Utilisation of visible/near-infrared hyperspectral images to classify aflatoxin B₁ contaminated maize kernels

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ARTICLE INFO

Article history:
Received 27 March 2017
Received in revised form
22 October 2017
Accepted 28 November 2017
Published online 15 December 2017

Keywords:
Aflatoxin B₁
Factorial discriminant analysis (FDA)
Maize kernel
Visible/near-infrared hyperspectral image

A visible/near-infrared (VNIR) hyperspectral imaging (HSI) system (400–1000 nm) was used to assess the feasibility of detecting aflatoxin B₁ (AFB₁) on surfaces of 600 kernels of four maize varieties from different regions of the U.S.A. i.e. Georgia, Illinois, Indiana and Nebraska. For each variety, four AFB₁ solutions (10, 20, 100 and 500 ppb) were artificially applied on kernel surfaces. Similarly, a control group was generated from 30 kernels of each variety treated with a solution of methanol. Principal component analysis (PCA) was used to reduce dimensionality of the HSI data followed by the application of factorial discriminant analysis (FDA) on the principal component variables. PCA results showed a pattern of separation between uncontaminated and contaminated kernels for all varieties except for Indiana and pooled samples. FDA showed the ability to predict AFB₁ contamination of each variety with over 96% validation accuracy while prediction for AFB₁ contamination group membership of pooled samples reached 98% accuracy in validation. Variation in the spectra of AFB₁ contaminated kernels could have caused the variation in the predicted AFB₁ contamination group membership. The PCA and FDA models where influenced by the chemical information from C–H, N–H and O–H bonds of VNIR spectral regions. This study presents the potential of using VNIR hyperspectral imaging in direct AFB₁ contamination classification studies of maize kernels of different varieties. The study further suggests that varietal differences of maize kernels may have no influence on AFB₁ contamination classification.

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1. Introduction

Maize is one of the major agricultural cereals grown worldwide. However, maize is susceptible to infection by toxigenic fungi, both in the field and during post-harvest storage. The fungal infections can reduce the quality and nutritive value of grains due to contamination with fungal secondary metabolites such as mycotoxins, which are toxic to both livestock and human beings. To prevent the contamination of maize kernels by aflatoxin B₁, the need for rapid and reliable detection and classification methods that can be used for on-farm or on-site screening of contaminated grain is very important.
Nomenclature

**Abbreviation** | **Definition**
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VNIR | Visible/near-infrared
NIRS | Near-infrared Spectroscopy
HSI | Hyperspectral Imaging
AFB1 | Aflatoxin B1
PCA | Principal Component Analysis
FDA | Factorial Discriminant Analysis
IARC | International Agency for Research on Cancer
HPLC | High Performance Liquid Chromatography
USDA-ARS | United States Department of Agriculture-Agricultural Research Services
MNF | Minimum Noise Fraction
ENVI | Environment for Visualising Images
ROI | Region of Interest
SNV | Standard Normal Variate
SGS | Savitzky–Golay Smoothing

Humans (Wu & Sun, 2013a). Aflatoxins are mycotoxins produced by Aspergillus species of fungi, such as *Aspergillus flavus* and *Aspergillus parasiticus* (Pearson, Wicklow, & Pasikatan, 2004; Shahin & Symons, 2011). Aflatoxin B1 (AFB1) is the most potent carcinogen and has been directly linked to long term adverse health effects, such as lung cancer in man and liver cancer in some animal species (Abrar et al., 2013). Consequently, around the world, organisations such as the International Agency for Research on Cancer (IARC) have recognised AFB1 as a Group 1 carcinogen for animals and humans (Fernández- Ibáñez, Soldado, Martínez-Fernández, & De la Roza-Delgado, 2009). At the same time, governments such as U.S.A., through the U.S. Food and Drug Administration, have long been strictly monitoring and regulating aflatoxin levels in human food and animal feed, and have set 20 ppb as the concentration limit for maize (Abbas, Cartwright, Xie, & Shier, 2006; Pearson et al., 2004). Therefore, the early detection of fungal infection and mycotoxin contamination of maize plants and kernels can be useful to prevent the entry of mycotoxins into the food chain (Del Fiore et al., 2010; Williams, Geladi, Britz, & Manley, 2012).

Commonly used analytical chemical methods to detect the toxicity in grains and feeds include enzyme-linked immuno-sorbent assay (ELISA), molecular identification approaches and chromatography techniques such as thin layer, gas and high performance liquid chromatography (HPLC). These methods have merit for being accurate and selective with very low detection limits. However, they are also expensive, destructive, and can be difficult and time-consuming. Hence, they are not well suited for handling a large number of grain samples. In order to detect mycotoxins on an industrial scale, it is critical and necessary to develop a rapid and non-destructive method. A faster, non-destructive method for detecting fungal and mycotoxin contamination is offered by spectroscopy. In this regard, spectroscopy related studies have been carried out, for example, Dowell, Ram, and Seitz (1999) used near infrared (NIR) spectroscopy to detect scab damage, vomitoxin and ergosterol in wheat. They showed that all scab-damaged kernels were correctly identified and more kernels with vomitoxin were identified by their method than did a visual inspection. In addition, vomitoxin and ergosterol were predicted with standard errors of 40 and 100 ppm, respectively. Pearson, Wicklow, Maghirang, Xie, and Dowell (2001) detected aflatoxin in maize with transmittance and reflectance spectroscopy. Using discriminant analysis and partial least squares regression, the authors analysed transmittance spectra (500–950 nm) and reflectance spectra (550–1700 nm) obtained on whole corn kernels that exhibited various levels of bright greenish-yellow fluorescence and reported that over 95% of the kernels were correctly classified as containing either high (>100 ppb) or low (<10 ppb) levels of aflatoxin. Similar results were obtained when using either transmittance or reflectance and when using either discriminant analysis or partial least squares regression. Dowell, Pearson, Maghirang, Xie, and Wicklow (2002) detected fumonisin in maize kernels infected with *Fusarium verticillioides* using reflectance and transmittance visible and near infrared spectroscopy. The authors reported that kernels with >100 ppm and <10 ppm were correctly classified as fumonisin positive or negative respectively, and models based on reflectance spectra had higher correct classification than models based on transmittance spectra. Wang, Dowell, Ram, and Schapaugh (2004) used a diode-array reflectance NIR spectrometer (400–1700 nm) to classify healthy and fungal-damaged soybean kernels and discriminate among various types of fungal damage. Partial least squares (PLS) and neural network models were developed by the authors and the highest classification accuracy was over 99% when spectra of 490–1690 nm were used with a two-class PLS model, whereas a five-class classification with neural networks yielded higher classification accuracy than PLS model. Berardo et al. (2005) detected kernel rots and mycotoxins in naturally and artificially contaminated maize samples using NIR reflectance spectroscopy and the samples were analysed for fungal infection, ergosterol and fumonisin B1 content. The authors indicated that the best predictive ability for global fungal infection and *F. verticillioides* was produced with a calibration model utilising maize kernels and maize meals, respectively. However, the problem with conventional spectroscopy is that it provides an average spectrum of a particular sample without any spatial information (Mishra et al., 2015; Wang, Heitschmidt et al., 2015), meaning that trace mycotoxins in a large sample may be missed (Folder, Van Der Heijden, Waalwijk, & Young, 2005).

Hyperspectral imaging (HSI) is an emerging technology that integrates both imaging and spectroscopy into one system capable of recording both spatial and spectral properties of a given sample (Ropodi, Panagou, & Nychas, 2016; Shrestha, Knapić, Zibrat, Deleuran, & Gislum, 2016; Siche et al., 2016; Wu & Sun, 2013b). Because HSI obtains a complete spectrum for each pixel in an image (Wu & Sun, 2013b), it is possible to identify unique spectral signatures at multiple locations within a given sample. These signatures are the result of the physical and chemical characteristics of the particular material analysed (Shrestha et al., 2016). The information from VNIR hyperspectral imaging has been used in varietal identification studies for maize kernels (Wang, Sun, Pu, & Zhu, 2016; Zhang, Liu, He, & Li, 2012) using principal component analysis.
(PCA) and factorial discriminant analysis (FDA) based on chemometric techniques. Furthermore, investigations involving VNIR hyperspectral imaging or short-wave NIR hyperspectral imaging have shown the feasibility of aflatoxin detection and fungal identification studies on maize (Pearson & Wicklow, 2006; Wang, Heitschmidt et al., 2015; Wang, Ni et al., 2015; Williams et al., 2012). However, these studies involved kernels from a single maize variety, thus requiring further investigation to examine the influence of the variation in kernels due to maize variety on aflatoxin or fungal detection.

NIR spectroscopy measures overtones and combination bands of the fundamental molecular vibrations found in the infrared region associated mainly with C–H, N–H, and O–H functionalities which are due to hydrogenic stretching, bending, or deformation vibrations (Berardo et al., 2005; Shenk, Workman, & Westerhaus, 2008).

Stretching vibrations occur at shorter wavelengths such as those used in this study (400–1000 nm). A sample material such as maize kernel or aflatoxin in this case, selectively absorbs NIR radiation that yields information about the molecular bonds within the material being measured. Shenk et al. (2008) stated that an NIR absorption band is produced when NIR radiation at a specific frequency (wavelength) vibrates at the same frequency (wavelength) as a molecular bond in the sample. Therefore, various functional groups at specific spectral bands may be correlated to the major maize kernel or aflatoxin constituents. According to Fernández-Ibáñez et al. (2009) and Del Fiore et al. (2010), the spectral information in the visible region of between 400 and 600 nm corresponds to colour changes especially of carotenoids and chlorophyll in cereal grains. Several infrared spectroscopic methods have been developed to identify aflatoxin and fungi in cereal grains (Fernández-Ibáñez et al., 2009; Pearson et al., 2004; Pearson et al., 2001; Wang et al., 2015a, b). Pearson et al. (2001) used the spectral ratio 735/1005 nm for aflatoxin detection to optimally separate highly contaminated corn kernels (>100 ppb) from the less contaminated (<10 ppb). Based on reflectance spectra (500–1700 nm) of yellow maize kernels, Pearson et al. (2004) used 750 nm and 1200 nm to correctly identify over 99% of kernels as aflatoxin contaminated (>100 ppb) or uncontaminated. Tripathi & Mishra (2009) reported that bands related to fungal infection were found between 870 and 1200 nm and associated with N–H bonds in most amino acids and aromatic rings. Wang et al., 2015a, b indicated that wavelengths between 670.2 nm and 985.8 nm played a role in separating normal from pure AFB1 contaminated maize kernels, in addition, the authors identified 606.8, 671.6, 869.4, 917.6, 953.5 and 978.6 nm as key wavelengths for differentiating pure AFB1 contaminated maize kernels based on their concentrations of AFB1.

As mentioned, aflatoxin is a by-product of growth and metabolism of A. flavus in maize and other cereals. Correspondingly, to detect changes of maize nutrients and tissue structure caused by the growth of fungi can be taken as an indirect way for aflatoxin detection. Berardo et al. (2005) reported the possibility of quantifying the infection from fungi and metabolites produced in maize grain and flour by Fusarium verticilloides using NIR spectroscopy. Fernández-Ibáñez et al. (2009) found that NIR spectroscopy was effective to detect aflatoxin presence at 20 ppb. A noteworthy observation is that there is limited literature related to the direct detection of fungal metabolites such as aflatoxin on the maize kernel surface by NIR spectroscopy. Hence, instead of detecting the metabolites produced by fungi in natural infected kernels, or the change in kernel nutritional composition of starch, proteins and lipids consumed by fungi, we would like to determine whether or not pure low level aflatoxin on/in clean maize kernels can be detected directly using VNIR hyperspectral imaging technology, and further we want to determine whether aflatoxin at concentrations as low as 10 ppb on/in clean maize kernels can be detected directly, and differentiated from the other aforementioned kernel attributes. Wang, Heitschmidt, et al. (2015) with VNIR reflectance measurement, showed that detection of pure aflatoxin artificially deposited on maize kernel surfaces was possible at concentrations as low as 10 ppb. However, Wang, Heitschmidt, et al. (2015) involved kernels from a single maize variety and the authors recommended that different maize varieties should be tested in order to build a more universal detection/classification model.

As stated above, most detection methods to date rely on indirect methods to detect aflatoxin, since the toxin’s distribution within the kernel matrix does not allow for direct detection. Although in naturally contaminated maize kernels, the distribution of AFB1 is not uniform over the maize kernels and this does not allow for direct detection, previous studies such as Chu, Wang, Yoon, Ni, and Heitschmidt (2017) reported that AFB1 is mainly distributed on the germ part of the maize kernels. It is noteworthy that Chu et al. (2017), using SWIR hyperspectral imaging, detected AFB1 in maize kernels artificially inoculated with A. flavus in the field. The authors developed a distribution map to visualise the possible distribution of AFB1 in the maize samples and reported that even though the distribution of AFB1 was non-uniform in the kernels, the AFB1 was mainly distributed on the germ part of the kernels. From another study, Fernández-Ibáñez et al. (2009) stated that AFB1 typically infects the maize kernel germ. The authors achieved natural infection of maize and barley kernels with growth of AFB1 at room temperature of 20 ± 2 °C for three months and analysed the samples with NIR spectroscopy. Pearson et al. (2001) reported that initially fungi such as A. flavus typically infects the kernel germ, uses the oil-rich germ for growth and metabolism and thus, the fungus should be more prevalent in the germ part. Therefore, it may be possible that the prevalence of fungi in the germ area may correlate to high distribution of aflatoxin in the germ part of the kernel, which was observed by Chu et al. (2017) and reported by Fernández-Ibáñez et al. (2009).

The objective of our study was to examine the potential application of VNIR hyperspectral imaging to classify AFB1 contaminated maize kernels of four varieties from four representative major producing areas of the United States and get the spectra characteristics of AFB1, as well as to try to verify the possibility of direct detection of aflatoxin on maize kernels. Hence, by applying AFB1 solution to the germ-side surface of kernels and extracting average spectra, this present study, was envisaged to show how artificially AFB1 coated kernels can possibly contribute to a rapid aflatoxin detection method development. The use of average spectra extracted
from the whole kernel germ-side region was expected to limit the influence of inhomogeneous distribution of AFB₁ encountered in naturally infected kernels on the detection accuracy of AFB₁. Considering the fact that conventional detection methods such as chromatography, and ELISA provide AFB₁ concentration value measured basing on whole kernel, the average spectra of the corresponding kernel may have direct correlation to the AFB₁ content in this kernel.

In this study, an experiment was conducted to demonstrate the feasibility of using VNIR hyperspectral imaging for directly detecting pure aflatoxin B₁ coated onto maize kernel surfaces with the specific objectives of twofold: (1) to establish a classification model to classify AFB₁ contaminated maize kernels of different varieties; and (2) to explain whether it is possible to directly detect the pure aflatoxin-coated maize kernel-surface using chemical information characterised by selected key wavelengths.

2. Materials and methods
2.1. Maize kernels and sample preparation

Yellow Maize kernels of four varieties (Fig. 1) typically originating from different regions of the U.S.A., i.e., Georgia (GA), Illinois (IL), Indiana (IN) and Nebraska (NE) were investigated in this study. Details about the types of kernels and number of samples of each variety used in the study are presented in Table 1. Some differences between varieties include: (1) the IL and IN kernels are darker yellow than GA and NE; (2) the size of embryos are different and reduce in this order NE > IL > GA respectively. All the maize kernels for the four varieties were grown in Tifton, Georgia, in the same soil and under the same growing conditions and harvested in 2010. The appearance of normal maize kernels from each variety is illustrated with colour images in Fig. 1. A sample of 150 maize kernels of about the same size, appearance, shape, and mass were selected from each variety to make a total of 600 samples. The kernels were provided by the Toxicology and Mycotoxin Research Unit, Russell Research Center, USDA-ARS (Spruce St., St. Louis, Mo., U.S.A.) with methanol, which, at the same time, was used to kill any residual mould spores in AFB₁.

Table 1 – The region of origin, type of kernels and number of samples.

<table>
<thead>
<tr>
<th>Region of origin</th>
<th>Type of Kernels</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Georgia</td>
<td>Dent</td>
<td>150</td>
</tr>
<tr>
<td>Illinois</td>
<td>Flint</td>
<td>150</td>
</tr>
<tr>
<td>Indiana</td>
<td>Medium dent/flint</td>
<td>150</td>
</tr>
<tr>
<td>Nebraska</td>
<td>Dent</td>
<td>150</td>
</tr>
</tbody>
</table>

The procedure for the preparation of stock solutions was adopted from Wang et al. (2014). The stock solutions were prepared as follows: initially, 5 mg of toxin were diluted with 5 ml of 100% methanol, which yielded 1 µg of toxin per µl of solution (1 µg/µl⁻¹). Then, two 1/10 serial dilutions were created by mixing 1 ml of solution of toxin with 9 ml of 20% methanol, yielding 0.01 µg of toxin per µl of solution (0.01 µg/µl⁻¹). After that, 2 ml of the 0.01 µg/µl⁻¹ were diluted with 0.4 ml of 20% methanol, yielding 0.00825 µg/µl⁻¹, and 20 µl of 0.00825 µg/µl⁻¹ toxin solution were deposited on each kernel to achieve 500 ppb (20 µl x 0.00825 µg of toxin = 0.165 µg of toxin per 0.33 g kernel). In order to achieve 100 ppb per 0.33 g kernel (0.033 µg of toxin per kernel), 4 µl of 0.00825 µg/µl toxin solution were deposited on each kernel. Lastly, a similar dilution process was applied to the 0.01 µg/µl⁻¹ solution to achieve 20 ppb and 10 ppb.

After solutions were prepared, the kernels of each variety were divided into five groups of 30 and put into five separate weighing boats. The first group, selected as a control, was treated with a 20 µl methanol-alone drop, whereas the rest of the groups were inoculated with 10 ppb, 20 ppb, 100 ppb and 500 ppb AFB₁ solutions. Prior to inoculation, the total mass of each group of 30 kernels was measured using a high resolution scale and the mass of the weighing boats was subtracted in the process. After recording the dry mass, next was the inoculation process where the kernels were removed from the weighing boats and placed on a Teflon® (polytetrafluoroethylene) plate designed to hold 30 kernels at a time in shallow elliptical wells on its surface. With a pipette, a small drop of various solutions was applied to the surface of kernels germ side up. Following inoculation, 120 kernels (four solutions x 30 kernels) were placed in a chemical hood for approximately 90 min to let the AFB₁ solutions dry. We assumed that, in the 90 min time interval prior to the imaging process, aflatoxin might still be entirely superficially localised and that aflatoxin could not have migrated into the interior of the kernel during the time of the experiment. In addition, we assumed that, as NIR wavelengths have a certain depth of penetration, even when the toxin might be internal or just

![Fig. 1 – Colour image of variety control samples showing arrangement of maize kernels in the experiment: GA–Georgia, IL–Illinois, IN–Indiana, NE–Nebraska.](image-url)
below the kernel’s pericarp or seed coat, the technology would still detect the aflatoxin.

For safety purposes, samples and solutions were handled with caution because aflatoxin is known to be carcinogenic. Before imaging, 30 kernels of the same concentration were placed on the Teflon® holder with elliptical wells of 6 rows x 5 columns. In addition, prior to imaging, the Teflon® sample holder was placed into an acrylic box of dimensions 8 inches x 8 inches x 2 inches with a 5 mm thick Borofloat® (Glass Fab Inc., Rochester, NY, USA) glass window in order to avoid exposure of laboratory personnel to AFB₁.

2.2. Hyperspectral imaging and spectral acquisition

In this study, a VNIR hyperspectral imaging system with a wavelength range between 400 and 1000 nm was used for image acquisition. The system was comprised of a scMOS PCO. EDGE camera (PCO-TECH, Romulus, MI, U.S.A.), spectrograph (V10M, Specim, Oulu, Finland), and front lens (Distagon T × 25 mm f/ 2.8, Zeiss, Oberkothen, Germany). Indirect lighting was facilitated by two soft-boxes with 500 W Tungsten–Halogen lamps (SilverDome® nxt: small, Photoflex, Watsonville, Calif., U.S.A.) positioned at about 45° angles above and lateral to the samples. The spectral calibration of the hyperspectral imaging system was accomplished using a series of pencil-style calibration lamps and lasers (Wang et al., 2014). Furthermore, image calibration was carried out with an image of a 75% Spectralon® reflectance panel (SRT-75-050, Labsphere, North Sutton, N.H., U.S.A.). Image acquisition and some pre-processing of the imagery were accomplished with HyperVisual® software (Phil-Lumina, Gulfport, Miss., U.S.A.). During image acquisition, the scanning process of the camera was controlled by the software while the samples remained motionless. HyperVisual® was also used to spectrally subset the imagery to a wavelength range between 400 and 1000 nm and to calibrate the imagery to percent reflectance. The inherent noise in the imagery was then removed using minimum noise fraction (MNF)/inverse MNF processing flow available in ENVI software (Harris GeoSpatial Solutions, Broomfield, Co., U.S.A.).

2.3. Spectral pre-processing

As concentration of aflatoxin was calculated in terms of kernel mass, to extract the spectra information from the kernel, regions of interest (ROI) were created from the whole surface of the kernel and the average spectra were adopted for subsequent analysis. In addition, ROIs of maize kernels were hand-digitised with the aid of the polygon function of the ROI tool available in ENVI software. The ROIs contained as many pixels as possible according to the size and shape of a particular kernel. The process of generating ROIs was the same for both the control and inoculated groups of each variety. The mean reflectance of pixels within each kernel’s ROI was extracted using ENVI software. Afterwards, the mean reflectance (R) data were transformed to absorbance units using log (1/R). Shenk et al. (2008) stated that absorption or log (1/R) spectra is the preferred form for collecting NIR spectra because in this form, spectra have a linear relationship with the concentration of a sample, in this case aflatoxin. The absorption spectrum is also useful because of its specificity which allows the possibility of determining qualitative information about a sample and its identification by comparing a measured spectrum with a library of reference spectra. As a pre-treatment process, standard normal variate (SNV) correction was applied to the data to reduce environmental effects caused by differences in kernel surface roughness and shape (Wang, Heitschmidt, et al., 2015). Then, Savitzky–Golay smoothing (SGS) was used to get rid of any spectral noise due to overlapping peaks (Kandpal, Lee, Kim, Bae, & Choi, 2015; Mishra et al., 2015). For this study, classification models were developed using Saisir software (Version 07/01/2009, France), a free package for chemometrics with MATLAB (The MathWorks, Inc., Natick, MA, U.S.A.).

2.4. Multivariate data analysis

The resultant spectra are really a mixture of pure aflatoxin and maize kernel surface and internal components and the macro chemical information in maize kernels may influence the trace aflatoxin information. Therefore, the aim of our data processing using chemometric techniques like PCA and FDA as well as pre-treatment methods such as SNV and SGS is to try to cancel or minimise that macro or useless information in order to highlight what we want, that is, aflatoxin information.

2.4.1. Principal component analysis (PCA)

The principal component analysis (Wu, Massart, & De Jong, 1997a) was used to visualise (Ropodi et al., 2016) the VNIR hyperspectral data for any patterns among the kernels for variety dissimilarities and variation due to AFB₁ contamination degree in each variety along with dimensionality reduction. The selection of optimal number of principal components (PCs) was based on the eigenvalues and variation explained by the components. The first 20 PCA principal components were used as input in developing the supervised FDA classification models (Karoui, Hammami, Rouissi, Steyer, & Steyer, 2009) was used to classify the maize kernels. The classification method exploits the subspace of the original variable space that best discriminates the m classes by maximising the inter-class variance with respect to the total variance. In addition, FDA estimates new synthetic uncorrelated variables called “discriminant factors”, which are linear combinations of the original variables that allow the best separation of the qualitative groups (Roger, Palagos, Guillaume, & Bellon-Maurel, 2005). Furthermore, for the FDA method, the preliminary transformation of the data into their principal components (PCs) is required because the method cannot be applied in a straightforward way to highly correlated hyperspectral data with multiple wavelengths.

For this study, the aim of the stepwise FDA was to predict to which of the five groups (control, 10, 20, 100, and 500 ppb) individual maize kernels belonged (Lin, Yang, & Kuo, 2012; Wang et al., 2014). FDA was applied on spectra data for each maize variety and later on pooled samples (all maize kernels), which were divided into a calibration and a validation set. The calibration set consisted of randomly selected 2/3 (20 of 30
kernels in each group) of the kernels while the validation set had the remaining 1/3 (10 of 30 kernels in each group) of the kernels (Wang, Heitschmidt, et al., 2015). Therefore, for each variety, the calibration set consisted of 100 samples and the validation set included 50 samples. This implied that 400 samples were available for calibration and 200 samples for validation in the pooled sample lot. The individual maize kernels were reallocated by FDA within the five groups i.e. the control, 10, 20, 100, and 500 ppb. The distance of each individual kernel from the various centres of gravity was calculated and the individual kernel was assigned to the group with the nearest centre of gravity. The classification results of FDA models calculated on spectra of kernels of each maize variety and pooled samples were evaluated in terms of accuracy and FDA discriminant factors (e.g. F₁, F₂, etc.). Because the FDA discriminant factors F₁, F₂ and F₃ are essential in the separation of maize kernels based on their concentrations of AFB₁, the beta coefficient curves of these discriminant factors were plotted and the key wavelengths were determined by inspecting the beta coefficient plot i.e. by extracting those wavelengths with high magnitude of beta coefficients (a wavelength at which the value of beta coefficient is an extreme is considered important) and by comparing with some wavelengths available in literature about aflatoxin.

3. Results and discussion

3.1. Original spectra analysis

The raw mean kernel reflectance spectra for each variety are presented in Fig. 2a–d. The average absorption spectra of normal and AFB₁ contaminated groups of each variety are illustrated in Fig. 3a–d. When compared to the normal kernels the average absorbance of contaminated kernels was higher for Georgia and Illinois varieties and lower for Indiana and Nebraska varieties in the visible region of around 400–600 nm. In addition, some differences in the spectra were revealed in the NIR range between 900 and 1000 nm for all four varieties. Salas and Henebry (2012) indicated that in the visible region, with spectral bands around 400–700 nm, pigments such as chlorophyll and carotenoids strongly absorb light that leads to generally lower reflectance which explains the shape of the spectral curves observed in this study in Fig. 2a–d of mean kernel reflectance. Furthermore, prominent peaks in the visible region (400–500 nm) in Fig. 3a–d may be related to spectral information associated with colour changes in the kernels due to residual chlorophyll and carotenoids within the kernel seed coat, perhaps because aflatoxin occurs in small concentrations and its effect on the spectra may be kept down by the more prominent kernel colour and therefore, more aflatoxin spectral information is expected in the NIR region of between 700 and 1000 nm (Wang, Heitschmidt, et al., 2015; Fernández-Ibañez et al., 2009).

3.2. Principal component analysis results of visible/near-infrared hyperspectral data

Results from PCA show the distribution of normal and contaminated maize kernels in Fig. 4a–d using the first three principal components i.e. PC₁, PC₂ and PC₃ which contained 64.4, 23.6 and 8.3% of the variance, respectively for Georgia, 75.5, 13.1 and 6.3% for Illinois, 63%, 19.4 and 7.4% for Indiana, and 61.9, 25.8 and 9.3% for Nebraska. Although the normal kernels could be generally separated from the contaminated ones, it was not possible to distinguish one contaminated group from another based on the aflatoxin concentration applied for the four varieties. Visual patterns of separation between normal and contaminated kernels were missing when PCA was applied on a pooled sample of all kernels from the four varieties (data not shown). The lack of a separation pattern between AFB₁ contaminated groups of maize kernels for each variety and the pooled sample lot might have been caused by the ineffectiveness of an unsupervised PCA technique to be used in building predictive models aimed at classifying samples in one or another group. However, the PCA results indicated that normal samples can be identified from contaminated ones on at least two varieties. Therefore, further classifications of contaminated samples were performed using a supervised stepwise FDA model.

3.3. Factorial discriminant analysis classification results

A stepwise FDA was performed on the first 20 principal components (PC) and the maximum number of principal components allowed to enter the FDA model was set to 12 because at this number of PCs the model was economical and satisfactory accuracy was achieved with retention of as much useful information as possible. The aflatoxin classification results of each variety and the pooled lot are shown in Table 2. Georgia samples were classified with 100% accuracy for both calibration and validation while the other three varieties (Illinois, Indiana and Nebraska) had relatively high classification accuracy in validation of 96, 96 and 98% respectively. Similarly, pooled samples reached high calibration and validation accuracies of 99.25 and 98%, respectively. This result from AFB₁ classification on the pooled sample lot suggests that variations in the maize samples due to variety may have insignificant influence on the outcome of the AFB₁ contamination classification.

It can be seen from Fig. 5a–d and Fig. 6 that control samples could be separated from aflatoxin-contaminated samples, and aflatoxin-contaminated groups were clearly discriminated from each other. A similar observation was reported by Wang, Heitschmidt, et al. (2015) who, using FDA and visible/NIR spectra of 150 yellow maize kernels, noted that FDA discriminant factor F₂ was helpful in separating control maize kernels from aflatoxin-contaminated kernels whereas discriminant factor F₁ was essential in differentiating maize kernels based on their aflatoxin B₁ concentrations (10, 20, 100 and 500 ppb). In addition, the authors achieved a validation classification accuracy of 98% and attributed the misclassification to spectral similarity. However, the roles of FDA discriminant factors F₁ and F₂ as described above from Wang, Heitschmidt, et al. (2015), were not identified in our study. In another related study, Kandpal et al. (2015) demonstrated the feasibility of SWIR spectra and partial least squares discriminant analysis (PLSDA) to detect aflatoxin B₁ coated on the surface of 195 kernels of each of the three maize varieties (yellow, white and purple). The authors obtained reliable
Results for the classification between the control and aflatoxin contaminated groups (10, 100, 500 and 1000 ppb) and the validation classification accuracy reached 86.6%, 80% and 91.1% for white, yellow and purple maize varieties respectively. However, the analysis of AFB1 contamination on a pooled sample of maize kernels of different varieties was not conducted in Kandpal et al. (2015). Clustering of non-contaminated kernels as well as different AFB1 contaminated groups was clearly observed in each variety (Fig. 5a–d) and the pooled sample (Fig. 6). The clustering of different groups of kernels suggests that kernels of the same cluster may have similar physical or chemical characteristics (Shrestha et al., 2016). The variation in these characteristics between different clusters of kernels creates differences in the spectra and by extension in FDA scores. It is worth noting that these differences in spectra may be due to differences in AFB1 intensity on the kernel surface (Pearson & Wicklow, 2006). The aforementioned phenomena is related to observations from Wang, Heitschmidt, et al. (2015) and Pearson et al. (2001) who showed that absorbance is generally much higher for kernels contaminated with high levels of aflatoxin. As mentioned, Wang, Heitschmidt, et al. (2015) detected pure aflatoxin artificially deposited on single maize kernels with visible/near infrared (400–1000 nm) hyperspectral imaging. On the other hand, Pearson et al. (2001) detected aflatoxin in single corn kernels by transmittance (500–950 nm) and reflectance (550–1700 nm) spectroscopy and the aflatoxin-contaminated kernels were prepared through A. flavus fungal inoculation rather than pure aflatoxin coating.

3.4. Spectral information about aflatoxin B1 contamination of different varieties

It was observed that the first three factors i.e. F1, F2, and F3 played a major role in the classification result, which could be seen from the distribution of validation kernels plotted in
Fig. 5a–d and Fig. 6 for the model constructed using individual variety data and pooled sample lot respectively. Not only could the non-contaminated kernels be separated from the AFB1-contaminated kernels completely, but also kernels with different concentrations of AFB1 could be separated clearly from each other. Thus, inspection of the beta coefficient curves for these FDA factors of the pooled sample lot (Fig. 7) can provide spectral information, by indicating which wavelengths significantly contribute to the observed differences. The selection of important wavelengths involved extraction...
The relationship between spectral and chemical characteristics of some selected wavelengths about AFB1 is reported in Table 3. The regions of 670–979 nm (Wang, Heitschmidt et al., 2015) and 850–950 nm (Del Fiore et al., 2010) that contain 710.1, 714, 735.9, 750.1, 790.3, 868.2, 968.4 and 981.4 nm were related to aflatoxin B1 and fungal contamination of maize kernels, respectively. The wavelength of 714 nm relates to the presence of benzene ring (Workman & Weyer, 2007), which can be attributed to either AFB1 or some amino acids like tyrosine in maize kernels. Some literature spectral values were close to our results (Table 3) such as 710 and 735 nm from Pearson et al. (2001) and 750 nm in Pearson et al. (2004). In this regard, Pearson et al. (2001) used 710 and 735 nm to identify highly aflatoxin-contaminated maize kernels (>100 ppb) from the less aflatoxin-contaminated kernels (<10 ppb) by using the reflectance spectra ratio at 710/760 and 735/1005 nm. In addition, Pearson et al. (2004) stated that the absorbance at 750 nm was able to correctly detect over 99% of yellow maize kernels as aflatoxin-contaminated (>100 ppb) or uncontaminated and like Pearson et al. (2001), the aflatoxin-contaminated kernels were prepared through A. flavus fungal inoculation instead of pure aflatoxin coating. Furthermore, the chemical information related to 790.3, 868.2, 968.4 and 981.4 nm (Table 3) was adopted from Stuart (2004, p. 86).

As stated in sub-section 2.1, AFB1 solution was applied to the germ-side surface of kernels. Although in naturally infected maize kernels, the distribution of AFB1 is not uniform over the maize kernels and this does not allow for direct detection, studies by Chu et al. (2017) and Fernández-Ibáñez et al. (2009) give accounts of how AFB1 is mainly distributed on the germ part of the maize kernels. In addition, only the average spectra from the germ side were used in our study, meaning that the distribution of AFB1 over the kernel does not influence the detection accuracy of AFB1 but factors such as AFB1 levels (0, 10, 20, 100 and 500 ppb) and variety of maize kernels affect the detection accuracy of AFB1. However, our results from the AFB1 classification of pooled samples (calibration and validation accuracy of 99.25 and 98%, respectively) suggest that changes in maize variety or kernel type are likely to have little influence on the detection accuracy.

The VNIR method proposed in our study focused on developing a general aflatoxin detection method which could possibly be used for online detection of aflatoxin by analysing average spectra from the germ side of the kernel, since the majority of aflatoxin in a naturally infected maize kernel is distributed around the germ part of the kernel. Although the results in our study were achieved with AFB1 coated kernels, the AFB1 was applied on the germ part of the kernels and in low levels like 10 ppb, 20 ppb etc by analogy with the natural infection of kernels where aflatoxin is not only found mostly around the germ part but also occurs in small concentrations. The correlation between fungus and aflatoxin distribution in maize kernels is the reason why the analysis of AFB1 spectra plays a role in the rapid aflatoxin detection method development. And, though our results can be seen as a starting point for the extension of the method to practical applications and as a suggestion, a lot of work is still required to test the proposed method on naturally contaminated kernels and include...
the inhomogeneous distribution of aflatoxin that is characteristic of naturally infected kernels in order to make the method more robust and stable.

The study showed a potential application of the VNIR hyperspectral imaging in AFB1 contamination classification of maize kernels. The classification accuracies for AFB1 contamination were substantial when each variety was analysed separately. Furthermore, the classification accuracies were considerable when all maize samples from different varieties and AFB1 contamination levels were pooled. This provides an indication of the variation between samples of different AFB1 contamination levels for each variety. The effect created by differences in AFB1 concentration on the kernel surface might be a source of variation among the maize kernels. Our results have demonstrated that variations in the maize samples due to variety insignificantly influence the outcome of the AFB1 contamination level classification results. However, factors such as year of harvest and moisture content may have influence on the outcome of the results. Therefore, the study advocates the need to investigate the influence of variation between harvest years and moisture content gradients for each variety on the AFB1 classification in order to build a more robust classification model.

4. Conclusions

The VNIR hyperspectral imaging technology and PCA/FDA statistical approach could differentiate uncontaminated and contaminated maize kernels with directly coated pure AFB1 and even discriminate between the contaminated kernels with different AFB1 levels. Detection of pure AFB1, artificially coated on maize kernel surfaces was possible at concentrations as low as 10 ppb. In addition, the study demonstrated the application of VNIR hyperspectral imaging system to examine the influence of varietal differences of maize kernels on AFB1 contamination classification. The classification accuracy results for pooled samples relative to AFB1 classification of each variety suggest that AFB1 contaminated kernels may be classified from normal kernels, as well as contaminated kernels separated based on the degree of contamination, irrespective of varietal differences. However, the influence of kernel orientation on the outcome of the results merits further research.

Acknowledgement

The authors would like to thank Dr. Charles W Bacon, Research Leader of the Toxicology and Mycotoxin Research Unit, USDA, ARS for providing maize kernels. Ms. Peggy Feldner, Food Technologist, Mr. Vernon Savage, Engineering Technician, Mrs. Jerrie Barnett, Biological Laboratory Technician, and Ms. Candace Betts, Physical Science Technician with the Quality & Safety Assessment Research Unit, USDA, ARS, were also thanked for their assistance with sample preparation, fabricating the sample holders, and image acquisition. This work was supported financially by the China National Science and Technology Support Program (Grant number: 2012BAK08B04).

R E F E R E N C E S

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