

Reflectance and Transmittance Spectroscopy Applied to Detecting Fumonisin in Single Corn Kernels Infected with *Fusarium verticillioides*

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ABSTRACT

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Reflectance and transmittance visible and near-infrared spectroscopy were used to detect fumonisin in single corn kernels infected with *Fusarium verticillioides*. Kernels with >100 ppm and <10 ppm could be classed accurately as fumonisin positive or negative, respectively. Classification results were generally better for oriented kernels than for kernels that were randomly placed in the spectrometer viewing area. Generally, models based on reflectance spectra have higher correct classification than models based on transmittance spectra. Statistical analyses

indicated that including near-infrared wavelengths in calibrations improved classifications, and some calibrations were improved by including visible wavelengths. Thus, the color and chemical constituents of the infected kernel contribute to classification models. These results show that this technology can be used to rapidly and nondestructively screen single corn kernels for the presence of fumonisin, and may be adaptable to on-line detection and sorting.

Fumonisin are mycotoxins produced by the fungi *Fusarium moniliforme*, *F. proliferatum*, and other fusaria and can be found in corn throughout the world (Munkvold and Desjardins 1997). Both *F. moniliforme* and *F. proliferatum* produce a series of toxins on corn of which fumonisins B₁ and B₂ are considered to have cancer-promoting activity (Bacon and Nelson 1994). The most abundant of these, fumonisin B₁, has been linked to reported incidences of leukoencephalomalacia (Ross et al 1991), pulmonary edema in pigs (Colvin and Harrison 1992), cancer in rats (Gelderblom et al 1992), and esophageal cancer in humans (De Nijs et al 1998).

The Food and Drug Administration currently is reviewing a draft of maximum allowable levels of total fumonisin in corn products destined for human or animal consumption. For human consumption, the allowable levels are 2–4 ppm for different corn food products. For animal consumption, the levels are 5–100 ppm for different animal feeds (U.S. FDA/CFSAN 2000). Recommended maximum fumonisin B₁ concentrations of 5–50 ppm for different livestock feeds (Neogen 1999) were adopted by the American Association of Veterinary Laboratory Diagnosticians.

Some of the existing methods for detecting fumonisin in corn and corn by-products include liquid chromatography (Rice et al 1995; Sydenham et al 1996); gas chromatography-mass spectroscopy (Pestka et al 1994); thin-layer chromatography (Schaafsma et al 1998); capillary zone electrophoresis (Maragos 1995); high-performance liquid chromatography with an electrospray mass spectrometer and evaporative light scattering detectors (Plattner et al 1996); immunosorbent assays (Pestka et al 1994); and immunofluorescence assays (Scott and Trucksess 1997). Simultaneous screening of mycotoxins also was developed using an image analysis system in combination with a line immunoblot assay (Abouzied and Pestka 1994). Miyahara et al (1996) developed an

ion pairing chromatographic separation with postcolumn derivatization with *o*-phthalaldehyde and subsequent fluorometric detection.

The more recent techniques require ≈30 min to process each sample. Some of the existing methods are considered expensive and laborious and may require extensive extraction, clean up, and derivatization procedures. Thus, studies to establish a fumonisin-detection procedure that is simple, rapid, and inexpensive are continuously evolving and developing.

Reflectance or transmittance visible and near-infrared (NIR) spectroscopy has been used to detect other single-kernel corn and wheat attributes such as class (Delwiche and Massie 1996), color (Dowell 1998), damage (Dowell et al 1999), protein (Delwiche 1995), aflatoxin (Pearson et al 2001), oil content (Orman and Schumann 1992), and levels of transgenic traits (Kramer et al 2000). Automated systems for detecting attributes of single seeds have been described by Pearson (1999) and Dowell et al (1998).

The objective of the research described here was to explore the potential of reflectance and transmittance visible and NIR spectroscopy for detecting fumonisin in single corn kernels infected with *Fusarium*. Real-time detection would allow identifying and removing fumonisin-contaminated single kernels from all corn destined for food or feed use. Rapid detection of fumonisin in samples would identify lots with significant fumonisin levels, thus providing information for segregating contaminated lots before they are commingled with lots with low or negligible fumonisin levels.

MATERIALS AND METHODS

Corn Samples

Pioneer 3394 corn kernels were harvested in 1999 from ears that were wound-inoculated with *Fusarium verticillioides* (Sacc.) Nirenb. (synonym = *F. moniliforme* Sheldon) NRRL 25457 in the late milk to early dough stage of kernel maturity (≈21 days after mid-silk; July 22, 1999) at the University of Illinois River Valley Sand Farm Experimental Station, Killbourn, IL. This strain of *F. verticillioides* was isolated from corn grown near Furman, SC (Wicklow et al 1998) and produces fumonisin B₁ (Bartelt and Wicklow 1999). Eighty ears were inoculated by making a knife cut along the axis of the ear (≈5 cm long and deep enough to cut into the kernels). The *F. verticillioides* inoculum was applied as a conidial suspension using a sterile pipe cleaner (8 cm) that was inserted into the wound and left there to mark the wound site at harvest.

Shortly after harvest on September 16, 1999, the ears were shelled by hand, the wounded kernels discarded, and the whole, nonwounded corn kernels examined for visible symptoms of *Fusarium* infection. Kernels then were separated into one of five categories based on visual characteristics: 1) no visible symptoms of fungal infection; 2) reddish-purple streaks restricted to the pericarp/aleurone;

¹ USDA-ARS, Grain Marketing and Production Research Center, 1515 College Avenue, Manhattan, KS 66502. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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3) a small portion of the endosperm (<20%) showing a white discoloration; 4) ≈40–60% of surface appearing shrunken and discolored; and 5) shriveling and discoloration over the entire surface. A total of 330 kernels (60–90 kernels from each category) were selected randomly, and each kernel was placed in an individual chamber of a plastic pill box.

The corn was stored in a freezer at ≈−7°C between experiments. Kernels were shipped overnight from the USDA ARS National Center for Agricultural Utilization Research (NCAUR), Peoria, IL to the USDA ARS Grain Marketing and Production Center, Manhattan, KS, for reflectance analysis. Kernels then were shipped overnight to the USDA ARS Western Regional Research Center, Albany, CA, for transmittance analysis. Finally, they were shipped overnight back to the NCAUR for chemical analysis.

Spectra Measurement

Transmittance spectroscopy. Whole-kernel transmission spectra from 550 to 1050 nm were measured using a fiber optic spectrometer (S2000, Ocean Optics, Dunedin, FL) with a sensor resolution of ≈0.31 nm and an optical resolution of ≈10.0 nm. Each kernel was randomly placed with the long axis of the kernel perpendicular to the sensor as shown in Fig. 1. The spectrometer collected 20 complete transmission spectra on each kernel and stored the average. The integration time of each sensor element was 5 msec.

The light source was a 100W quartz-tungsten-halogen lamp coupled to a 3-mm diameter fiber optic bundle (77501, Oriol, Stratford, CT). The transmittance spectra were measured from light emitting from an ≈3-mm diameter circle in the middle of each kernel that was lying flat, as shown in Fig. 1. Transmitted light through the kernel was collected by a collimating lens and transmitted to the spectrometer with a fiber optic. A light standard and dark standard were obtained between sampling each kernel. The dark standard was obtained by blocking the light source with a steel shutter. The light standard was obtained by replacing the sample with a glass neutral-density filter with a transmission of 0.1% (35-5941, Ealing, Holliston, MA). The enclosure around the kernel was fabricated from square steel tubing (2.54 cm × 2.43 cm × 5 cm long) painted black. The tube was closed by welding a steel cap to one end, and a piece of rubber was held to the other end while spectra were being sampled.

Reflectance spectroscopy. Whole-kernel reflectance spectra from 400 to 1700 nm were measured using a diode-array, NIR spectrometer (Pertin Instruments, Springfield, IL) as shown in Fig. 2. The spectrometer measures absorbance using an array of silicon (7 nm

resolution) and indium-gallium-arsenide sensors (11 nm resolution). The spectrometer sampled 15 spectra and stored the average in <1 sec. The spectra were stored for subsequent analysis using GRAMS/32 software (Galactic Industries Corp., Salem, NH).

The spectra of 330 single corn kernels with various fumonisin amounts were collected at both germ-down (germ facing the sensor) and germ-up positions. The kernels were placed manually on a bifurcated interactance probe attached to the spectrometer and light source. The viewing area was 17 mm in diameter, the illumination bundle was a 7-mm diameter ring, and the reflectance probe bundle was 2 mm in diameter.

Chemical Analyses

Total fumonisin (B₁, B₂, and B₃) was measured with a fluorometer after extracts were purified with immunoaffinity columns (Fumonitest, Vicam, Watertown, MA) using the procedure recommended for corn, sorghum, and 17% protein poultry feed (Ware et al 1994). Kernels were weighed individually and then placed in groups of five according to their classification based upon symptoms of fungal infection and numerical sequence within each pill box. The fumonisin level of each five-kernel group then was assigned to each individual kernel from that group. Kernels were analyzed in groups instead of individually to reduce cost and analysis time.

The kernels were placed within an envelope of weighing paper and crushed using a hammer; the fragments were transferred to a silitated 4-dram vial. The extraction procedure followed the manufacturer's instructions but was scaled appropriately for a smaller sample weight. Then NaCl (0.1 × sample weight) was added to the fragmented corn kernel sample followed by a volume of 80% methanol and water (v/v) that was equivalent to twice the sample weight. The samples were vortexed for 1 min and filtered through Whatman 2V filter paper into a clean test tube. The extract was diluted (1:4 v/v) with phosphate buffered saline (PBS)/0.1% Tween-20 Wash Buffer and pushed through a Whatman GF/B 1.0-μm syringe filter into another clean test tube. Based on preliminary analyses of the fumonisin levels determined for larger subsamples of each category of *F. verticillioides* infected kernels, the quantity of diluted extract passed through the column was adjusted (i.e., 0.1, 1, 2, or 5 mL) so as not to exceed a column capacity of 8 ppm of fumonisin. The column then was washed once with 10 mL of PBS/0.1% Tween-20 and washed a second time with 10 mL of PBS. The fumonisin was eluted from the affinity column using 1.0 mL of HPLC grade methanol and collected into a glass cuvette to which 1 mL

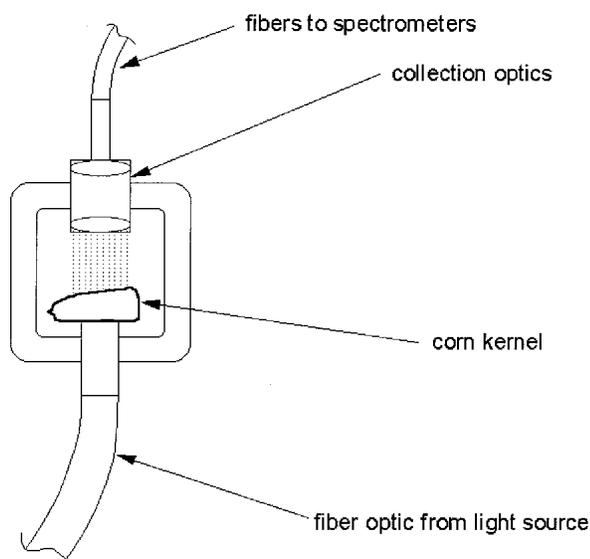


Fig. 1. Transmittance sampling apparatus.

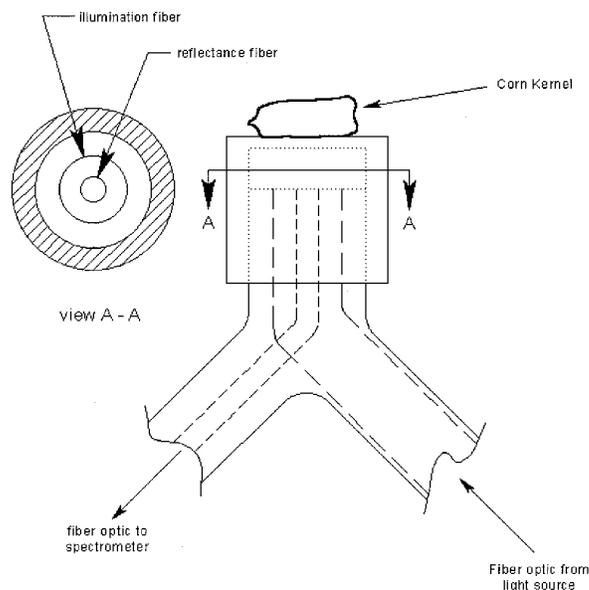


Fig. 2. Reflectance sampling apparatus.

of Fumonitest Developer A and B mixture also was added. The cuvette was vortexed and placed into a calibrated fluorometer to read the fumonisin concentration.

Data Analyses

Partial least squares (PLS) analysis. A PLS regression (Martens and Naes 1989) with a sequential one-sample-out cross-validation was used to classify kernels as fumonisin positive (≥ 10 ppm) or fumonisin negative (< 10 ppm). The wavelength regions used were 600–1,050 nm for the transmittance analysis and 550–1,700 nm for most reflectance analyses. Beta coefficients were obtained to determine the influence of wavelength regions on classification models. All data were mean centered before analysis.

Discriminant analysis. After acquisition of all spectra, all possible ratios of two absorbance values were computed using absorbance values at increments of ≈ 5 nm for the transmittance spectra and ≈ 15 nm for the reflectance spectra. This resulted in totals of 4,656 ratio values for each kernel using the transmittance spectra and 3,157 ratios using the reflectance spectra. Feature sets of wavelength ratios from each spectra type (reflectance or transmittance) were analyzed separately. However, only a few of these features can be measured economically during a high-speed sorting operation. An exhaustive search was performed for the best single feature and combination of two features to classify kernels as fumonisin positive or negative. Kernels were considered fumonisin positive if their measured fumonisin concentration were greater than or equal to one of three different threshold levels (10, 50, and 100 ppm). Discriminant analysis was used as the classification procedure with both pooled and nonpooled covariance matrices (Huberty 1994). The Mahalanobis distances were computed from each kernel to the fumonisin positive and negative groups. A kernel was classified into the group with the lowest corresponding Mahalanobis distance. Sample means and covariance matrices for each group were computed using odd-numbered samples only. The feature set

that obtained the lowest classification error rate for each fumonisin threshold level on the even-numbered samples was recorded.

RESULTS AND DISCUSSION

Table I shows the distribution of fumonisin from the five-kernel groups in the various categories of visual defects. All groups containing fumonisin > 100 ppm were from the 100% shriveled and discolored category. Most (83%) of the groups with an average fumonisin of 10–100 ppm were from the 40–60% shriveled and discolored category. However, all groups of asymptomatic kernels had measurable fumonisin, with a mean of 1.8 ppm. Because these asymptomatic kernels were harvested from *Fusarium* inoculated ears, levels of fumonisin contamination might have exceeded background levels for asymptomatic kernels from noninoculated ears. Some individual kernels might have had higher or lower fumonisin levels than indicated by the five-kernel group averages.

The asymptomatic kernels had the highest average weight (0.36 g) and the lowest fumonisin concentration (1.8 ppm). The kernels that were 100% shriveled and discolored showed the lowest average weight (0.13 g) and the highest fumonisin concentration (476.7 ppm). The other three categories had similar average kernel weights, but fumonisin concentrations varied considerably (3.6, 6.1, and 42.6 ppm). Although lighter weight kernels tended to have higher fumonisin concentrations, kernel weight alone may not be a consistent indicator of fumonisin levels.

Spectral Characteristics of Infected Kernels

Plots of the average transmittance ($\log[(S_0 - D)/(S - D)]$ where S_0 is the light standard spectrum, D is the dark spectrum, and S is the measured spectrum through the sample) and germ-up reflectance ($\log(1/R)$) spectra are shown in Figs. 3 and 4, respectively, for kernels with fumonisin levels > 100 ppm, 10–100 ppm, and < 10 ppm. The average reflectance spectra of kernels with the

TABLE I
Summary by Category of Kernel Weight and Fumonisin Concentration of Corn Kernels Used for Collecting Reflectance and Transmittance Spectra

Category	n	Kernel Weight ^a (g)		Fumonisin Concentration ^b (ppm)		Distribution of Fumonisin (%)			
		Average (SD)	Min-Max	Average (SD)	Min-Max	nd ^c	0–10 ppm	10–100 ppm	>100 ppm
Asymptomatic	60	0.36 (0.05)	0.22–0.54	1.8 (1.7)	0.5–7.0	0	100	0	0
Red purple streak	60	0.28 (0.08)	0.09–0.47	3.6 (3.8)	0.0–14.0	8.3	83.4	8.3	0
<20% discolored	60	0.31 (0.07)	0.20–0.46	6.1 (9.5)	1.0–37.0	0	91.7	8.3	0
~50% discolored	90	0.28 (0.07)	0.10–0.44	42.6 (24.0)	1.6–72.0	0	16.7	83.3	0
~100% discolored	60	0.13 (0.05)	0.04–0.25	476.7 (115.0)	280.0–610.0	0	0	0	100
Total	330			100.4		1.5	54.5	25.8	18.2

^a Kernel weight was based on individual kernel weights of the 330 corn kernel samples.

^b Fumonisin concentration was based on averages of five-kernel groups.

^c Not detectable.

TABLE II
Fumonisin Classification Results Using Ratios of Two or Four Wavelengths in a Discriminant Analysis (DA)^a or All Wavelengths in a PLS Analysis^b

Kernel Orientation	Analysis Type	Wavelengths (nm) Used for DA Ratios or PLS Analysis	False Positive Errors (%) ^c		False Negative Errors (%) ^c		Total Kernels Incorrectly Classed (%)
			nd ^d	1–10 ppm	10–100 ppm	>100 ppm	
Trans.	Random	DA 845/955, 815/935	0	1.9	73.0	1.7	20.2
		DA 640/910	0	5.8	43.5	0	14.4
		PLS 600–1,050 (3) ^e	0	5.6	60.0	0	18.5
Refl.	Germ-up	DA 715/790, 1,315/1,615	0	2.2	31.8	0	9.4
		DA 625/1,555	0	5.0	31.8	0	10.9
		PLS 550–1,700 (2)	0	0.6	40.0	0	13.6
	Germ-down	DA 745/775, 1,555/1,660	0	7.2	23.5	0	10.0
		DA 835/1,030	0	2.8	48.3	0	14.0
		PLS 550–1,700 (2)	0	2.2	64.7	0	17.9
Random	DA 745/925, 820/880	0	3.3	37.7	0	11.5	
	DA 730/1,360	0	2.2	51.8	0	14.5	

^a Covariance matrices pooled for all discriminant analyses except for 2-wavelength reflectance analyses.

^b Results calculated from transmitted and reflected energy.

^c False positive kernel < 10 ppm of fumonisin classified as fumonisin positive. False negative kernel ≥ 10 ppm of fumonisin classified as fumonisin negative.

^d Not detectable.

^e Number of PLS factors used in calibrations.

germ-down (data not shown) were very similar to those of the kernels with the germ-up. The reflectance spectra showed that absorbances of kernels with high levels of fumonisin were generally higher in the visible region of the spectrum (<750 nm), and the opposite was seen at longer wavelengths. On the other hand, the transmittance spectra absorbances of fumonisin-contaminated kernels were generally higher at wavelengths <900 nm. These results agree with those achieved from studies of single kernels infected with *Aspergillus flavus* and contaminated with aflatoxin (Pearson et al 2001). Apparently, the fungus causes the kernel to darken and reduces the vitreousness. The increase in absorption caused by the kernel discoloration would explain the increase in transmittance and reflectance absorbances at visible wavelengths. In the NIR region, the scattering caused by fungal damage may cause more energy to be reflected back toward the light source and, thus, not transmitted through the kernel. This would be measured as more absorption by transmittance spectroscopy because light does not pass through an infected kernel as readily as through a healthy vitreous kernel. However, this scattering would be measured by reflectance spectroscopy as less absorption in the NIR region because infected kernels scatter light back toward the sensor. Although trends related to fumonisin levels are seen in average transmittance and reflectance spectra, variations between single kernel spectra prevent using absorbance values at one wavelength in classifications.

Spectra from kernels with >10 ppm of fumonisin were compared to spectra from kernels with >10 ppm of aflatoxin obtained from Pearson et al (2001). PLS cross-validation results showed 97% of the kernels could be correctly classified as containing fumonisin or aflatoxin. The beta coefficients showed that wavelengths at ≈450–500 nm contribute most to classifications. This region corresponds to the blue-green region of the spectrum, and implies that the color of *Fusarium* and the aflatoxin-producing fungus *Aspergillus flavus* differ significantly. Other influences such as crop year and cultivar were not studied but may not affect the predictive ability of the models if these factors are included in the calibrations.

Classification Results

In all cases, the lowest errors were achieved using a fumonisin threshold of 10 ppm to define kernels as fumonisin positive or negative. The classification results when using threshold values of 50 and 100 ppm to consider kernels as fumonisin positive or negative did not yield as accurate results as the 10-ppm threshold (data not shown). Classification results were excellent (almost no errors) for kernels with no detectable fumonisin and with >100 ppm of fumonisin (Table II). Kernels with 1–10 ppm of fumonisin (<7%) were classified incorrectly as fumonisin positive. Classification was generally

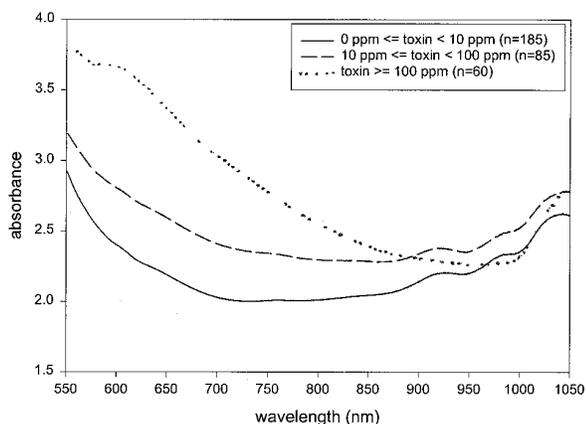


Fig. 3. Plot of average absorbance spectra when analyzing transmitted energy for corn containing different levels of fumonisin. Transmittance was measured as $\log[(S_0 - D)/(S - D)]$ where S_0 is the light standard spectrum, D is the dark spectrum, and S is the measured spectrum through the sample.

poor for kernels with 10–100 ppm of fumonisin (error rates 23.5–73.0%). Generally, the lower the rate of misclassified <10 ppm kernels, the higher was the rate of misclassified 10–100 ppm kernels. Thus, selecting a different classification threshold can improve classifications in the 10–100 ppm region while decreasing the classification accuracy for kernels with lower fumonisin levels. In all cases, classification results using discriminant analysis were best when using linear discriminant functions (pooled covariance matrices).

Classification results would likely improve if fumonisin had been measured in each kernel instead of measuring fumonisin in five-kernel groups. For example, an individual kernel giving a false positive error may actually have >10 ppm of fumonisin if the fumonisin levels in the other four kernels of that group caused the average to be <10 ppm.

Kernel orientation effects. Classification results were generally better for oriented kernels than randomly placed kernels, which was expected because orientation variability is removed from the classification models. However, there were no clear trends as to which orientation, germ-up or germ-down, resulted in best classifications.

Reflectance vs. transmittance. The single-feature (ratio of two wavelengths), two-feature (two ratios of two wavelengths), and PLS classification models based on reflectance were generally better than the respective models based on transmittance. However, more experimentation with a larger data set would be needed to determine which of the classifications (one or two features, reflectance or transmittance spectra) is most robust.

Wavelength regions of interest. The PLS beta coefficients for transmittance spectra (data not shown) indicated broad regions at ≈650, 710, 935, and 990 nm contributing to classification models. These regions generally agreed with those in the discriminant analysis (Table II). The PLS beta coefficients for reflectance spectra collected from kernels placed germ-up or germ-down were almost identical to each other, which was expected, with wavelength regions at ≈590, 995, 1,200, and 1,410 nm contributing to PLS reflectance classification models. Also, for the reflectance analysis, some wavelengths indicated by PLS beta coefficients occurred at wavelengths selected in the one- or two-feature discriminant analysis, whereas other wavelengths used in the discriminant analysis occurred in regions with minimal influence on PLS calibrations. Most classification models utilized some information from visible wavelengths (<700 nm), which indicates that the visible damage to the kernel by the fungus is related to fumonisin levels. However, all classification models also included some NIR wavelengths, which indicates that chemical constituents in asymptomatic and infected kernels are different. Thus, the presence of the fungus and the subsequent production of fumonisin could affect such kernel characteristics as color, vitreousness, protein structure, or oil content. Any of these changes would be manifested as

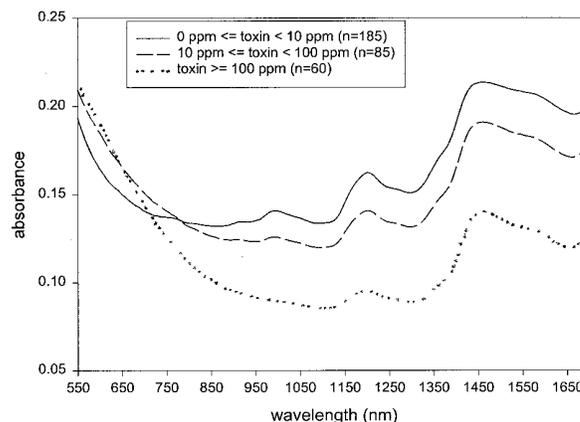


Fig. 4. Plot of average absorbance spectra when analyzing reflected energy for corn kernels placed with the germ facing away from the sensor. Absorbance was measured as $\log(1/R)$.

a difference in reflectance or transmittance spectra obtained from healthy versus damaged kernels. Neither the spectra nor the beta coefficients indicated that the ability to predict high and low levels of fumonisin is due entirely to scattering. Thus, the calibrations likely are relying on changes in kernel color and kernel composition related to fumonisin levels. Fumonisin itself probably is not influencing NIR spectra because constituents present at the ppm level do not absorb detectable amounts of NIR energy.

Potential Applications

Commercial sorting machines are available that can measure transmittance (Anzai et al 1993) or reflectance (Satake USA, Houston, Texas; Sortex, Newark, CA) of whole corn kernels at two discrete wavelengths and compute their ratios in a high-speed sorting operation (5,000–10,000 kg/hr). However, instrumentation that utilizes reflected versus transmitted energy generally is easier and more cost-effective to implement. Implementation of a two-feature discriminant function utilizing four wavelengths or full wavelength PLS calibration would require some hardware and software development for online applications. Further study would be needed to warrant development of a sorting system capable of utilizing additional wavelengths. Technology does exist for developing a system to sort samples using reflected or transmitted energy and with calibrations utilizing all wavelengths at a rate of ≈ 1 kernel/sec.

Because fumonisin is not uniformly distributed among all kernels and may occur only in a few highly contaminated kernels within a sample (Whitaker et al 1998), bulk-sample NIR analysis will not be as sensitive to fumonisin contamination as single kernel analysis. Although this single kernel technique is not sensitive at the average FDA allowable levels (2–4 ppm), there will likely be several kernels with high levels of fumonisin in samples with low average fumonisin levels. These kernels with high fumonisin levels would be detected by the single kernel system and thus indicate that the entire lot may have an average fumonisin level exceeding FDA levels. The number of kernels that would need to be analyzed would be determined by the allowable sampling error and time constraints for specific situations. Thus, this research shows that reflectance or transmittance spectroscopy may have practical applications toward detecting fumonisin in single corn kernels for screening samples or for online detection.

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