Detection of mycotoxins and toxigenic fungi in cereal grains using vibrational spectroscopic techniques: a review

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Received: 12 August 2019 / Accepted: 17 September 2019

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OPEN ACCESS

REVIEW ARTICLE

Abstract

Nutrition-rich cereal grains and oil seeds are the major sources of food and feed for human and livestock, respectively. Infected by fungi and contaminated with mycotoxins are serious problems worldwide for cereals and oil seeds before and after harvest. The growth and development activities of fungi consume seed nutrients and destroy seed structures, leading to dramatic declines of crop yield and quality. In addition, the toxic secondary metabolites produced by these fungi pose a well-known threat to both human and animals. The existence of fungi and mycotoxins has been a redoubtable problem worldwide for decades but tends to be a severe food safety issue in developing countries and regions, such as China and Africa. Detection of fungal infection at an early stage and of mycotoxin contaminants, even at a small amount, is of great significance to prevent harmful toxins from entering the food supply chains worldwide. This review focuses on the recent advancements in utilising infrared spectroscopy, Raman spectroscopy, and hyperspectral imaging to detect fungal infections and mycotoxin contaminants in cereals and oil seeds worldwide, with an emphasis on recent progress in China. Brief introduction of principles, and corresponding shortcomings, as well as latest advances of each technique, are also being presented herein.

Keywords: vibrational spectroscopy, toxic secondary metabolites, infrared spectroscopy, Raman spectroscopy, hyperspectral imaging

1. Introduction

As the most common food staple, cereals and oil seeds collectively provide major sources of dietary calories, including carbohydrates, fatty acids, and proteins, for human and livestock. Cereal grains in form of maize, rice, wheat, and barley constitute a majority of daily sustenance (Orina *et al.*, 2017). Despite many improvements and efforts that have been made in cultivation, harvesting, and storage of cereals and oil crops for a long time, fungal infection remains a serious and challenging problem. The fungi present in grains can cause quality changes, such as dull appearance, musty odour, germ damage, and poor seed germination, leading to reduced edible and huge economic losses (Senthilkumar *et al.* 2016a,b).

Mycotoxin contamination in cereals and oil crops is caused by secondary metabolites produced by fungi, mainly belonging to species of the genera *Aspergillus*, *Fusarium* and *Penicillium*, and pose serious threats to the health of human and livestock (Wagacha and Muthomi, 2008; Jin *et al.*, 2009). Highly prevalent and critically important mycotoxins include aflatoxins (AFs), ochratoxins (OTs), deoxynivalenol (DON) and fumonisins (FBs), among other toxins described by Pascale (2009) and Selvaraj *et al.* (2015). The United States Food and Drug Administration (FDA), the European Union, the FAO as well as many other countries, including China (Zhang *et al.*, 2014), have regulated mycotoxin concentration levels both in food and feed.

AFs are a group of carcinogens associated with liver and lung cancer in humans and mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Yao *et al.*, 2010). Among the 18 types of AFs that have been identified, aflatoxin B₁ (AFB₁) is considered to be the most toxic, and together with aflatoxin B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) constitutes the four major AFs that contaminate cereals and oil crops (Gupta *et al.*, 2013; Turner *et al.*, 2009). According to previous aflatoxin outbreaks it is estimated that, when consumed for 1 to 3 weeks, a dose of 20 to 120 µg AFB₁/kg body weight per day is acutely toxic and potentially fatal. OTs are a group of secondary metabolites that are mainly produced by different *Aspergillus* and *Penicillium* species. Distinguished by chemical structures, the ochratoxin family contains three members: ochratoxin A (OTA), B, and C (Todscato *et al.*, 2014). OTA is considered to be the most prevalent and harmful, and can cause immunosuppression and immunotoxicity in animals (Lea *et al.*, 1989). DON, also known as vomitoxin, is one the major secondary metabolites produced by *Fusarium* species. It is predominantly found in cereal grains, such as wheat, barley, and maize (Liu *et al.*, 2009). DON not only decreases grain yield and quality, but also poses a great health risk to human and livestock who have consumed DON-contaminated food or feed products (Leonard and Bushnell, 2004). The most sensitive physiological parameter for animals to low-level DON exposure is the emetic response (Pestka and Smolinski, 2005). FBs, another group of mycotoxins derived from various species of the genus *Fusarium*, are commonly found on cereals grown in the northern temperate regions of America, Europe, and Asia (Creppy, 2002; Yazar and Omurtag, 2008).

A number of reliable, specific and sensitive analytical methods, such as culture and colony techniques (Gourama and Bullerman, 1995), the PCR-methods (Burlakoti *et al.*, 2007; Ur-Rahman *et al.*, 2020), chromatographic technologies (Martos *et al.*, 2010; Thiex *et al.*, 2009), mass spectrometry (MS) (Sforza *et al.*, 2006), and enzyme-linked immunosorbent assays (ELISA) (Liu *et al.*, 2016), have been developed to detect fungal infection and/or mycotoxin contamination in food and feed. However, these traditional methods tend to be time consuming and labour intensive, and require skilled personnel. These methods also need tedious sample preparation before carrying out, making them unsuitable for large-scale non-destructive detection or rapid online sorting applications. To meet the needs of detecting fungal infection, mycotoxin contamination and/or mycotoxin concentration in cereals and oil seeds, rapid and non-destructive technologies have received extensive attention.

Vibrational spectra result from the interaction of vibrational motions of a molecule with electromagnetic radiation, including the infrared spectrum and the Raman spectrum (Siesler, 2011). They are fingerprints of adsorbed molecules

and can be used for chemical identification. Recently, vibrational spectroscopy and imaging techniques, such as infrared spectroscopy (Gaspardo *et al.*, 2012; Hernandez-Hierro *et al.*, 2008; Kaya-Celiker *et al.*, 2015), Raman spectroscopy (Lee *et al.*, 2014a,b), and hyperspectral imaging (Siripatrawan and Makino, 2015; Teena *et al.*, 2014; Wang *et al.*, 2015a,b) have been investigated for application in detecting of toxigenic fungi and/or mycotoxins in food and feed. A brief introduction of these spectroscopic techniques is given in Table 1. This manuscript provides an overview of working principles, applications, advances, and limitations of the aforementioned non-destructive techniques that have been utilised in rapid detection of mycotoxins and toxigenic fungal contamination in major cereal and oil crop varieties (including maize, wheat, barley, rice and peanut). Most reviewed applications in this paper will focus on recent studies that have been conducted in China, but relevant studies performed in other countries will also be selectively presented.

2. Principles and application of vibrational spectroscopic techniques

Near infrared spectroscopy

NIR spectroscopy is a type of vibrational spectroscopy with spectral range from 780 to 2,500 nm (12,500–4,000 cm⁻¹). The NIR spectrum is produced by the energy absorption of molecular overtones and combination vibrations of chemical bonds, such as C-H, N-H, O-H, and C-O (Cen and He, 2007). All molecules containing these chemical bonds will have a measurable NIR spectrum, making NIR spectroscopy a suitable technique to provide complex structural and chemical component information of a large range of organic materials (Manley, 2014). NIR spectra can be collected in transmittance and reflectance modes according to the type, property, and component of samples under examination (Porep *et al.*, 2015). In order to obtain a spectrum with high signal to noise ratio (SNR), averaging multiple scan results is a common practice. However, due to the complicated chemical composition of organic samples, obtained spectra in NIR frequencies often consist of many overlapping peaks (also known as multicollinearity) (Cozzolino *et al.*, 2013; Manley, 2014). Furthermore, both chemical composition and physical characteristics of samples under investigation will be reflected on the acquired spectral data (Wu *et al.*, 2018). Preparing samples with proper moisture content, uniform particle size, and surfaces as flat as possible, and so on, are of great importance for reducing spectral noises. Grinding samples into powder before analysis may seem a good solution, however, taking the large amount and complex conditions of samples into account, grinding all samples is obviously unrealistic. To overcome this disadvantage of NIR spectroscopy, in addition to the use of advanced computer hardware and high-precision spectroscopy equipment,

Table 1. Brief description of near-infrared (NIR), mid-infrared (MIR), and Raman spectroscopic, and hyperspectral imaging techniques (HSI).

	NIR spectroscopy	MIR spectroscopy	Raman spectroscopy	HSI
Spectral range	780-2,500 nm (12,500-4,000 cm ⁻¹)	4,000-400 cm ⁻¹	4,000-50 cm ⁻¹	380-2,500 nm (26,316-4,000 cm ⁻¹)
Phenomenon characteristic	Absorption	Absorption	Scattering	Absorption
Information	Overtone and combination bands	Fundamental vibrations	Fundamental vibrations or rotations	Overtone and combination bands
Type of sample analysed	Organic and inorganic	Organic and inorganic	Organic	Organic and inorganic
Advantages	Little or no sample preparation needed Low cost Low absorption coefficient: - high penetration depth permitted - strongly absorbing and highly scattering samples allowed	Little or no sample preparation needed Stronger absorption bands than NIR spectroscopy High specificity, and few overlaps	Narrow bands, and few overlaps: - highly specific like a chemical fingerprint of a material - good signal-to-noise ratio (SNR) Little or no sample preparation needed Basically impervious to water Fast for complete analysis	Little or no sample preparation needed Both spatial and spectral information available Suitable for qualitative and quantitative analyses
Disadvantages	Broad and overlapping absorption bands 10-100 times weaker than the corresponding fundamental MIR absorption bands Influenced by chemical and physical sample properties	Poor sensitivity and accuracy for low concentrations Not comparable to NIR spectroscopy in quantitative analysis Not sensitive to some substances (e.g. Ne, Ca ²⁺ , H ₂)	Weak Raman effect: - sensitive and highly optimised instruments needed - unsuitable for fluorescent samples Laser source may destroy samples Expensive experiment materials	Huge data cube: - advanced hardware needed - data pretreatments needed - effective chemometric algorithms needed Expensive instrumentations

chemometric algorithms are indispensable (Osborne *et al.*, 2006). Chemometric algorithms aim to remove noisy signals as much as possible without losing critical information, and encompass a broad range of methods, such as spectral preprocessing methods, informative wavelength extraction, pattern recognition, and calibration model development (Cen and He, 2007; Cozzolino, 2009; Roggo *et al.*, 2007). Detailed descriptions on these statistical methods can be found in previous studies, ranging from Guyon and Elisseeff (2003) to Cheng and Sun (2015) and Wang *et al.* (2017).

Fusarium Head Blight (FHB) infection, a major destructive wheat disease, can damage structure and nutrients of wheat, and produce DON. In order to understand the relationships between *Fusarium*-damaged kernels (FDKs) and DON concentration, Jin *et al.* (2014) compared single-kernel near-infrared (SKNIR) estimated FDKs and DON concentration with visually estimated FDKs and gas chromatography-mass spectroscopy (GS-MS)-measured DON, respectively. They found that the correlation coefficient between SKNIR-estimated FDKs and SKNIR-estimated DON was the highest ($r=0.95$; $P<0.001$), followed by between

GS-MS-estimated DON and visual-estimated, as well as SKNIR-estimated FDKs, with r -values of 0.74 ($P<0.001$) and 0.72 ($P<0.001$), respectively. Therefore, both visual and SKNIR-estimated FDKs could be used to predict DON concentration in wheat kernels. In their study on using NIR spectra to detect mouldy rice grains, Jin *et al.* (2016) investigated two variables: temperature and moisture content. The results showed that either low temperature (5 °C) or moisture content (<13%) would inhibit fungal growth. However, when the moisture content exceeded 16%, the total number of fungal colonies still increased rapidly, even in a low temperature environment, suggesting that low moisture content is key to preserve quality during grain storage. The established multiple linear regression (MLR) model could predict the number of fungal colonies with a high coefficient of determination (R^2) value of 0.897.

Compared to NIR spectroscopy mentioned above, Fourier transformed Near Infrared Spectrum (FT-NIR) spectroscopy provides stable spectra and further improves the spectral resolution (Wu *et al.*, 2018). Previous studies have used FT-NIR spectroscopy to detect mycotoxins in

cereal grains (De Girolamo *et al.*, 2009, 2019; Gaspardo *et al.*, 2012; Peiris *et al.*, 2017). Zhang *et al.* (2014) applied FT-NIR spectroscopy ranging from 10,000–4,000 cm^{-1} (1000–2,500 nm) for the rapid detection of AFB₁ in rice grains. Moisture content influences fungal growth and aflatoxin production, and by using FT-NIR they found that less than 13% moisture is a critical threshold to prevent fungal growth when rice is kept at room temperature. FT-NIR spectroscopy proved to be a suitable technique for the detection of AFB₁ with concentrations not lower than 20 $\mu\text{g}/\text{kg}$. The partial least squares regression (PLSR) model established by standard normal variate and detrending (SNVD) pretreated spectra showed the best performance with $R^2 = 0.87$, and a standard error of prediction (SEP) of 3.211%.

Huang *et al.* (2015) chose FT-NIR (10,000–4,000 cm^{-1}) in conjunction with chemometric algorithms to discriminate healthy, mouldy, and budding peanuts. Due to nutrient consumption during fungal growth and peanut germination, contents of lipids, proteins, and other substances in mouldy or budding peanuts often changed. Original spectra of different sample groups clearly showed differences in spectral ranges of 8,321–8,560 cm^{-1} , 9,041–9,280 cm^{-1} , and 9,521–9,760 cm^{-1} , which were sensitive to second overtone of N-H and C-H groups. The best discriminate model established by the *k*-Nearest Neighbour (KNN) pattern recognition method obtained an identification rate of 98.84% for identifying the different peanut groups.

In the research of Peiris *et al.* (2009), two NIR systems were used to detect NIR absorption spectra of DON, and of healthy and *Fusarium*-damaged wheat kernels. The spectra of DON with concentrations ranging between 0.5 to 2,000 mg/kg in acetonitrile solution showed three peaks at 1,408, 1,904, and 1,919 nm. Single healthy and *Fusarium*-damaged wheat kernels were scanned and recorded in a spectral range of 950–1,650 nm. Shifts of peak positions between the spectra of these two types of samples were observed, which may be caused by changes in the levels of grain structural compounds and contamination of DON (Peiris *et al.*, 2009). These results show that sound kernels and FDKs with different DON levels have distinguishable characteristics in the NIR spectral ranges. Peiris *et al.* further analysed single wheat kernels contaminated with FHB and DON (Peiris *et al.*, 2010). A PLSR model was developed to estimate DON levels and to divide FDKs into groups of having low (<60 mg/kg) or high (>60 mg/kg) DON contamination. DON levels of high DON single kernels could be estimated with an $R^2=0.87$ and a SEP of 60.8 mg/kg .

Recently, Sirisomboon *et al.* (2013) developed calibration models using raw and pretreated NIR spectra in conjunction with a PLSR algorithm, to predict the percentage of fungal infection found in rice. Models developed from original

spectra for total fungal infection and from maximum normalisation spectra for yellow-green *Aspergillus* infection, provided the highest prediction accuracies with an *r* of 0.668 and 0.437, and SEP of 28.874% and 18.723%, respectively. Evidently, these unsatisfactory results showed that moisture and starch content in rice could have influenced overall extent of fungal infection (Sirisomboon *et al.*, 2013).

Lim *et al.* (2018) used the spectral range between 1,175–1,700 nm to discriminate between normal and *Fusarium*-contaminated hulled barley, naked barley, and wheat. The correct classification rate (CCR) of the developed discrimination models using raw spectra was over 98% for all samples, while second and third-order derivative spectral pretreatments improved the accuracy. Therefore, when designing an experiment, in order to obtain a higher accuracy, different parameters should be considered and controlled within the proper ranges.

Mid-infrared spectroscopy

MIR spectroscopy, covering the spectral range of 4,000–400 cm^{-1} (2,500–25,000 nm), is also sensitive to some groups (C-H, O-H, N-H, C-O, and C=O) in molecules and often refers to IR spectroscopy. The MIR spectral region can be divided into four regions: the X-H stretching region (4,000–2,500 cm^{-1}), the triple bond region (2,500–2,000 cm^{-1}), the double bond region (2,000–1,500 cm^{-1}), and the fingerprint region (1,500–400 cm^{-1}) (Karoui *et al.*, 2010). The absorption in MIR range relies on the fundamental frequency of molecular vibrations (Shen *et al.*, 2018). Very few compounds have the same MIR spectrum, making MIR spectroscopy suitable for qualitative and quantitative determination of chemical components of samples (Su *et al.*, 2017). With the development of modern interference IR spectroscopy techniques, the availability of advanced computer technology, and the emergence of powerful chemometric algorithms, IR spectroscopy reappeared as a means of characterizing microbial samples (Amiel *et al.*, 2000; Greene *et al.*, 1992; Kaya-Celiker *et al.*, 2014). FTIR spectroscopy in combination with non-invasive reflectance equipment, such as photoacoustic spectroscopy (PAS), diffuse reflectance spectroscopy (DRS), and attenuated total reflectance (ATR) technique has been developed to identify fungal infections and mycotoxin or other residual contaminations in different types of samples (Kaya-Celiker *et al.*, 2016; Kos *et al.*, 2016; Makio *et al.*, 2007; Mariey *et al.*, 2001; Maquelin *et al.*, 2002).

Shen *et al.* (2016) applied ATR-FTIR to detect AFB₁ in brown rice. Rice samples were divided into four categories, containing AFB₁ concentrations of ≤ 5 $\mu\text{g}/\text{kg}$, between 5 and 50 $\mu\text{g}/\text{kg}$, between 50 and 300 $\mu\text{g}/\text{kg}$, and >300 $\mu\text{g}/\text{kg}$, respectively. The mean spectra of these four categories did not show significant differences, but in specific spectral ranges, e.g. 1,760–1,730 cm^{-1} , spectral absorption values

decreased with an increase in AFB₁ content. When predicting AFB₁ concentrations, the PLSR model performed the best, with a regression coefficient of validation (r_v) of 0.970 and root mean-square error of prediction (RMSEP) of 70.8 µg/kg.

Later, Shen *et al.* compared the applicability of FT-NIR (12,000–4,000 cm⁻¹) and MIR spectroscopy (4,000–600 cm⁻¹) for classification and quantification of total AFs (AFB₁, AFB₂, AFG₁, and AFG₂) in brown rice (Shen *et al.*, 2018). Similar to Shen *et al.* (2016), rice samples containing different levels of AFs were divided into (five) groups. For the classification of samples with varying AFB₁ concentration values, the linear discriminant analysis (LDA) model obtained CCR of 96.9 and 90.6% for NIR and MIR spectroscopy, respectively. The PLSR model for simultaneous detection of AFB₁, AFB₂, AFG₁, and AFG₂ in rice also obtained satisfactory results for both NIR (r_v =0.936–0.973) and MIR (r_v =0.922–0.970), showing these two techniques could be used as suitable tools for rapid detection of AFs in rice both qualitatively and quantitatively. Jiang *et al.* (2017) artificially infected peanut kernels with *A. flavus*, *A. parasiticus*, and *Aspergillus ochraceus*, and counted fungal colonies in mouldy samples using ATR-FTIR technique. During the growth of fungi, fatty acids, proteins, and carbohydrates in peanuts were consumed, and the total number of colonies increased accordingly, causing changes to the spectrum intensity and location. Principle components analysis (PCA) was carried out for further analysis. Several bands (e.g. 1,743, 1,726, 1,695–1,543, 1,460, 1,378 and 1,131 cm⁻¹) related to nutrient composition in peanuts had large weight coefficient in the first three principle components (PCs). The established PLSR model could predict the total number of colonies of multiple strains with an R^2 for prediction set of 0.9157 and root mean-square error (RMSE) of cross-validation of 0.2080 (lg (cfu/g)). Shen *et al.* (2019) applied ATR-FTIR for rapid detection of DON concentration in wheat and wheat products. He showed that samples with different DON content had different absorption values at bands of 1,740, 1,648 and 1,549 cm⁻¹, making these wavebands suitable for quantitative detection of DON. Both PLSR and stepwise multiple linear regression (SMLR) models could effectively predict DON content in samples with an R^2 and RMSEP for prediction set of 0.86 and 0.426 mg/kg, and of 0.86 and 0.438 mg/kg, respectively.

Kos *et al.* (2002) was the first to utilise the ATR-FTIR technique for classification of maize infected by *Fusarium graminearum*. PCA was applied to separate DON-contaminated and ergosterol-contaminated samples from the blank ones. Investigated concentration ranges for ergosterol and DON were 0.73–4.5 mg/kg and 0–2.6 mg/kg, respectively. Samples with toxin content above 0.31 mg/kg could be successfully detected with CCR values between 75–100%. In further research, Kos *et al.* (2003) used a spectral range from 1,800 to 800 cm⁻¹ for all analyses. The detected

concentration ranges were 0.31–2.60 mg/kg for DON and 0.88–3.60 mg/kg for ergosterol. The CCR value could be up to 100% for the classification model built by the KNN algorithm. In order to apply these methods in real-life environments and to reflect the inherent variability of the acquired dataset, Kos *et al.* (2016) chose a large amount of DON-contaminated maize samples with concentrations at the regulatory limit. The bootstrap-aggregated decision tree classifier was able to classify DON-contaminated maize samples at 1,750 µg/kg (set by EU for unprocessed maize) and 500 µg/kg thresholds with an accuracy of 79 and 85%, respectively. When analysing FTIR-ATR spectral characteristics of healthy and *Fusarium*-damaged wheat kernels, Peiris *et al.* (2012) noted considerable variation between their pericarp spectra. Spectra of *Fusarium*-damaged pericarp exhibited higher peak height at 1,160, 1,203, 1,313, and 1,375 cm⁻¹ bands, which were likely caused by the existence of DON and fungi. When endosperm sections of healthy kernels and moderately infected kernels were compared, the variation in band peak characteristics were very limited. It can be concluded from this research that IR spectroscopy can be used to detect DON in the surface tissues of FDKs.

Raman spectroscopy

Raman spectroscopy was discovered by the Indian physicist Sir Chandrasekhara Venkata Raman, and ranges from at least 50 cm⁻¹ to 4,000 cm⁻¹. This technique involves hitting samples with a monochromatic light source (i.e. laser) and detecting the scattered light, as shown in Figure 1 (Yang and Ying, 2011). Most of the scattered radiation, which is known as Rayleigh or elastic scattering, has the same frequency as the incident beam (Egerton and Hardin, 1975). When an inelastic collision between incident photons and sample molecules occurs, the vibrational or rotational energy of molecules changes, resulting in Raman scattering.

In this process, if some molecules are in their ground energy state and the incident photons lose energy, then scattered photons will be shifted into longer wavelengths (Stokes scattering with lower frequency) (Scotter, 1997; Smith and Dent, 2019). Otherwise, if some molecules are

Figure 1. Energy level diagram of Rayleigh and Raman scattering.

already in vibrationally excited states, then those molecules can contribute energy to the incident beam, resulting scattered photons that contain high energies (shorter wavelengths) than the incident radiation. They contribute to the anti-Stokes bands of the spectrum. Under normal experimental circumstances, the number of molecules in their ground energy state is greater than those in excited states. Therefore, Stokes scattering signals are much more intense than the anti-Stokes scattering signals (Egerton and Hardin, 1975).

Raman scattering relies on changes in the polarisability of chemical bonds, making it sensitive to covalent bonds in non-polar functional groups, such as C=C, C-C and S-S (Taabu *et al.*, 2015). A typical Raman spectrum is featured by a series of narrow and nonoverlapping peaks. Different compounds have different vibrational spectra, making Raman spectroscopy a powerful tool for qualitative identification through its molecular fingerprint signals (Phillips *et al.*, 1999). Raman spectroscopy can also be used for quantitative determination, because the intensity of Raman bands increases linearly with the analyte concentration (Yang and Ying, 2011). That is, when the molecules to be tested are poor scatterers and/or at low concentration, the intensity of Raman bands will be very weak. To suppress noisy signals (e.g. fluorescence and phosphorescence) and to amplify Raman signals, different Raman spectroscopy systems, such as dispersive Raman spectroscopy, Fourier transform Raman (FT-Raman) spectroscopy, surface-enhanced Raman (SER) spectroscopy, and spatially offset Raman (SOR) spectroscopy are developed and applied for various applications (Kizil *et al.*, 2002; Matousek 2006; Vankeirsbilck *et al.*, 2002; Wu *et al.*, 2012).

Raman spectroscopy has been used as a potential tool to detect fungi or mycotoxins in cereals in recent years. Liu *et al.* (2009) first explored the feasibility of FT-Raman spectroscopy for rapid screening of DON-contaminated wheat and barley meal. They chose a 1,064 nm NIR excitation laser to reduce interference from fluorescence and photodecomposition of chemical components in wheat and barley. No obviously different peaks could be found between spectra of wheat (or barley) with low DON and high DON, indicating that DON contents at mg/kg level did not change the major functional groups and chemical constituents in wheat and barley. For the purpose of separating samples with low and high DON contents, two Raman bands at 1,560 and 904 cm^{-1} were selected to develop an intensity-intensity plot. By setting proper threshold intensities for 1,560 and 904 cm^{-1} bands, 11 of 12 wheat samples with DON >2 mg/kg and all wheat samples with DON <1 mg/kg, and 10 of 11 barley with DON >6 mg/kg and all barley samples with DON <1 mg/kg could be correctly classified separately, which is in agreement with their PCA results. The feasibility of a portable Raman-

system based SER scattering for rapid detection DON in cereals was investigated in the study of Yuan *et al.* (2017). For pure DON solutions, a wide range of detected concentrations between 10^{-7} - 10^{-2} M and a limit of detection (LOD) at 100 nM could be reached. When they applied this technique to analyse DON-contaminated maize, the achieved LOD was less than the Chinese standard limits, with a value of 10^{-6} M.

Lee *et al.* (2014a) applied Raman spectroscopy ranging from 200 to 3,500 cm^{-1} to qualitatively and quantitatively analyse AFs in ground maize samples. Kernels with an aflatoxin concentration range of 0-1,206 $\mu\text{g/kg}$ were divided into five groups. Samples with aflatoxin concentration below 20 $\mu\text{g/kg}$ were treated as the non-contaminated. Just like the Raman spectral characteristics shown in the study of Liu *et al.* (2009), Raman spectra of maize samples with different AFs concentrations had spectral similarities. Only some bands in several regions showed differences in relative intensity and position. This phenomenon indicates that the effects of physical, chemical, and structural characteristics of grains on the band intensity and sharpness may be more significant than the effects of mycotoxin concentration levels. The best discriminant models developed by LDA did not misclassify any sample with AFs concentration above 20 $\mu\text{g/kg}$ as aflatoxin negative, and had CCR values in the range of 94-100%. PLSR models showed the highest R^2 (0.941-0.957), but performed poorly when AFs concentration was very low. Later, Lee *et al.* (2014b) applied SER spectroscopy to detect AFs in maize. The best classification models developed by KNN yielded CCR values in range of 74.3-91.4% for the validation set, which were not higher – and in fact were even lower – than the classification results in their previous study (Lee *et al.*, 2014a). Lee and Herrman (2016) also analysed the potential and limitation of SER spectroscopy in qualitative and quantitative detection of FB (0-209 mg/kg) in maize. KNN models yielded a slightly better predictive accuracy than other chemometric classification models with CCR for the validation set in ranges of 70.6-79.4%. Quantification models established by a MLR algorithm also yielded acceptable predictive ability, with a R^2 of 0.900-0.940 and RMSEP of 11.162-16.777 mg/kg.

Raman spectroscopy can provide high-sensitive fingerprints for samples, but the high cost of system components, instability of nanostructures, variation in sample batches, and other factors, are still challenges to applying Raman spectroscopy for the detection of mycotoxin-contaminated cereals.

Hyperspectral imaging

Unlike spectroscopic techniques mentioned in previous sections that can only provide information in spectral domain, hyperspectral imaging (HSI) is able to acquire

three-dimensional (3-D) datasets that contain two spatial dimensions and one spectral dimension (El Masry *et al.*, 2012b). A 3-D hyperspectral image can be collected in four modes: one pixel at a time, one line at a time, one image of the same wavelength at a time, and the whole data cube at a time. Typically, the line by line collection mode is more often used in the fields of food and agricultural products. The characteristic of being able to provide image information makes hyperspectral imaging a suitable technique for detection of heterogeneous samples. A hyperspectral image may contain hundreds of wavelengths, and each image at the same wavelength usually contains hundreds of pixels. Hardware and software with excellent performance are needed to store and deal with such huge data cubes (El Masry *et al.*, 2012a). More problematic are the irrelevant noises (e.g. background, edge shadows, remained labels, sample holders) and redundant information that are inevitably collected. In order to extract useful information from the acquired hyperspectral images, the following steps of processing (though not covering all aspects and the order is not fixed) are usually needed to go through: (1) calibration with white and black reference images captured from a standard diffuse reflectance surface and a blocked camera lens environment, respectively; (2) cropping out peripherally irrelevant areas and removing low SNR wavebands; (3) image filtering to suppress system and random noises; (4) image segmentation and region of interest (ROI) selection; (5) extraction of spectral and/or spatial features; and (6) further data analysis by applying chemometric algorithms. Those who are interested in detailed descriptions about how to preprocess a hyperspectral image before further analysis can refer to previous studies (e.g. Chen and Qin, 2008; Ravikanth *et al.*, 2017; Vidal and Amigo, 2012). Originating from remote sensing, hyperspectral imaging has enjoyed great growth over the past decades in detection of food safety and quality issues, such as in meat (Barbin *et al.*, 2012; Jia *et al.*, 2017; Zhu *et al.*, 2013), fruit and vegetables (El Masry *et al.*, 2007; Taghizadeh *et al.*, 2011; Yu *et al.*, 2014), cereal grains (Mahesh *et al.*, 2008; Ravikanth *et al.*, 2015), milk (Fu *et al.*, 2014; Munir *et al.*, 2018), and processed foods (Ariana and Lu, 2010; Talens *et al.*, 2013). With regard to detecting fungi and/or mycotoxin contamination in cereal grains, several studies are available (Chu *et al.*, 2017; Wang *et al.*, 2014, 2015b; Williams *et al.*, 2010).

Wang *et al.* (2014) attempted to detect AFB₁ (with concentrations of 10, 20, 100 and 500 µg/kg) on maize surfaces directly using HSI (1000-2,500 nm) accompanied with PCA and stepwise factorial discriminant analysis (FDA). A minimum classification result of 88% was achieved for the independent validation set, showing that it was possible to discriminate AFB₁ at a low concentration of 10 µg/kg on clean maize kernel surfaces. Afterwards, the feasibility of using HSI to detect AFB₁ in a single maize kernel inoculated with conidia in the field, was assessed (Wang *et al.* (2015b). As shown in Figure 2, interactive

analysis based on scatter plot of PC score images was proven to be an effective way to locate and to remove noisy pixels caused by detectors. There are three prominent peaks on the loading line of PC₁₀₋₁₂ (Figure 2A), which are located at the same wavelength locations as unusual serrated mutations on the original spectral curves. The 3-D score plots of PC₁₀₋₁₂ were rotated and two stray parts of pixels along the axis of PC₁₀ and PC₁₂ were found; these two pixels groups are marked with different colours (Figure 2B), respectively, and exported as ROIs in the PC₁ score image. As shown in Figure 2C, lines with the same colours as ROIs could be found in PC₁ score image. By masking these lines, noises in original spectra could be eliminated. Taking into account that information of AFs is trace compared to the information of maize nutrients, researchers reckoned without the first three PCs and chose PC_{4-PC₈} to select input data for the spectral angle mapper (SAM) classifier. In the research of Wang *et al.* (2015b), PC₇ tended to be essential in the selection of wavelengths, such as 1,729 and 2,344 nm, which corresponded with AFB₁, and the PC₇ score image resembled SAM classification result image. Classification accuracies for kernels that contained AFB₁ higher than 100 µg/kg or lower than 10 µg/kg, all reached values above 80%. However, performance of the classifier for classifying kernels with an AFB₁ concentration between 10 and 100 µg/kg was less satisfactory. Therefore, it could be an option to combine multiple techniques for practical detection and/or separation kernels.

In order to identify mouldy peanuts using NIR hyperspectral images (970-2,570 nm), PCA was applied twice in the research of Jiang *et al.* (2016). Background areas were easily masked due to the great differences between reflectance of background and peanuts. The first PCA was carried out to determine the mouldy-sensitive bands, which had local minimum or maximum values in the eigenvector curves. The first four PCs (denoted by 1PC₁, 1PC₂, 1PC₃ and 1PC₄ in this research) contained most of the information (99.82%). However, since 1PC₁ mainly reflected information of peanut itself, only eigenvectors of 1PC₂₋₄ were used to select the mouldy-sensitive bands. A second PCA was then carried out on the selected mouldy-sensitive bands. Again, the first four PCs (denoted by 2PC₁, 2PC₂, 2PC₃ and 2PC₄) contained the majority of information. 2PC₂ and 2PC₄ were chosen for further analysis as these two PCs explained the 'mouldy' information of interested. The researchers proposed the following equation to fuse multiple PC images into one in this study:

$$\text{Image} = \sum \frac{1}{i \times \lambda_i} \times 2PC_i \quad (1)$$

Where i is the order of the chosen PCs, λ_i was the i -th eigenvalue, and $2PC_i$ was the corresponding PC score image selected from the second PCA. In the resulting image, the

Figure 2. Eliminating noises in hyperspectral imaging using PCA. (A) loading lines of PC₁₀₋₁₂; (B) pixel clusters in the 3-D score image space, (C) corresponding noise lines in the PC₁ score image (adapted from Wang *et al.*, 2015b).

pixel value was proportional to the degree of mould. Therefore, mouldy and healthy pixels could be separated by setting a proper threshold α to the image. Taking the fact that healthy pixels may be miscalculated as mouldy ones into account, it is better to set another threshold β to assist define a peanut as healthy or mouldy. When ratio of mouldy pixels to total sample pixels exceeds the threshold β , this peanut could be considered as a mouldy kernel. In the research of Jiang *et al.* (2016), values for α and β were 0.6 and 15%, respectively. Classification accuracy reached 87.14% in the learning image and 98.73% in the validation image, showing that the proposed method to merge spectral and spatial information together could be a feasible way to identify healthy and mouldy peanuts. Similarly, Qiao *et al.* (2017) tried to identify fungus-contaminated peanuts by utilising spectral-spatial features in hyperspectral images. They chose the feature selection method of analysis of variance (ANOVA) and feature extraction method of nonparametric weighted feature extraction (NWFE) to concentrate favourable information together, so that contaminated and healthy peanuts could be separated more

easily. In this study, each pixel was classified using a support vector machine (SVM) algorithm. When labelling a sample by calculating the ratio of mouldy pixels to total peanut pixels, the threshold-value β was also 15%. The pixel-wise classification accuracies were all above 94% both in learning and validation images.

The early detection of toxigenic fungi on cereal grains is of great importance to prevent these harmful materials from moving up the food chain. In order to investigate changes caused by fungal contamination on maize kernel surfaces, spectra of different maize hybrids that artificially contaminated with different fungus species were analysed in the research of Del Fiore *et al.* (2010). Spectra of uncontaminated maize kernels with different moisture values were also obtained and no obvious spectral differences were found, confirming that moisture content did not affect spectral features in the Visible-NIR spectral range. They also noted that kernel colour had a strong impact on spectral signals located in the NIR spectral range. To identify fungal contamination, spectra with

wavelengths greater than 600 nm were recommended in order to minimise the influence of pigmentation of kernels. Finally, PCA followed by discriminant analysis allowed discrimination of *Aspergillus niger* strains on the kernel surface after 2 d of inoculation. The potential performance of NIR HSI (1000-2,500 nm) for detection of fungal contamination and activity in maize was evaluated in the research of Williams *et al.* (2012). Eight images T_0 - T_8 (with T_5 absence due to technical issues) were successfully collected at eight specified time intervals (0, 17, 20, 23, 26, 43, 69, and 90 h) after *Fusarium verticillioides* spore inoculation. These images together with an image of the control group were merged into a mosaic data cube and PCA was applied to the noise removed image. In the score image of PC_4 , there were three distinct clusters from left to right which corresponded to pixels from control kernels to inoculated kernels for 90 h, respectively. When these clusters were divided into green, black, and red categories, their corresponding pixels in score image would be control, T_0 - T_1 , and T_2 - T_8 , respectively. PLSR models for the prediction of the degree of infection obtained R^2 values in the range of 0.8-0.98 for all treatments, indicating that it was possible to monitor changes in fungal infected maize over time.

For the early detection of *Fusarium* infection in wheat using visible-NIR HSI (400-1000 nm), it was found that spectra over 750 nm were not relevant to disease detection (Bauriegel *et al.*, 2011). During the whole wheat head development period before harvest, the start of the mid-milk stage was recommended as the best disease determination time. In order to classify healthy and diseased tissues, Bauriegel *et al.* (2011) proposed a new head blight index (HBI), which indicated the relevant spectral differences in spectral ranges of 550-560 and 665-675 nm. Classification of diseased ears can be performed in a very short time using HBI, making HBI a suitable outdoor classification method. Shahin and Symons (2012) also applied visible-NIR HSI to detect FDks in Canadian wheat samples. Partial least squares discriminant analysis (PLSDA) was used to separate all samples with varying degrees of infection ranging from sound through mild to severe, into sound and damaged categories. The best PLSDA model could reach high classification results with an overall FDks detection rate of 90% and false positive (misclassified sound kernels into damaged group) of 9%. In their research on automatic detection of FHB in wheat kernels, Barbedo *et al.* (2015) proposed an algorithm to segment single kernels from each other and to calculate a *Fusarium* index (FI). Mean pixel values of sound and damaged kernels were calculated and a proper threshold was given to separate these two groups. The value of this threshold needs to be adjusted according to the different research environments. FI, which calculates the proportion of pixels in a kernel with values larger than the given threshold, was defined as an indicator of the likelihood of kernels being infected by the FHB.

Combined with PCA, this algorithm overcame factors, such as position, direction, shadow, and cluster of kernels, and finally reached a classification accuracy above 91%.

Before fungi form large colonies, they are invisible to the naked eye. Therefore, scanning electron microscopy (SEM) was used to observe symptoms of *Aspergillus oryzae* infection on rice in the study by Siripatrawan and Makino (2015). They found that mycelia first appeared on the embryo side and then the endosperm part, which may be due to the higher level of nutrients (e.g. protein, vitamins, and minerals) contained in the embryo. Combined with a self-organising map (SOM), HSI was able to monitor the degree of *A. oryzae* development even when samples were mould-free to the naked eye. PLSR also obtained a good result when predicting fungal development, with a R^2 value equal to 0.97.

Kandpal *et al.* (2015) applied PLSDA models to detect AFB₁ concentration in maize with different surface colours. Savitzky-Golay first derivative preprocessed spectra obtained acceptable accuracy for all three varieties and the highest overall CCR value was 96.9% for the purple variety. Though a slight misclassification may occur when the AFB₁ concentration is lower than 10 µg/kg, aflatoxin-free samples could readily be separated from contaminated ones. Senthilkumar *et al.* (2016a) was the first to apply HSI to the detection of OTA in stored wheat. After carrying out PCA, based on the highest factor loadings of PC_1 , 1,280, 1,300, and 1,350 nm were identified as the three significant wavelengths that corresponded to fungal (*Aspergillus glaucus* and *Penicillium* spp.) contamination. Again, in another report by Senthilkumar *et al.* (2016b) to detect fungal infection (*A. glaucus* and *Penicillium* spp.) and OTA contamination in stored barley, 1,260, 1,310, and 1,360 nm were identified to be the wavelengths to detect fungal infection damage in kernels from factor loadings of PC_1 . These two reports confirmed that 1,480 nm was only related to OTA in contaminated samples. Statistical and histogram features calculated from significant wavelengths were subjected to classifiers, and the quadratic classifier achieved a higher CCR than linear and Mahalanobis statistical classifiers. OTA-contained wheat and barley samples could all be completely distinguished from OTA-free samples. Satisfied classification accuracies showed that HSI could be a feasibility tool for the detection of different stages of the fungal infection process, as well as different levels of OTA accumulation.

3. Other techniques

Due to nutrient consumption, especially protein hydrolysis which often happens in mouldy cereals and oilseed crops, a dull appearance and unsatisfactory odour produced by the infected samples are unavoidable in most cases. Also, AFs will exhibit fluorescence when excited with ultraviolet (UV)

light sources or NIR laser excitation. Therefore, many other non-destructive detection techniques, e.g. an electronic nose based on electronic sensors to identify specific odour components (Paollesse *et al.*, 2006), fluorescence spectroscopy which analyses the fluorescence properties of certain samples (Hruska *et al.*, 2013; Yao *et al.*, 2013), and image-processing based machine vision (Ataş *et al.*, 2012) could also be used to identify and evaluate fungal growth and mycotoxin contamination. Except for these aforementioned techniques applied in a laboratory environment, researchers have devoted a lot of efforts to the development of non-destructive sorting equipment that can be well adapted to factory scale or online testing environment. In the high-volume grain sorting market, there are different kinds of sorting machines based on colour discrimination, spectral analysis, image processing, and so on. Among this kind of grain sorting equipment, the majority is the colour-based. According to the mode of material transmission, colour sorter machines can be divided into two categories: vertical type and crawler type. Both kinds of equipment can detect the colour changes caused by fungal development and remove them effectively. A colour sorter machine usually consists the following parts: feeding devices, transmission devices, light source, detecting system, controller, and sorting devices.

At the start of the 21st century, colour sorters dominating the Chinese market were imported from Japan (e.g. Satake and Anzai), Switzerland (e.g. Bühler), South Korea and other countries. In recent years, many Chinese brands (e.g. Meyer and Wesort Optoelectronic) that are mainly produced in the cities of Hefei and Shenzhen, begin to meet the Chinese market needs of rapid and non-destructive grain-sorting technology. After decades of development, the techniques for traditional photoelectric colour sorters and charge-coupled device (CCD)-based colour sorters have been relatively mature. However, a critical fact that cannot be ignored is that mycotoxins may be present in some seeds, even if there is no obvious appearance of fungal damage. As in actual storage, it would be a difficult task to prevent cross-infection. Colour sorters will be incompetent to recognise these mycotoxin contaminated seeds.

Therefore, new infrared- and ultraviolet fluorescence-based sorting machines, which can detect both the appearance outside and the chemical composition information inside a seed (or a kernel), are being developed at a fast pace to meet the market demand for improved technologies. In addition to the mainstream structural designs in the market, some research teams are also experimenting with new designs aiming at detecting different samples. For instance, the crawler-type fungal-damaged and toxin-contaminated grain sorting machine shown in Figure 3 (Jiang *et al.*, 2018). This machine takes the flat shape of maize into account, as fungi will only grow on one side of a kernel. An arc-shaped turnover device in conjunction with

two belts layers was designed to turn maize kernels over, so both kernel sides can be examined. The core component of this sorting equipment is the photodiode-based data acquisition card installed in the detection system. LED lights with different wavelengths are arranged in circle to give uniform illumination. Thus, the photodiode arranged in centre of the lights is able to collect data on the samples. According to previous studies (e.g. Pearson *et al.* 2004), light sources with different wavelengths can be used in high speed sorting machines to help identify mycotoxin-contaminated grains. Thus, by changing the wavelengths of the LED lights, this sorting machine could be used to detect and remove different kinds of mycotoxins contaminating grain.

4. Conclusions and future trends

Benefitting from the exciting recent advances in hard- and software technology, vibrational spectroscopic techniques, including infrared spectroscopic and Raman spectroscopic technique have greatly evolved in the past decades. It is apparent that research on rapid detection of fungi and mycotoxins by vibrational spectroscopic techniques started later in China compared to the western countries. However, this special and fast-growing research field still offers many opportunities for contributions by Chinese scientists.

All the spectroscopic techniques have shown satisfactory performances in the laboratory environment, but for large-scale applications of these technologies or online automatic applications, there is still a long way to go. The major obstacles that need to overcome are the high cost of the equipment, huge datasets, lengthy processing procedures, and the demand of high detection limits. To fully utilise these spectroscopic techniques further in a commercial market, developing high-speed and low-cost equipment, improving the appropriate modelling robustness in data processing, and increasing the detection sensitivity to meet the demands of detection limits are necessary. In addition, combining multiple techniques in designing new instrument, e.g. X-ray, fluorescence spectroscopy, nuclear magnetic resonance, electronic nose, could be a prudent approach to overcome the shortcomings of an individual technique.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/WMJ2019.2510>.

Table S1. Summary description of studies using near-infrared, mid-infrared, Raman spectroscopic, and hyperspectral imaging techniques.

Figure 3. The schematic diagram (A) and photo (B) of a belt-based double-side sorting machine for detecting mouldy and toxin-contaminated maize kernels, developed by the Non-destructive Detecting Laboratory for Agricultural and Food Products in the College of Engineering at the China Agricultural University.

Acknowledgements

This work was financially supported by the National Key Research and Development Program of China (2018YFC1603506).

Conflict of interest

The authors declare no conflict of interest.

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