accounted for 85% of the data variability. Tallada et al. [20] also found better predictions constituents measured in corn kernels (protein, oil, and soluble sugar) when absolute values were used and no preprocessing applied, averaging the spectra of 30 kernels and using bulk reference values. Calibrations developed with absolute units may benefit of less spectral preprocessing or even not preprocessing at all compared with measurements with relative units (%) [19,20]. However, there are other researchers who found that concentrations or relative units worked best for their applications or obtained mixed results. Spielbauer [19] utilizing the same instrument as Tallada et al. [20] got better results with relative units (mass %) when predicting oil and protein, but better results when working with absolute values when predicting starch. Dowell et al. [55] also found that working with relative units (ppm instead of ng) was the best when predicting DON or ergosterol in wheat kernels. The impact on working with mass percentage (relative units) or absolute content units at the end depends on the instrument, application, and seeds characteristics (commodity and physicochemical traits). But even if calibration precision could be improved by

working with absolute values, producers and breeders prefer percentage mass units. The simultaneous prediction of seed mass and compound in absolute units could be a good alternative to obtain predictions expressed in % mass. However, the errors from both calibrations and transformation to % would be added in the final predictions and turn them in unacceptable.

5. NIR measuring modes and instrumentation

Although the way to assure the best calibrations is by assembling a device optimized for single seed analysis, most of the literature reporting successful single seed applications used commercial instruments, initially designed for bulk grain samples or other general purposes. Some commercial brands have designed adapters for small samples in order to gain instrument versatility. Those work well for single seeds. NIR technologies most utilized in the literature include dispersive devices such as grating monochromators (GM) and diode arrays (DA), Fourier Transform (FT), and chemical imaging units (CI). Diode array instruments measure the signal from all the wavelengths simultaneously and are usually the cheapest and the most suitable for fast measurements in rougher environments (i.e. on a field) because they do not contain mechanical parts. Fourier Transform instruments, which measure all the wavelengths at the same time as well but in frequency domain, are mostly seen as laboratory instruments because its higher complexity. FT advantages over diode arrays and monochromators are well known (i.e. higher signal to noise ratio, higher precisions, higher resolution etc.) but those do not generally lead to significant overperformance when working in the NIR region, especially when working with agriculture samples. Resolution, for instance, does not need to be especially high as biochemical complexity of agriculture samples translates in broad spectra with no sharp peaks to be resolved [56]. Chemical imaging is a relatively newer technology in NIR spectroscopy, which provides an additional spatial dimension to the NIR multivariate data (also known as data hypercubes). This allows both identifying and mapping NIR biochemical information. The feature is useful when seed characteristics or compounds under study are located in specific regions of the seeds. For instance, when analyzing oil content in corn kernels, only pixels belonging to the germ region should be considered as most of the oil is located in that region [16]. On the negative side, chemical imaging units are generally slower, have lower signal to noise ratios (SNR), and have lower penetration of the radiation in the sample. The data generated in a single image is over 70,000 times larger than the data from a conventional NIR instrument and need of additional pattern recognition algorithms for selecting the pixels of interest, therefore chemical imaging units require higher computing power.

Both transmittance and reflectance measurements modes have been employed in NIR single seed analyses, but there is no overall best mode when analyzing seeds with homogeneous composition as Dowell et al. [15] and Daun et al. [57] concluded in their study with wheat kernels and canola seeds, respectively. However, when analyzing heterogeneous kernels such as corn, transmittance measurements may not be the best choice. Baye et al. [41] could not predict oil in corn kernels with transmittance and Cogdill et al. [14] were not very successful using imaging transmittance as the calibrations were close to be valid for rough screening (RPD around 1.2). Orman et al. [13] also got similar cross-validation results when working with a traditional single point transmittance instrument (SECV = 1.2% R^2 = 75%). Although it is logical that traditional reflectance instruments report significantly better performance when the kernel germ faces the detector, there are also slight differences in performance due to kernel positioning in transmittance mode. However, the difference between calibrations with germ facing the detector and facing the light source has been reported to be of 0.2 percentage points and cannot be considered significant [13].

When process automation, speed, and flexibility must be taken in account, reflectance mode technologies will be always superior and preferred. Therefore, any application for monitoring or on-line must rely on reflectance diode-arrays. For in-lab applications where analysis time is not a relevant constraint, the use of Fourier-transform or gratting instruments, either reflectance or transmittance, can be considered. Scanning the seeds on movement gives better results than static measurements [23,58], most probably because during seed movement scattering effects are minimized, reducing the variability due to kernel shape and size. Janni et al. [23] observed significantly better results with a patented measurement method [58] where the kernel was tumbling by air flow while the measurement was taken. A similar approach was proposed by Armstrong [59]. His USDA proprietary instrument can scan up to 10 seeds per second if vacuum is applied, while seeds are tumbling in an illuminated silica tube. The whole seed is scanned in movement. That approach eliminates effects from sample positioning while giving an homogeneous illumination of the entire seed and providing a high quality signal. In a recent study carried by Agelet et al. [44] the USDA instrument overperformed four other instruments (Fourier transform transmittance, grating monochromator reflectance, and diode array reflectance) when predicting protein and oil in soybean seeds. Instruments collecting data on a moving seed gave lower prediction errors than the instruments taking static measurements, similar to what Janni et al. [23] reported.

Averaging several spectra from a single seed will increase the signal to noise ratio (SNR). For static measurements, the spectra averaging should not be done on spectra taken respotting the kernels because changes in light scattering due to small changes in position overcome the benefits of spectra averaging [13]. When trying to speed up seed analysis, averaging spectra is not possible, so the quality of the spectra and calibration accuracy will depend on the initial SNR of the device.

6. Quantitative single kernel analysis

It is known that compositional difference within seeds exist within the same plant and even within the same spikelet, in the case of wheat. Wheat kernels located at the below the head of spikelets have higher protein content on average than kernels located on the top [60]. Environmental and field-related characteristics bring compositional changes among samples of the same variety [61,62]. As a result, the variability of major compounds in seeds can be quite large within a batch of kernels harvested from a same field. Quantification of those compounds in single seeds by NIRS aims to a high speed segregation of seeds and grains to narrow down their range and to increase batch uniformity.

Wheat and corn kernels are two of the commodities that have been the most extensively studied at single level by NIRS. Their world-wide significance in human and animal consumption together with the impact of their physiochemical composition on the quality of the end products, raised the interest in having these commodities properly analyzed and sorted. The oldest studies from the 90s focused on wheat kernels and were carried out by The United States Department of Agriculture (USDA). Table 1 summarizes all single seed quantitative applications which were developed scanning whole individual seeds and using single kernel reference data: the range of standard error of prediction and determination coefficients (minimum–maximum) when more than one research or algorithm/preprocessing methods are available, and the best calibration use based on the highest reported validation RPD from all studies applying the suggested thresholds by Williams [7]. When RPD was not available and could not be calculated from the published data, the highest determination

coefficient achieved in validation was used to determine the calibration use based on the correlation given by Kovalenko et al. [43]. SEPs expressed as % come from calibrations developed with % of the measured compound mass over seed mass (dry, as is, or at fixed moisture rate), unless specified. The technology or technologies that were utilized for each application are mentioned using the abbreviations explained at the bottom of the table.

Comparing the results of several researches is complicated for several reasons besides any difference due to the instrumentation. Firstly, the samples used to develop the calibrations and to validate them differ between researches. The NIR spectral variability between seeds coming from different regions, crop-year, seasons, and varieties can be large. If few samples and seeds were involved in the calibration and validation process, the chances of having isolated, not extrapolable results are high because the calibration is not robust and representative of the prediction sample set. Most probably, another research using the same number of seeds will report significantly different errors of prediction and calibration predictive performances. The minimum number of seeds and varieties to be included in the calibration will depend on the characteristics of the commodity, instrument, and use of the calibration (i.e. quality control or screening, the extension and location of the seeds to be analyzed in the future etc.).

Secondly, the reported statistics by several authors may not be comparable with each other. When NIRS technologies were still under development, some researchers would only report the correlation coefficient between predicted and real values, which although providing an idea of calibration performance, it does not give information about the expected error in future predictions. Comparing cross-validation statistics with independent validation statistics, as already mentioned, may also be misleading. On the other hand, a calibration with lower prediction errors does not have to be always better. A study which shows the lowest SEP does not mean it has the best performance if the compound covered range by the calibration or validation set is significantly shorter than the one covered by other studies. Last but not least, there are other differences to be considered when comparing calibration statistics such as prediction units, reference methods, calibration algorithms, or moisture basis among others.

Across all commodities moisture is not surprisingly the attribute measured with most success (best calibration suitable for any purpose and quality control). Protein and oil have been predicted with low errors and allowed the best reported calibrations to be used for quality control for some of the commodities. Corn, due to its heterogeneity, is one of the commodities that report the lowest precision for those compounds. Soybean seeds, on the other hand, report calibrations with the best performance. Starch can only be screened. Starch is also measured with higher errors in bulk samples, similar to what has been seen in the Iowa State University Grain Quality Laboratory for corn (data not shown). Seed mass is another attribute which have been measured with relative success. Although most researchers reported mass calibration performance for screening for most commodities (RPD=2.5), soybean seed dry mass can be predicted with high accuracy (Table 1). It is expected that using dry mass as reference values or mass at current moisture level lead to different results. Any increase in moisture content in the seeds may lead to swelling, and since mass measurement seems to be correlated to seed shape [44] a negative impact and decrease in accuracy may be expected. While protein and oil calibrations including high moisture samples have been successful for bulk samples, the effect of moisture in predicting attributes such as oil, protein, and mass in single seeds has not been reported yet.

There are studies that have not reported successful results for the same applications as in Table 1, most likely due to instrumental and sample limitations. For instance, Delwiche et al. [85] worked in predicting protein in soybean seeds with a monochromator transmittance device using absolute units. The obtained calibration was unusable (RPD=1.2, $R^2 < 0.50$, RMSEP=13.93 g/kg). In that same work an attempt to predict inorganic phosphorous was carried with no success as well in transmittance (SEP=568.6 mg/kg, RPD=1.2), but worked better for reflectance (SEP=248 mg/kg, RPD=2.8). Because NIRS does not quantify inorganic compounds, it was probably indirectly measured through other compounds. Hom et al. [38] attempted to measure total aromatic compounds but had extremely low R^2 (36%), and SECV=0.34 μmol/g of dry mass. On the other hand, some of the reported studies may be overoptimistic due to the small data sets analyzed and deficient validation process, or have some controversy involved. The quantification of mycotoxins by NIRS is one of the studies that has brought most attention and controversy at the same time. Several studies have shown that mycotoxin contamination does not occur evenly in all the kernels and location within a batch, but rather a small fraction of highly contaminated kernels [25,86–91]. That means that if there was a fast and non-destructive method to detect and remove the highly contaminated kernels entire batches of corn could be still recovered and used as livestock feed. Some feasibility studies have shown promising results, but it has not been proved that developing a stable model involving several varieties, crops, and crop-years is possible. Even if detection of indirect changes on corn kernels due to fungi and mycotoxin can be done, the high variability from both corn kernels and fungi strains put this application under a big question mark.

7. Qualitative and discriminative applications

Chemical imaging units are more utilized for qualitative studies than for quantitative, probably because the mapping capabilities and the advantage of analyzing several individual seeds at the same time while targeting compounds that are not distributed homogeneously in the seed (i.e. oil in corn kernels), diseases that happen in specific seed areas (i.e. wheat black tip), and monitoring biological processes in the seed. Most of the applications tested with NIR chemical imaging have been also tested with single-point technologies with comparable results. For instance, classification of vitreous or hard-soft kernels has been done with traditional single point instruments, but the use of chemical imaging allowed Manley et al. [12] to have a closer look to the biochemical properties of the kernels and to find a third class of endosperm in corn that had combined features of flourly and vitreous endosperms together. Therefore, chemical imaging is shown to be a powerful tool in qualitative applications and provides additional understanding of biological processes of seeds. Some of the qualitative or discriminative applications are the detection of aflatoxins [92], fungi infection and damage [11,93–104], germination [105–109], discrimination of grain varieties, seed types, or impurities [110–115], insect damage [105,116–119], color using short wavelengths [120,121], and characterization of single grains and seeds [19,122–126]. Although most of those applications are reported to be successful, Manley et al. [127] advise that previous to develop any single seed application with chemical imaging, the variation of the seed shape and texture has to be evaluated versus the change of the chemical compound of interest, as the major sources of variability in chemical imaging spectra come from seed shape and texture.

Tables 2 and 3 summarize the main qualitative and discriminative applications of NIR chemical imaging and ‘conventional’ single-point technologies on single seeds, respectively. Note that there are over 4 times more discriminative applications with chemical imaging than quantitative applications with chemical imaging. If more than one class is discriminated in the study, the overall sorting accuracy is the average of the partial sorting accuracies. If there is more than one citation for an application, or different algorithms/preprocessing methods have been tested leading to more than one sorting accuracy, the range of achieved

accuracies is displayed. When no specific accuracy was reported, the research was mainly qualitative, or retrieving results was not possible it is indicated with 'n.a.'. Table 3 shows in the third column all NIR single-point technologies that were used for each application according to current literature, using abbreviations explained at the bottom of the table.

Among the studies listed in both tables, the ones focused on detecting and sorting by mold damage and insect infestation have the most extensive related literature. Chemical imaging technologies allowed early detection and tracking of fungi development on kernels when wavelengths from the visible region were also used [11,93,95]. Discrimination of insect-damaged or infested kernels is another well-studied application. Some insect-damaged kernels are easily removed by cleaning, but insects growing inside the grain are invisible for methods based on visual inspections [166]. Accuracies in segregating kernels contaminated with insects depended of the size of the larvae. Kernels with large larvae could be discriminated with accuracies up to 94%, small larvae lead to accuracies of only 63% [154]. When the larvae is large enough, high discrimination accuracies were possible using a short wavelength range [151]. The most relevant wavelengths in insect infestation were the ones related to water (involved in the metabolic processes of the insects), protein, lipids, phenolic compounds, and carbohydrates because of the absorption of chitin from the insect cuticle and a decrease of starch levels in the grain [151–153]. Other type of damage such as heat or frost have also been analyzed with NIRS. Wang et al. [138] analyzed several damage types in soybeans. Heat damage results (accuracies over 95%) agreed with those from Agelet et al. [135] for corn kernels. However, while soybeans damaged by frost could be successfully discriminated with accuracies above 95% when using non-linear classifiers such as Artificial Neural Networks (ANN) [138], corn kernels damaged by frost could not be discriminated by any of the tested algorithms [135]. Early frost damage in corn mainly affects the germ and any changes caused by cold seem not detectable by NIRS. The early detection of seed viability or plant abnormality is not possible with conventional single-point technologies [135], which agrees with Wang et al. [138] with soybean seed sprouting. For pine seeds, the results of Lestander [161] in sorting pine seeds by their viability were high probably because they used artificially killed seeds for the non-viable class, instead of seeds non-viable due to aging such as Agelet et al. [135] Chemical imaging technologies, on the other hand, have been more suitable to identify early sprouting according to the available studies for wheat kernels [105–108].

Discriminating varieties or wheat classes is an indirect way to improve the quality of grain batches. The overall accuracies for discriminating kernels from different wheat varieties can be very high (Tables 2 and 3), especially when varieties with different colors are scanned on the visible region together with the NIR region. When analyzing all wheat classes, durum is the class that is classified from the rest with the highest accuracy. Classes that have the same color (i.e. Hard red spring vs. hard red winter) will often lead to higher misclassified seeds compared to discriminative models involving classes with different colors (red vs white), even if the visible region is not involved in sorting because soft wheat varieties have a specific texture which NIR can identify [62]. Spectral differences may arise in NIR spectra between seeds which cannot easily be determined by conventional reference analysis. For that reason, unsupervised sorting based on specific variability sources of scanned grains, even if the source of variability is unknown in advance, can improve the quality of wheat batches and the resulting flour [167].

Sorting and discrimination can be also based on quantifiable attributes such as protein, oil or moisture after setting sorting thresholds according to quality targets and calibration error. Calibrations which errors are large for accurate quantification but are acceptable for screening, can still be utilized to discriminate kernels based on 'high' and 'low' amount of the measured compound. For breeders, farmers, and industrial applications, segregating seeds according to high-low content of a specific compounds allows shifting the overall average of the batch. This is often good enough to improve plant genetics or the quality of end-products. Current studies have also shown a good repeatability of the low-high classification results. Using the information of the visible wavelengths in addition of NIR, Delwiche et al. [140] segregated wheat samples between high and low protein with sorting accuracies ranging 78–98% – with lowest accuracies coming from discriminating kernels within a same class. Dowell et al. [65] sorted wheat kernels from four bins according to

Table 2
Major commodities and qualitative/discriminative applications developed with chemical imaging NIRS at single seed level: overall sorting accuracies (a range is reported if different researches and methods are available, 'n.a.' indicates no specific data avilable) and main references.

Commodity	Sorting application	Overall Sorting accuracy (%)	Mode	Citation
Corn	Different varieties	99	Reflectance	[110]
	Hardness	n.a.	Reflectance	[12,122]
	Aflatoxin	n.a.	Reflectance	[92]
	Fungal infection	94	Transmittance, Reflectance	[93,94,95]
Soybeans	Genetically modified soybeans	77–90	Reflectance	[29]
	Classes	87–100	Reflectance	[111]
	Mold infection	0–100	Reflectance	[97]
	Mold: Scab	83–95	Reflectance	[11,96,98–102]
	Black tip	95	Reflectance	[103]
	Ergot	99.9	Reflectance	[104]
	Sprouting	97–100	Reflectance	[105–108]
	Insect infestation	85–100	Transmittance, Reflectance	[105–119]
Wheat	Color and stain	n.a.	Reflectance	[120,121]
	Vitreousness	94–100	Reflectance	[102,123,124]
	Viability	n.a.	Reflectance	[125]
	Moisture behavior	n.a.	Reflectance	[128,129]
Barley	Sprouting	37–77	Reflectance	[109]
	Viability	n.a.	Reflectance	[125]
Rice	Classes &Varieties	80–100	Reflectance	[112–114]
Sorgum	Viability	n.a.	Reflectance	[125]
Seed mixtures	Oats vs groats	97–99	Reflectance	[115]
Spinach	Mold infected	26–88	Reflectance	[126]

Table 3
Major commodities and qualitative/discriminative applications developed with single-point NIRS at single seed level: overall sorting accuracies (range is reported if different researches and methods are available, 'n.a.' indicates no specific data avilable), NIR technologies used, and main references.

Commodity	Sorting application	Overall Sorting accuracy (%)	Technology[a]	Citation
Corn	Haploid vs hybrids	92	GMT	[130]
	Fumonisin	80–90	DAR, DAT	[15,25,131,132]
	Aflatoxin	25–99	DAR, DAT	[25,131,132]
	Fungal infection	85–100	GMR, DAR	[133,134]
	Frost damage	60–68	DAR	[135]
	Heat damage	88–99	DAR	[135]
	Viability	38–51	DAR	[135]
Soybeans	Viability (1) and vigor (2)	(1) 48.5–62, (2) n.a.	DAR	(1) [135], (2) [136]
	Aging	60–100	DAR	[137]
	Genetically modified soybeans	72–98	DAR, GMR	[28–30]
	Fungal contamination	83–100	DAR	[138,139]
	Frost damage	72–97	DAR	[138]
	Heat damage	84–97	DAR	[138]
	Sprout	54–64	DAR	[138]
	Weather	61–98	DAR	[138]
Wheat	Class separation	65–100	DAR, GMR, PGT	[140,141]
	Hardness	n.a.	DAR	[142]
	Vitreousness	72–100	DAR	[26,27,143]
	Mold: Scab	77–97	DAR	[144–149]
	Mold damage	95–98	DAR	[145]
	Color	99–100	DAR	[63,150]
	Insect infestation	62–99	DAR, GMR	[63,151–154]
	Waxy kernels	47–95	DAR,GMR	[70,155,156]
	Heat damage	91–100	DAR	[157]
Rice	Pecky rice	99–100	DAR	[158]
Pine and tree seeds	Empty seeds	92–100	GMT, GMR	[159,160]
	Viable	90–100	GMT	[160,161]
	Vigour	75–100	GMT	[162]
	Parents and Origins	91–96	GMR	[163]
	Insect damage-infested	90–100	GMR	[160,164]
Seed mixtures	Weed, wheat, sunflowers, stubble	n.a.	GMR	[165]

[a] Abbreviations: Diode array reflectance (DAR), Fourier-Transform reflectance and transmittance (FTR and FTT, respectively), Gratting Monochromator reflectance and transmittance (GMR and GMT, respectively), prism gratting transmittance (PGT), Dual beam transmittance (DBT).

hardness and protein content and were able to segregate 4 bins with increasing protein fraction intervals of 1%. The average difference of bins with high and low protein was 3.1% points. For hardness, the average difference between bins was of 5.7 hardness units, and the maximum difference between the lowest and highest hardness bins was 19.9 h.u. Some authors have also worked with few wavelengths for discriminating wheat kernels according to their protein content, with just multiple linear regression (MLR) [168] or setting signal threshold at specific wavelengths [148]. However, using few wavelengths when discriminating multiple classes of wheat (hard red, durum etc.) is not accurate enough [140]. Pasikatan and Dowell [168] using wavelengths 920 and 1660 nm discriminated high protein ($>12.5\%$ at 12% moisture weight basis) and low protein ($<11.5\%$, 12% m.b.) wheat kernels. With a maximum of two consecutive sorting processes, initial blends of 95:5 could lead to a final protein concentration of the batch equal to the dominant protein class. Pearson et al. [148] developed a LED sorting system working with just few wavelengths from the NIR region (840, 940, 1070 nm) and Vis region (470, 527, and 624 nm). The system achieved sorting speeds of 20 kernels/s at low cost (roughly 1000 dollars). In that case, the instrument was just calibrated to identify kernels with potentially high protein and to identify potentially contaminated kernels with *Fusarium* head blight. The final sorted sample, 40% of the initial unsorted batch, achieved a protein average of one percent point higher than when unsorted.

8. Conclusions

Near infrared technologies have been utilized for in single seed analysis, mostly targeting corn, wheat, and soybeans. Quantitative analysis of major organic compounds such as moisture, oil, and protein lead to most calibrations usable for any purpose. The predictive ability of the calibration is mainly given by the commodity (kernel size and heterogeneity) and the instrumentation characteristics. Although no measurement mode (reflectance, transmittance) have lead to the best reported calibrations, when dealing with heterogeneous seeds reflectance is the best working mode. On the other hand, scanning seeds while tumbling or spinning aid in developing calibration with higher precisions and overcome any other difference between instruments (measurement mode, average of scans, technology etc.). Preprocessing methods such as SNV, MSC, detrending and derivatives work well when developing calibrations using relative units such as percentage. However, when predicting seed mass no preprocessing works better than plain apparent absorbance. Good results have been also achieved when working with absolute units for compounds such as protein or oil, but the use of either relative or absolute units will be given by the instrument and characteristics of the samples and compound to be measured. Calibration models suitable for screening may still be used for discrimination of seeds with low and high compound concentrations. Those models can successfully disaggregate wheat mixtures, increasing the overall homogeneity of the seed batch.

The future of NIR single kernel sorters will be driven by the needs of specific applications. Optimization and customization of optical components and sampling systems are required to ensure the success of an application on a specific commodity. On the other hand, while high sorting speeds may be desirable, accuracy and precision are often sacrificed when speeding up the analyses. Similar to what happens already with NIT bulk analyzers, most

probably NIR single kernels analyzers will continue being built based on potential end users: Breeders and laboratory devices (lower speed, higher accuracies, more human interaction required) and farmers, traders, and food industries (high speed, high robustness, high degree of automation, lower accuracy).

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