Exhibit 6a, #56

Automated Nondestructive Detection of Internal Insect Infestation of Wheat Kernels by Using Near-Infrared Reflectance Spectroscopy

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ABSTRACT Wheat kernels infested internally with larvae of 3 primary insect pests of grain, the rice weevil, *Sitophilus oryzae* (L.); the lesser grain borer, *Rhyzopertha dominica* (F.); and the Angoumois grain moth, *Sitotroga cerealella* (Olivier), were scanned with a near-infrared spectrometer incorporated into a single kernel characterization system to determine differences in absorption due to the presence of larvae. The single kernel characterization system delivers kernels into the spectrometer viewing area at the rate of 1 per 4 s. We were able to differentiate uninfested kernels from kernels infested with larvae of all 3 species by using this automated system. Moisture content, protein content, or wheat class did not affect classification accuracy. The calibration included spectral characteristics in the wavelength ranges of 1,000–1,350 and 1,500–1,680 nm. Larval size was a factor in the sensitivity of the system, with 3rd and 4th instars rice weevil being detected with 95% confidence. In contrast to many other procedures used to detect internal insect infestations in grain, this system could be incorporated into the current grain inspection process and provide the grain industry with quantitative data on internal insect infestations in wheat.

KEY WORDS rice weevil, lesser grain borer, Angoumois grain moth, detection, near-infrared, wheat

POSTHARVEST GRAIN LOSSES caused by pests and poor storage practices total more than $1 billion per year in the United States (Cuperus and Krischik 1995). Grain is inspected for the presence of insect infestations to minimize losses associated with insect damage during storage and transportation in both domestic and export markets. The U.S. standards consider wheat, *Triticum aestivum* L., to be infested if ≥2 live insects injurious to wheat are found in a 1-Kg sample (USDA 1991). In addition, inspectors consider kernels insect damaged if boring or tunneling by insects on the kernel surface is observed. The wheat grade is reduced to U.S. Sample Grade if ≥32 insect-damaged kernels are found in a sequential sampling of a total of 100 g of wheat. These labor-intensive inspection procedures identify the presence of adult insects outside the wheat kernels but may not detect immature insects developing within kernels. Thus, a sample may appear uninfested or undamaged although it contains kernels with a developing population of immature insects. Storey et al. (1982) reported that an initial inspection found 4% of wheat samples obtained from 79 U.S. elevators were infested with insects. However, after incubating the samples for 4–6 wk, 16% of the samples were discovered to be infested. This indicates that many grain samples contain hidden larvae although no adult insects are detected at the time of inspection. These hidden infestations can cause serious quality losses during subsequent storage and transportation.

Although sieving easily detects adult insects feeding outside wheat kernels, internal infestations are more difficult to detect. Several procedures have been developed to identify infested kernels. These include staining kernels to detect weevil egg plugs (Frankenfeld 1948, Milner et al. 1950), density separations (Milner 1958), crushing kernels between ninhydrin-impregnated paper (Dennis and Decker 1962), detection of carbon dioxide produced by insects (Bruce et al. 1982), detection of uric acid by using high-performance liquid chromatography (Wehling and Wetzel 1983), nuclear magnetic resonance spectroscopy (Chambers et al. 1984), x-ray analysis of kernels (Schatzki and Fine 1988), acoustical sensors to detect insects feeding within grain kernels (Hagstrum et al. 1990), and enzyme-linked immunosorbent assays of kernel extracts (Schatzki et al. 1993). All these procedures can detect internal insects or larvae in kernels with varying degrees of success. However, these techniques are slow; labor-intensive; difficult to automate; require expensive, specialized equipment; or are applicable only to specific insect species. Therefore, a better method is needed to detect insects inside wheat kernels.

Near-infrared spectroscopy (NIRS) is a procedure that can rapidly detect and measure the chemical composition of biological materials. Molecules comprising organic matter vibrate at discrete frequencies corresponding to specific wavelengths in the infrared

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Table 1. Description of samples used for insect larval detection tests

Variable tested	Wheat class	Moisture content, %	Protein content, %	Insect species
Class	Hard red winter	11.3	11.3	Rice weevil
	Hard red spring	12.3	12.1	Rice weevil
	Soft red winter	10.4	11.2	Rice weevil
	Soft white	12.5	12.3	Rice weevil
	Hard white	12.3	12.3	Rice weevil
Protein	Hard red winter	13.2	11.3	Rice weevil
	Hard red winter	12.8	16.2	Rice weevil
Moisture	Hard red winter	10.0	11.3	Rice weevil
	Hard red winter	11.3	11.3	Rice weevil
	Hard red winter	13.2	11.3	Rice weevil
Species	Hard red winter	11.3	11.3	Rice weevil
	Hard red winter	11.6	11.3	Lesser grain borer
	Hard red winter	11.6	11.3	Angoumois grain moth

region. When the wavelength of the incident infrared energy corresponds to the frequency of vibration of a specific molecule, this energy is absorbed by the molecule. Optical sensors measure this absorption and the amount can be related to the concentration of a particular constituent. Thus, NIRS can measure concentrations of components of different molecular structure such as protein, water, or starch (Murray and Williams 1990).

Ridgway and Chambers (1996) used NIRS (1,100–2,500 nm) to detect insect larvae in single wheat kernels and external infestations in bulk samples. Their study showed differences in absorption possibly due to insect cuticular chitin present in the kernels. Although they showed that NIRS can detect larvae in kernels, the minimum size of larvae detectable and the effects of moisture, protein, wheat class, and insect species on the sensitivity and accuracy of the method were not studied. The objective of the current research was to determine how these variables affect the detection of hidden insects in wheat by using an automated, single-kernel NIRS measurement system.

Materials and Methods

Wheat samples were selected to test the effect of class (hard red winter, soft red winter, hard red spring, soft white, and hard white), protein content within class, moisture content within class, and 3 insect species infesting a single class on the accuracy of detecting hidden insect larvae of different sizes by automated NIRS of individual kernels. Table 1 lists the moisture and protein contents for each of these samples.

Wheat Class. One cultivar with similar protein content from each of the 5 classes was selected to test for the effect of wheat class. The moisture contents of these cultivars were adjusted to ≈12.0% by adding an appropriate amount of water followed by equilibration for 2 wk over a saturated solution of sodium bromide (NaBr) at 25°C resulting in an equilibrium relative humidity of 58% (Greenspan 1977). Equilibrium moisture contents were determined with the single kernel characterization system (Martin et al. 1993).

Protein Content. To determine the effect of protein on detecting internal larvae, the moisture content of 2 cultivars of hard red winter wheat with significantly different protein contents (11.3 and 16.2%) were equilibrated as described above. The Federal Grain Inspection Service determined the protein content of the samples by using standard NIRS procedures.

Moisture Content. The effect of moisture content on internal larval detection was determined by adjusting samples of a hard red winter wheat to 3 different moisture contents by the addition of water followed by equilibration over saturated solutions of potassium carbonate (K_2CO_3), NaBr, and sodium chloride (NaCl) at 25°C. This resulted in equilibrium relative humidities of 43, 58, and 75% (Greenspan 1977) and measured equilibrium moisture contents of 10.0, 11.3, and 13.2%, respectively.

Insect Species. To determine the detection performance of the automated system for immature stages of different internal insect species, a hard red winter wheat was selected and equilibrated to ≈12% moisture as described above. The test insects were 3 primary storage pests: the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae); the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); and the Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae). Stock cultures of these species were maintained on whole wheat at 27°C and 50–60% RH.

Infestation Procedure. To obtain wheat kernels with different-sized larvae of the rice weevil, 20-g samples of wheat were placed in clear, plastic vials (16 dram, 3.2 by 8 cm) with screen lids, and equilibrated for 2 wk at the appropriate temperature and relative humidity conditions. Four adult female rice weevils (≈1 wk old) were removed from stock cultures and placed in each vial. The adult weevils were removed after 14 d, and a 2nd equilibrated 20-g wheat sample was infested with 4 new female weevils. For the other 2 species, 10 adult lesser grain borers or 10 Angoumois grain moths were placed on 20 g of equilibrated wheat in the vials. The adults were removed at weekly intervals and fresh samples infested each succeeding week for 4 wk. Using these infestation methods, we obtained all possible ages of larvae for detection analysis after 4 wk because egg-to-pupal development requires ≈28 d at 27°C for each of these species (Birch 1945, Sharifi and Mills 1971b, Strong et al. 1967). This infestation procedure was used for all tests.

X-ray Procedure and Size Determination. Wheat kernels from each treatment were x-rayed (Throne 1994), and radiographs were examined on a light table to identify infested kernels. About 100 infested kernels, containing approximately equal numbers of small, medium, or large larvae, were selected from each treatment. Larval size was estimated by an area measurement calculated from the length and width measured on the radiograph by using a 7× micrometer. We also x-rayed and selected kernels not exposed

to insects, and kernels exposed to insects but not infested with larvae for each treatment to use in the detection analysis. Each measured kernel was uniquely identified and NIR spectra collected within 8 h of x-raying to minimize changes in larval growth or kernel moisture content.

Spectra Collection and Data Analysis. A diode-array, near-infrared spectrometer integrated with a single kernel characterization system (Perten Instruments, Reno, NV) was used to automatically collect spectra from single wheat kernels. The spectrometer measures absorbance from 400–1700 nm by using an array of silicon and indium–gallium–arsenide sensors and collects data at a rate of 30 spectra per second. The system automatically delivered and randomly positioned a single kernel in the spectrometer viewing area. Eight spectra were collected from a kernel before another kernel was delivered to the viewing area. The 8 spectra were averaged to reduce noise and recorded in 5-nm increments. Kernels were delivered at a rate of 1 kernel per 4 s, although 2 kernels per second is achievable. The spectra were stored on a hard-disk for subsequent analysis by using GRAMS/32 software (Galactic, Salem, NH).

Spectra were analyzed using partial least squares (PLS) regression, a spectral decomposition technique similar to principal component regression (Martens and Naes 1989). The PLS regression uses concentration data during the decomposition process and includes as much information as possible into the first few loading vectors. It also takes advantage of the correlation between the spectral data and the constituent concentrations. All data were mean centered before analysis. Half of the kernels in each treatment were used to develop a calibration and the remaining kernels were used for validation.

Our 1st step in analyzing the spectra was to determine which wavelengths were most important in detecting insect-infested kernels. We plotted correlations between absorbance and larval size, enabling us to select wavelength regions with the highest correlation coefficients. We then compared results of PLS regression by using data from all wavelengths to results with selected wavelengths. This enabled us to minimize the number of wavelengths used in the calibration while maintaining classification accuracy. Slopes, intercepts, and coefficients of determinations (r^2) were compared using procedures described by Steel and Torrie (1980) and Rice (1989). The maximum attainable r^2 was calculated using the method of Draper and Smith (1981), which is based on the premise that no equation can explain variation among observations measured at the same x-value.

Results and Discussion

System Calibration and Classification. The results obtained when correlating absorbance to larval size showed that little classification information resulted from absorbance in the visible or very near infrared (<1,000 nm) region (data not shown). A comparison of classification accuracies (r^2 and standard error values) resulting from calibrations with and without these regions confirmed this observation (Table 2). Excluding wavelengths below 1,000 nm probably does not improve classification for 2 reasons. First, absorbances from 700–1000 nm are primarily caused by weak 3rd overtones of fundamental absorbances that are difficult to measure (Murray and Williams 1990). Secondly, there are no visible differences between infested and uninfested kernels so no differences would be evident in the spectra from 400–700 nm. Thus, wavelengths below 1,000 nm were not used in final calibrations.

Table 2. Comparison of classification results using various wavelength ranges

Wavelength range, nm	No. factors[a]	r^2	SEP
450–1,680	18	0.63	0.94
1,000–1,680	17	0.66	0.90
1,000–1,350, 1,500–1,680	17	0.67	0.89

SEP, standard error of a prediction.
[a] Number of classification factors in the partial least squares regression analysis.

Kernels with internal larvae are typically higher in average moisture content than kernels without larvae because of water present in the larva itself and because insect respiration indirectly raises kernel moisture (Ridgway and Chambers 1996). However, kernel moisture does not always indicate infestation because kernels with low moisture content that contain larvae could absorb radiation similarly to high-moisture kernels with no larvae. Excluding wavelengths near ≈950 nm and between 1,350–1,500 nm improved the classification because this excluded most wavelengths at which water absorbs radiation (Murray and Williams 1990). Tests on samples with various moisture ranges supported this observation. When the calibration did not exclude moisture effects, larvae in samples with lower moisture content were predicted with an r^2 value as low as 0.10. Excluding moisture effects improved the r^2 to 0.63.

Based on the calibration studies, the wavelength ranges selected for analyzing the wheat kernels were 1,000–1,350 nm and 1,500–1,680 nm. Although these ranges include wavelengths at which protein absorbs radiation (1,100–1,200 and 1,500–1,680; Murray and Williams 1990), tests with wheats of the same class (hard red winter) but of different protein levels (11.3 and 16.2%) showed little effect of protein on the detection of internal insect larvae (Table 3).

Spectra of ground samples of cuticle from 5th instars of tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), and also the β(1-4)-linked hexasaccharide of 2-acetamido-2-deoxy-D-glucopyranoside derived from crab chitin (Sigma, St. Louis, MO) were analyzed to determine if absorbance peaks derived from insect cuticle or chitin are similar to wavelengths found useful for detection. Spectra of both compounds showed absorbance peaks at ≈1,178 nm and 1,500 nm, which correspond with wavelength ranges selected for classification. Thus, kernels containing larvae may have absorbed more radiation at

Table 3. Relationship between predicted and actual larval size by using a 17-factor partial least squares calibration over the wavelength ranges of 1,000–1,350 and 1,500–1,680 nm

Variable tested	Treatment	n	r^2	% Max r^2	Slope	SE	Intercept	SE
	All samples	577	0.70[a]	84	0.73[a]	0.02	0.43[b]	0.04
Protein	11.3%	60	0.73a	79	0.63a	0.05	0.56a	0.11
	16.2%	60	0.65a	74	0.66a	0.06	0.35a	0.14
Moisture	10.0%	60	0.61a	72	0.61a	0.06	0.36a	0.10
	11.3%	59	0.77a	80	0.64a	0.05	0.69a	0.10
	13.2%	60	0.73a	79	0.63a	0.05	0.56a	0.11
Species	AGM	60	0.79a	82	0.85a[c]	0.06	0.29ab	0.20
	LGB	60	0.57a	62	0.87a	0.10	0.05a	0.11
	RW	59	0.77a	81	0.64a[c]	0.05	0.69b	0.10
Class	Hard red winter	59	0.77a	81	0.64a	0.05	0.69a	0.10
	Soft red winter	56	0.58a	81	0.61a	0.07	0.58a	0.15
	Hard red spring	41	0.66a	73	0.69a	0.08	0.52a	0.14
	Hard white	60	0.72a	75	0.60a	0.05	0.62a	0.12
	Soft white	60	0.56a	59	0.75a	0.09	0.75a	0.16

Means in a column for the same group and followed by the same letter are not significantly different at the $P = 0.05$ level, unless otherwise noted. AGM, angoumois grain moth; LGB, lesser grain borer; RW, rice weevil.

[a] No r^2 values or slopes were significantly different from those for all samples ($P = 0.05$).

[b] No intercepts were significantly different from those for all samples ($P = 0.02$).

[c] Angoumois grain moth and rice weevil slopes are not significantly different at $P = 0.02$.

these wavelengths due in part to the chitin present in insect tissues (Kramer and Koga 1986).

Using 1st or 2nd derivatives of spectra (Hruschka 1990) did not improve detection of internal larvae. The number of PLS factors selected (17) for the calibration model was the optimum recommended by PLS analysis. Adding or deleting several factors did not significantly affect detection results. We used the calibration equations to predict larval size in the validation data sets, and regressed predicted larval size on actual larval size to determine the accuracy of predictions (Table 3). The PLS calibration that included samples representing all variables (combined samples across protein and moisture contents, insect species, and wheat classes) was the best for predicting the size of larvae. The equation attained an r^2 of 0.70, which was 84% of the maximum possible r^2.

Figure 1 shows a plot of the actual versus predicted larval size for the calibration that included all samples. Protein or moisture content, insect species, or wheat class did not affect classification accuracy (Table 3). The r^2 values did not differ significantly ($P = 0.05$) within treatment groups or from the overall value. Thus, the variation in the data explained by each model is similar for all variables examined. No slope of any of the treatment groups differed significantly ($P = 0.05$) from the slope for all samples, and no intercept differed significantly ($P = 0.02$) from the intercept for all samples (Table 3). There was a difference between intercepts of the insect species with the rice weevil having a greater y-intercept than the lesser grain borer. This results in rice weevil larval size being slightly overpredicted at values less than ≈ 2 mm^2 and under-predicted at values greater than ≈ 3 mm^2 by using the equation for all variables combined.

Spectra of kernels exposed to insects but containing no larvae were compared with kernels not exposed to insects. The test samples originated from the same wheat sample and were subjected to the same environmental conditions. No difference existed between exposed and unexposed kernels, as indicated by a low r^2 of 0.13 ($n = 336$).

Larval Size. The equation based on all variables detected larvae as small as 2 mm^2 with 95% confidence (Table 4). The minimum detectable size was 1.1 mm^2 for lesser grain borers, 2.0 mm^2 for rice weevils, and 2.7 mm^2 for Angoumois grain moths. Although Angoumois grain moth larvae must be 2.7 mm^2 or larger to be predicted with 95% confidence, mature Angoumois grain moth larvae are larger than larvae of the other 2 species.

Because infested samples would typically contain larvae of all ages, one would expect to detect infested samples with greater confidence than predicting the larval size within an infested sample. In most sampling

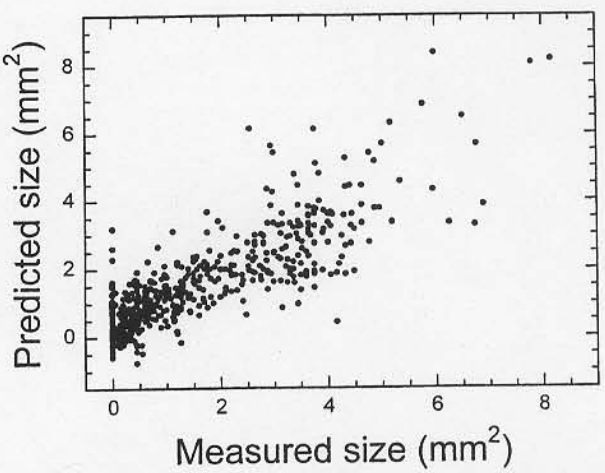

Fig. 1. Actual versus predicted larval sizes resulting from a 17-factor PLS calibration over the wavelength ranges of 1,000–1,350 and 1,500–1,680 nm ($r^2 = 0.70$, slope = 0.73, intercept = 0.43). Larvae were combined across all treatments: moisture contents, protein contents, wheat class, and insect species.

Table 4. Minimum larval size (width × length) detectable in individual wheat kernels at specific confidence level

Variable	Treatment	Min. detectable size (mm^2) at				
		99%	95%	90%	80%	50%
	All	2.4	2.0	1.7	1.5	1.0
	All without LGB	2.5	2.0	1.8	1.5	1.0
Protein	11.3%	2.2	1.8	1.6	1.4	1.0
	16.2%	2.4	1.9	1.7	1.4	0.9
Moisture	10.0%	1.9	1.5	1.4	1.1	0.8
	11.3%	2.2	1.8	1.6	1.4	1.1
	13.2%	2.2	1.8	1.6	1.4	1.0
Species	AGM	3.4	2.7	2.3	1.8	1.1
	LGB	1.4	1.1	0.9	0.7	0.4
	RW	2.4	2.0	1.7	1.5	1.1
Class	Hard red winter	2.4	2.0	1.7	1.5	1.1
	Soft red winter	2.8	2.2	1.9	1.7	1.2
	Hard red spring	2.2	1.8	1.6	1.4	1.0
	Hard white	2.3	1.9	1.7	1.5	1.1
	Soft white	3.3	2.7	2.3	2.0	1.4

AGM, angoumois grain moth; LGB, lesser grain borer; RW, rice weevil.

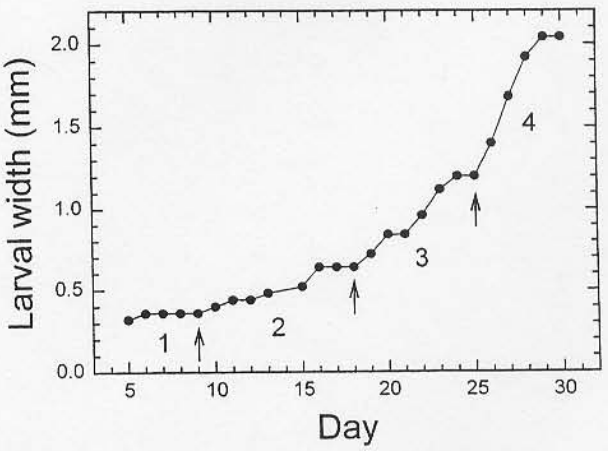

Fig. 2. Maize weevil larval width measured by radiography as a function of day of development. Arrows indicate end of molt and numbers indicate instar.

situations, the ability to detect infested samples is more important than predicting larval size. If lesser grain borers are suspected in samples from regions where the rice weevil or Angoumois grain moth are not typically found, then the minimum detectable larval size for 95% confidence could be reduced to 1 mm^2.

Correlation of Measured Size to Instar. The length of rice weevil larvae was correlated with their width (slope = 1.7 ± 0.0095; intercept set = 0; $r^2 = 0.98$; $n = 758$). A larva that measured 2 mm^2 would be 1.1 mm wide and 1.8 mm long. Sharifi and Mills (1971a) determined mean head capsule widths of rice weevil larvae that corresponded to the 4 instars. Because head capsule width for their largest larvae (0.6 mm) was only 1/3 the size of the body width of our largest rice weevil larvae (1.8 mm), their results were not useful for relating our measurements to instar. Throne (unpublished data) used x-rays to study the daily development of larvae of the maize weevil, *Sitophilus zeamais* Motschulsky, in corn, (*Zea mays* L.), kernels. The maximum width of maize weevil larvae (2.0 mm) was approximately the same as the maximum width of rice weevil larvae (1.8 mm) observed in our study. These data for maize weevils were used to determine rice weevil instar in the current study. Molting periods were assumed to occur when larval width did not change for ≥1 d. A rapid change in larval width follows this period (Fig. 2). The mean maximum widths of 1st, 2nd, and 3rd instars were 0.37, 0.57, and 1.22 mm, respectively. The standard errors of the maximum widths of 1st, 2nd, and 3rd instars were 0.01, 0.03, and 0.06 mm, respectively. Corresponding maximum lengths for these instars would be 0.63, 0.97, and 2.1 mm, respectively. Corresponding maximum width × length would be 0.23 mm^2, 0.55 mm^2, and 2.56 mm^2 for 1st, 2nd, and 3rd instars, respectively. Thus, the NIRS method can detect 3rd and 4th instars in wheat kernels with 95% confidence.

A major source of classification error is likely to be the random placement of the kernel in the instrument viewing area. Related research on wheat color class measurement showed that orientation (e.g., crease-up versus crease-down) can influence classification (Dowell 1997). However, a commercial automated larval detection device probably would not orient kernels. Thus, these results should represent the potential for larval detection with a commercial unit.

This automated method of nondestructively detecting internal insect larvae could be integrated into the current grain inspection process. The single kernel characterization system currently analyzes a 300-kernel sample in under 3 min. Addition of the NIRS would not slow the operation of the single kernel characterization system and could provide important information about the presence of insect-infested kernels, information that the industry needs to monitor and manage insect infestations in wheat.

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