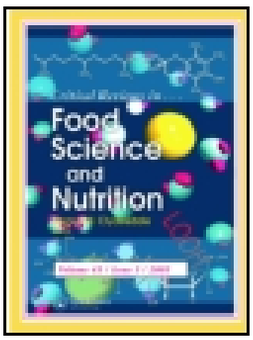

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Mycotoxin Research with Advanced Molecular Spectroscopy

Advanced synchrotron-based and globar-sourced molecular (micro) spectroscopy contributions to advances in food and feed research on molecular structure, mycotoxin determination and molecular nutrition

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ABSTRACT

Mycotoxin contamination has been a worldwide problem for food and feeds production for a long time. There is an obviously increased focus of the food and feed industry toward the reduction of mycotoxin concentration in both the raw materials and finished products. Therefore, both effective qualitative and quantitative techniques for the determination of mycotoxins are required to minimize their harmful effects. Conventional wet chemical methods usually are time-consuming, expensive, and rely on complex extraction and cleanup pretreatments. Synchrotron-based and globar-based molecular spectroscopy have shown great potential to be developed as rapid and non-destructive tools for the determination of molecular structure, molecular nutrition

and mycotoxins in feed and food. This article reviews the common types of mycotoxins in feed and food, their toxicity, as well as the conventional detection methods. The principle of advanced molecular spectroscopy techniques and their application prospects for mycotoxin detection are discussed. Recent progress in food and feed research with molecular spectroscopy techniques is highlighted. This review provides a potential and insight into how to determine the structure and mycotoxins of feed and food on a molecular basis with advanced Synchrotron-based and globar-based molecular (micro) spectroscopy.

KEYWORDS

Molecular structure, synchrotron radiation, mycotoxin detection, molecular spectroscopy

ACCEPTED MANUSCRIPT

I. Introduction

1.1. *General*

As toxic compounds produced by certain fungal genera, mycotoxins could cause serious illnesses and death in humans and animals (Hussein and Brasel, 2001; Zain, 2011). They commonly exist in agricultural commodities and could be produced at various stages (before and after the harvest of cereals, during processing and transportation, etc.). The plant-based raw stuff may contain a high content of mycotoxins, while that in end product may be lower (Marin et al., 2013).

From a chemical point of view, mycotoxins exhibit numerous structures and have different biological effects on humans and animals, such as carcinogenicity, teratogenicity, mutagenicity, neurotoxicity, or immunotoxicity (Hussein and Brasel, 2001; McKean et al., 2006; El-Sayed and Khalil, 2009). More than 300 mycotoxins have been found, and only those have proven to be highly toxic received extensive scientific attentions (Zain, 2011). According to their different biological origins and structures, mycotoxins could be classified into cyclopeptides, terpenes, nitrogenous metabolites and polycetoacids (Bhat et al., 2010). Human exposure to mycotoxins usually results from eating contaminated food, including both plant-based and animal-derived food (Richard et al., 2003). Many factors can affect the magnitude of toxicity of animals or humans ingesting of mycotoxin contaminated feedstuffs or foods, such as species, modes of action, and defence mechanisms (Hussein and Brasel, 2001).

Mycotoxin contamination results in severe quality reductions of economically important commodities and brings great economic loss. To prevent mycotoxins from being a threat to humans and animals, both producers and governments are spending huge amounts on mycotoxin management, which mainly including research, testing and insurance. In the United States, the contamination of aflatoxin (maize and peanuts), fumonisin (maize), and deoxynivalenol (wheat) resulted in estimated annual loss ranging from $ 0.5 million to over $ 1.5 billion (Robens and Cardwell, 2003).

1.2. Common mycotoxins in feed and food

A variety of crops is suitable for the growth of mycotoxigenic fungi. Ochratoxins, aflatoxins, fumonisins, ergot alkaloids, and patulin are regarded as the common types found in feed and food (Bhat et al., 2010; Marin et al., 2013).

As highly carcinogenic and mutagenic toxins produced by fungal species of *Aspergillus flavus* and parasiticus, Aflatoxins appear primarily in humid and hot climates and can infect many agricultural commodities like rice, peanuts, maize, fish meal, and meat meals (Ellis et al., 2000; Stroka and Anklam, 2002; Bhat et al., 2010). Cleaning procedures can remove mould-damaged kernels from cereals or nuts, which may result in more than 40% reduction of aflatoxins (Park, 2002). To date, scientists have identified nearly 18 kinds of aflatoxins, among which aflatoxin B_1 (AFB_1) and M_1 (AFM_1) are considered as most important ones (Bhat et al., 2010). AFB_1 is usually regarded as the most hepatotoxic and hepatocarcinogenic and presents in a variety of agricultural commodities (Marin et al., 2013). The regulatory limits of AFB_1 (2 ppb) and total aflatoxins (4 ppb) in grains, dried fruits and peanuts for human consumption were set up by

European Committee Regulations (Stroka and Anklam, 2002). As the major mono hydroxylated derivative of AFB_1, AFM_1 is formed in the liver via cytochrome P450-associated enzymes (Marin et al., 2013). AFM_1 contamination in milk products is of great significance, since most infant and young animal are relying on milk to obtain basal nutrition for growth and development, and they are more sensitive to toxins than adults.

Ochratoxins, mainly including ochratoxin A (OTA), ochratoxin B (OTB), ochratoxin C (OTC) and ochratoxin α (OTα), are toxins produced by fungi of *Aspergillus* and *Penicillium* genera (Ringot et al., 2006). OTA is considered as the most potent among them (Bhat et al., 2010; Marin et al., 2013). Many factors, such as water activity (a_w), environment temperature, and medium composition, could change the physiology of OTA-producing fungi, thereby affect their production of OTA (Ringot et al., 2006). In temperate and cool regions, *Penicillium verrucosum* and *P. nordicum* are major producers of OTA (Pitt, 1987; Larsen et al., 2001; Castella et al., 2002). The *P. verrucosum* prefer to contaminate grains, while *P. nordicum* are mainly detected in milk and meat products (Larsen et al., 2001). Interestingly, a previous study suggested that some sort of competition may exist between OTA and AFB_1 either in their absorption rate in the digestive tract or at the production level in foods since AFB_1 was always absent or present at low concentrations whenever the high content of OTA was detected (Zain, 2011). The common food processing procedures are unable to destroy OTA since it's a stable compound. Previous research showed that keeping temperatures above 250 °C for several minutes could decrease the concentration of OTA (Marin et al., 2013).

Fumonisins (FBs) are a group of toxins synthesized primarily by several species of *Fusarium* molds, among which the *Fusarium proliferatum* and *Fusarium verticillioides* are regarded as the most common producers (Miller, 2008; Marin et al., 2013). The long-chain hydrocarbon unit of them is important to their toxicity (Zain, 2011). They are frequently detected mycotoxins in maize, which can be formed before and after harvest (Marin et al., 2013). As the most toxic member of this group, Fumonisin B_1 (FB_1) is frequently detected in naturally contaminated corn or fungal cultures (Orsi et al., 2000). The FBs concentration in foods could be reduced by some thermal or extrusion treatments (Marin et al., 2013).

Ergot alkaloids are toxic compounds produced by fungal genera of *Claviceps* that contaminate a broad range of grass species, including some cereals, at the time of flowering (Krska and Crews, 2008; Schmale and Munkvold, 2009). The developing grain could be replaced by the fungus with ergot body that containing ergot alkaloids (Krska et al., 2008). Damp, warm and rainy seasons like springs and summers favor ergot infestations (Caporael, 1976). The total ergot alkaloids concentration in sclerotia ranges from 0.01 to 0.5 (w/w) (Lorenz and Hoseney, 1979; Schoch and Schlatter, 1985). The sclerotia are usually harvested together with grains and could contaminate cereal-based feed and food with ergot alkaloids (Krska and Crews, 2008). Ergometrine, ergocristine, ergocornine, ergotamine, ergocryptine, and ergosine are the six major high toxic ergot alkaloids frequently detected in feed and food (European Feed Safety Authority, 2005).

Patulin is a toxic fungal metabolite included in the smallest group of toxic metabolites (Bhat et al., 2010). It is produced by a variety of molds belonging to the species of *Paecilomyces*,

Penicillium, *Byssochlamys*, *Aspergillus*, and *Eupenicillium* genera (Marin et al., 2013). As an important mycotoxin often associated with fruits, patulin is commonly detected in apple and apple products (Murillo-Arbizu et al., 2009). Patulin was also detected in ruminant feedstuffs. Previous study reported that typical neurotoxic signs in animals could rarely be caused by the lower content of patulin found in silage, but the ruminal microflora might be detrimentally affected due to the antimicrobial activity of patulin (Schneweis et al., 2000).

1.3. Toxicological aspects of the common mycotoxins

Similar to all toxicological syndromes, mycotoxicoses also can be categorized as chronic or acute (Zain, 2011). Chronic toxicity is the result of long-term exposure to low-dose toxins. Acute toxicity is distinguished from chronic toxicity, which usually has a swift onset and noticeable symptoms.

Aflatoxicosis is toxic hepatitis which can result in jaundice or even more severe cases, death (Zain, 2011). In the1960s, the epidemic of turkey "X" disease showed the toxicity of aflatoxins, for the first time (Spensley, 1963). The feed contaminated with *A.flavus* caused mass mortality in turkey farms in UK. AFB_1 can act synergistically with HBV infection and has been extensively related to primary hepatic carcinoma in human, therefore it has been categorized as Group 1 human carcinogen (IARC, 1993). The magnitude of response in vertebrates to exposure to AFB_1 could be affected by numerous factors, such as dietary types, nutrient supply, gender, as well as other environmental factors (Eaton and Groopman, 2013).

Ochratoxin A is regarded as nephrotoxic, carcinogenic, teratogenic, and immune-suppressive (Bhat et al., 2010), and has been classified as Group 2B human carcinogen (IARC, 1993). As a potent nephrotoxin, OTA takes kidney as the major target organ and is frequently associated with severe kidney diseases (Marin et al., 2013). OTA may also have harmful effects on other body systems in addition to renal symptoms. Broilers fed with OTA contaminated feed (0.3-1 mg/kg) showed reduced glycogenolysis and a dose-dependent glycogen accumulation in the liver was observed (Zain, 2011).

According to the previous study, FBs could cause both acute and chronic toxicity such as carcinogenicity and cardiovascular toxic effects in many animal species (Gelderblom et al., 1991). The major target organs of FBs are kidney and liver, and the response to the dose can be affected by sex, species, and strain-dependent differences (Voss et al., 2007). A link between the high morbidity of human esophageal cancer and the consumption of maize severely contaminated with fumonisins and *F. verticillioides* in South Africa was also suggested by a previous study conducted by Marasas et al. (2004). Consuming FB_1 contaminated feeds could result in pulmonary oedema in swine, which has many clinical signs including decreased feed intake, weakness, cyanosis, dyspnoea, and death (Marin et al., 2013).

Ergotism is also known as ergot poisoning, which is resulting from intaking of ergot alkaloids contaminated food or feed (Caporael, 1976). A previous study reported that ergot alkaloids intoxication has several symptoms, such as burning skin sensation, abdominal pains and anhypnia, etc (Caporael, 1976). The latest outbreak of gangrenous ergotism was occurred in Ethiopia, according to a laboratory studies carried out by Urga et al. (2002). Nowadays, ergotism

is still regarded as a significant veterinary problem, especially in pigs, chicken, horses and domestic ruminants (Bennet and Klich, 2003).

Patulin is regarded to be carcinogenic and genotoxic, might cause apoptosis in human leukemia cells and induce gene mutations in cultured in mammalian cells (Schumacher et al., 2006). Patulin has inhibitory activity on many enzymes, mainly due to its strong affinity for sulfhydryl groups (Marin et al., 2013). The functions of kidney, gastrointestinal tissues, liver, and immune system can be harmed by patulin, according to previous studies (Wichmann et al., 2002; Bhat et al., 2010).

1.4. Basic idea of molecular spectroscopy techniques

When Chemical bonds in molecules encounter infrared light with the certain vibrational frequencies (same as the bonds' characteristic vibrational frequency), vibrations occur. Many factors, including the mass of atoms, firmness of bonds, as well as other features of molecules could affect the vibrational frequency of bonds (Karoui et al., 2010). As a branch the rotational-vibrational spectroscopy, infrared (IR) spectroscopy is one of the most important molecular spectroscopy techniques available to scientists. The IR radiation induced transitions between different vibrational energy levels could be measured by IR spectroscopy spectrometers (Larkin, 2011). A spectrum can be generated by passing infrared light through specific material and recording how much energy is absorbed by the molecules at each wavelength.

Infrared absorption bands are characterized by the specific wavelength (energy), shape (environment of bonds), and intensity (polar character or polarizability) (Stuart, 2005; Larkin,

2011). Each molecule has unique vibrational energy levels, so the IR spectrum offers the "fingerprint" of a particular molecule, and contains information related to the molecular environment, dynamics and structure (Stuart, 2005; Larkin, 2011; Rao, 2012).

Mid-infrared (MIR) region (4000-400 cm^{-1}) contains fundamental vibration of molecules, while the Near-infrared (NIR) spectra (700-2500 nm) contains overtone information and combinations of the fundamental vibrations, making them more difficult to be interpreted (Shiroma and Rodriguez-Saona, 2009). Therefore, MIR spectroscopy is more sensitive to the chemical composition of samples compared to NIR (Bellon-Maurel and McBratney, 2011).

Molecular spectroscopy plays an import role in modern science and has a very long history (Rao, 2012). With the improvement of radiation sources and the development of chemometrics, more and more important aspects of molecular spectroscopy techniques have been demonstrated (Stuart, 2005; Dumas et al., 2007; Yang and Yu, 2017).

II. Conventional methodology to detect mycotoxin

2.1. Screening methods and quantitative methods

The requirements to monitor mycotoxins and prevent them from posing a great risk to humans and animals have prompted the development of a variety of detecting methods for screening and quantification of mycotoxins in agricultural commodities. The varying concentration ranges (from < 1 ppb to > 10000 ppb) of mycotoxins in various agricultural commodities, and their chemical diversity present huge challenges to analytical scientists (Krska et al., 2008).

Gas chromatography (GC), high-performance liquid chromatography (HPLC), and Liquid Chromatography with Tandem mass spectrometry (LC-MS/MS) are conventional methods for quantitative analyzing of mycotoxins in feeds and foods. Semi-quantitative or qualitative results can be provided by thin layer chromatography (TLC), which is frequently applied for screening of mycotoxin contaminated samples. Another rapid screening technique is enzyme-linked immunosorbent assays (ELISA).

2.2. High-performance liquid chromatography

The HPLC with UV-diode array detection or fluorescence detection (HPLC-FD) has been a powerful technique for mycotoxins determination (Trucksess, M. W., 1998). Most of the HPLC protocols applied for mycotoxins analyze are similar (Turner et al., 2009), and many of them have been adopted as the official AOAC methods (Kralj Cigić and Prosen, 2009). Some types of mycotoxins, which have natural fluorescence (OTA, citrinin and aflatoxins) could be directly determined by HPLC-FD methods (Valenta, 1998). But other mycotoxins which don't have a suitable chromophore need derivatisation before their determination (Shephard, 1998). Studies showed that pre-or post-column with proper reagents could be employed for derivatisation purpose (Neely and Emerson, 1990; Chiavaro et al., 2001). In practice, HPLC-FD methods are mainly applied for the detection of OTA in food, such as rice, dried fruits, and green or roasted coffee (Zinedine et al., 2007; La Pera et al., 2008). The HPLC method based on diode array UV detector can be used to analyze 182 structurally different mycotoxins was also developed (Frisvad and Thrane, 1987). However, only the mycotoxins produced in fungi can be analyzed

by this method, and it's unfit for detecting mycotoxins in feedstuff and food (Pittet, 2005; Bueno et al., 2015).

2.3. Gas chromatography

Gas chromatography is a widely used chromatography technique for separating and detecting substances that can be vaporized without decomposition. In GC, a capillary or packed column is used as stationary phase, and an inert gas usually works as the moving phase (Grob and Barry, 2004). The separation of components is based on their relative affinity with the stationary phase. Combined with different detection methods, such as UV detection, electron capture detection, mass spectrometry, or fluorescence detection, GC is extensively adopted in application for food and feedstuff analysis (Krska et al., 2008; Köppen et al., 2010). GC is designed for the detection of the compounds which are volatile or semi-volatile, non-polar or semi-polar, and thermally stable. Derivatisation procedure is needed for most mycotoxins before GC detection since they are polar and non-volatile molecules (Scott, 1995). GC has been used as a popular method for quantitative analyze of trichothecenes in feeds and food (Bueno et al., 2015). Very limited studies can be found for the detection of patulin, OTA, citrinin, or ZON by GC techniques (Köppen et al., 2010). The first disadvantage is only volatile components or those that can be converted into volatile components, are able to be analyzed (Bueno et al., 2015). Another problem is the thermal stability of the components since heating sometimes degrades the components (Turner et al., 2009).

2.4. Liquid Chromatography-tandem mass spectrometry

Co-contamination with different mycotoxins is frequently observed under natural conditions (Streit et al., 2012; Wang et al., 2016). According to the result of multi-mycotoxin studies, 75%-100% of the samples were co-contaminated with many mycotoxins, which could be detrimental to animal performance at already low contents (Streit et al., 2012). Consequently, increased studies have been conducted to explore techniques which can detect multiple mycotoxins simultaneously.

Taking advantage of both the separation power of LC and excellent sensitivity of mass-spectrographic techniques, LC-MS/MS has gained more and more popularity in recent years (Gros et al., 2006; Beltran et al., 2009).

Electrospray ionization (ESI) is a frequently used ionization method for LC-MS/MS, another important one is atmospheric pressure chemical ionization (APCI) (Berthiller et al., 2007; Bueno et al., 2015). Through the adoption of ESI or APCI, ionization polarities could be switched within the same chromatographic run to achieve the best possible ionization yields for different mycotoxins (Berthiller et al., 2007).

As a very promising technique, LC-MS/MS can determine multiple mycotoxins simultaneously without the need for derivatisation (Bhat et al., 2010; Bueno et al., 2015). A variety of feed and food samples have been analyzed for multi-mycotoxins by LC-MS/MS, such as corn, corn flakes, rye flour, wheat, barley, tall fescue, grass, complex feed, rice, peanuts, biscuits, figs, apple, orange, sweet cherry, tomato, milk, cheese, etc (Sulyok et al., 2007; Berthiller et al., 2007; Krska and Crews, 2008; Wang et al., 2016; Hu et al., 2016). Recent representative applications of LC-MS/MS in food and feed studies are listed in Table1.

2.5. Enzyme-linked immunosorbent assay

The spatial structure of the mycotoxin can be distinguished by a certain antibody, and the ELISA method was developed based on this principle (Zheng et al., 2006). Commercial ELISA kits have been designed for major types of mycotoxins, and this method has been applied routinely for rapid screening many types of mycotoxins, especially for raw materials (Gilbert and Anklam, 2002; Krska et al., 2008). Most of the ELISA kits rely on a heterogeneous, competitive format in which the mycotoxin from a feed or food sample competes with the labelled mycotoxin for certain quantity of antibody-binding sites (Bueno et al., 2015). High concentration of mycotoxin in substrate could result in reduced binding of the labelled mycotoxin and weaker signals generated by the assay.

ELISA kits are sensitive, rapid, simple, and portable for practical application in the field (Trucksess, 2001). However, there are also several obvious disadvantages of ELISA methods. For example, the target compounds are no longer antigens, but mycotoxins, so the compounds which have similar chemical groups are also able to bind with the antibodies, and this may result in overestimated or underestimated concentration of mycotoxins (Zheng et al., 2006; Bueno et al., 2015). What's more, they can only be applied to the matrices for which they were validated due to the insufficient validation of ELISA methods (Gilbert and Anklam, 2002).

2.6. Thin layer chromatography

As a rapid and cost-efficient method for mycotoxin detection, TLC can analysis multiple mycotoxin-contaminated samples simultaneously, but it is unable to make precise or sensitive measurements (Bueno et al., 2015). Traditionally, TLC is the most popular methods for

mycotoxin measurements, many of the pioneering research on mycotoxins were conducted using this technique (Pittet, 2005). Today, TLC is still widely used for the analysis of mycotoxins, due to its low cost, simple operation, using of UV-VIS spectral analysis, high throughput (Turner et al., 2009). In developing countries and poverty-stricken areas, TLC offers a cost effective alternative to LC-based technique and has an important role for control of regulatory limits and surveillance purpose (Gilbert and Anklam, 2002).

III. Globar-sourced advanced molecular spectroscopy

Infrared spectroscopy techniques are promising tools for the fast and intact detection of mycotoxins in feed and food (Krska et al., 2008). They only need minimum sample preparation and have the potential to be joined with on-line detection systems. The NIR spectroscopy (750-2500 nm) is widely used in many areas in feed and food industries particular for grain quality monitoring (Wang and Paliwal, 2007). Nevertheless, the combination bands and overtones of fundamental vibrations measured by NIR technique generally produce weak and broad peaks (Lee et al., 2015). In contrast to NIR, most of the peaks in MIR region are sharp and narrow due to molecular vibrations of the matrix compounds (Yang and Irudayaraj, 2001). What's more, the degree of resolution of MIR spectra is much higher than that of NIR spectra; as a consequence, MIR bands could be assigned to specific chemical groups (Roychoudhury et al., 2006). The plentiful information contained in MIR spectra may benefit the constructing of acceptable calibration models for mycotoxins detection.

3.1. Concept and principal of infrared molecular spectroscopy

Infrared region extends from the red edge of visible spectrum (around 700 nm) to the short microwave portion (100000 nm) (Lasch and Naumann, 2015). The IR spectroscopy can quantify the vibrational modes of IR active molecular bonds, offering a unique, label-free tool for investigating molecular structure and dynamics without perturbing the sample (Baker et al., 2014; Griffiths and De Haseth, 2007). The Fourier transform infrared (FTIR) spectrometer is a commonly used device to produce such spectra, and it circumvented obvious defects of traditional dispersive IR spectroscopy technique (Griffiths and De Haseth, 2007; Lasch and Naumann, 2015). FTIR spectrometer is no longer working like dispersive IR spectrometer, which uses monochromator (grating or prism) to separate the wavelength of IR light (Lasch and Naumann, 2015). A hot white light source (globar), Michelson interferometer (split the light into two arms), detector, and computer are the basic components of a typical FTIR spectrometer (Lasch and Naumann, 2015; Liu and Yu, 2016). Compared to dispersive spectrometer, FTIR equipment is more sensitive and could detect the entire IR region simultaneously and yields high spectra with excellent resolution (Griffiths and De Haseth, 2007; Liu and Yu, 2016).The typical FTIR spectra of barley wheat samples are shown in Figure 1.

3.2. Important IR bands in feed and food research

The biological components of feed or food have many different molecular bonds. IR spectroscopy could detect the light absorption of specific molecular bonds at their charateristic frequencies, and unknown components in the sample could be identified by mining the information contained in the spectrum (Messerschmidt and Harthcock, 1988; McKelvy et al., 1998; Liu and Yu, 2013). MIR spectrum (ca. 4000-400 cm^{-1}) mainly consists of four parts,

including the X-H stretching region (ca. 4000-2500 cm^{-1}), the triple bond region (ca. 2500-2000 cm^{-1}), the double-bond region (ca. 2000-1500 cm^{-1}), and the fingerprint region (ca. 1500-600 cm^{-1}) (Stuart, 2004; Liu and Yu, 2013). Some characteristic bonds of certain biological components have also been revealed by previous studies. For example, in the mid-IR spectrum, the amide Ⅰ (its peak is found around ca. 1650 cm^{-1}) and amide Ⅱ bands (its peak is located at the region around ca. 1550 cm^{-1}) are characteristic bands of peptide bonds which can be used to explore the secondary structure of protein (Jackson and Mantsch, 1995; Marinkovic et al., 2002; Yu, 2004); the typical bands of C = O ester (around 1738 cm^{-1}), CH_2 (near 2925 and 2853 cm^{-1}), and CH_3 (around 2961 and 2871 cm^{-1}) are considered as characteristic bands of lipids (Jackson and Mantsch, 1995; Wetzel and LeVine, 1999; Marinkovic et al., 2002; Yu, 2004); carbohydrates are composed of variety of bonds and have complex IR absorption bands, and usually generate intensity bands around ca.1550-800 cm^{-1} (Wetzel et al., 1998; Yu, 2004). These representative bands could be used as suitable probes to investigate the changes or differences in protein, lipid, and carbohydrate molecular structures. Typical MIR absorption frequencies recorded of cereal materials include hydroxyl, methylene, carbonyl (CO) stretching, NH bending and CO stretching vibrations; the CO and NH vibrations can further be related to protein and carbohydrates present in the samples (Kos et al., 2003).

Due to the limited sensitivity of conventional spectroscopy-based techniques, low concentration contaminants including mycotoxins in a complex matrix could not be directly detected (McMullin et al., 2015). Nonetheless, mycotoxigenic fungi infection may cause changes

in the protein and carbohydrate content, as well as other physical alterations, which can be detected by examining differences in specific bands (McMullin et al., 2015; Kos et al., 2016).

3.3. Univariate and multivariate statistical methods

To investigate the characteristics of IR spectra, both univariate and multivariate statistical techniques are needed (Yu, 2005; Liu and Yu, 2016). Quantitative analysis of spectral characteristic parameters (e. g. band frequencies, intensities, and band intensity ratios) is mainly completed by univariate analysis methods (Yu, 2006). After converting the spectra to absorbance display mode, the absorbance value for each wavelength could be obtained and used to investigate the chemical composition or other parameters of specific samples. For instance, the molecular structures of protein between different grains and dried distillers grains with solubles (DDGS) was successfully investigated by univariate analysis (Liu et al., 2012) and the protein characteristics of bioethanol products after dry fractionation process (Zhang et al., 2012).

Unlike univariate statistical technique, which is mainly for analyzing single variables of interest at a time, multivariate statistics can evaluate relationships among multiple objects. The data sets obtained from IR spectra is always large and complicated, thus multivariate analysis techniques are more appropriate for these complicated situations. The hierarchical cluster analysis (CLA), principal component analysis (PCA), linear discriminant analysis, and artificial neural networks are the major multivariate statistic techniques applied in IR study (Dumas et al., 2007).

The PCA and CLA are frequently used in spectral data analysis. They are used for transforming large sets of correlated variable into a smaller number of uncorrelated variables (Liu and Yu, 2016). PCA could reduce the dimensionality of original data sets by deriving a new data set which composed of uncorrelated variables called principal components (PCs) (Yu, 2005). A score (eigenvector) is assigned to each spectrum to define its relationship to the specific principal component. A two or three-dimensional scatter plot could be created using the eigenvector to show the similarity between the spectra. A typical score/score plot of PCA analysis calculated from preprocessed (standard normal variate transformation) FTIR spectra data of barley and wheat samples is shown in Figure 2.

The CLA aims to build a hierarchy of clusters and reveal relatively homogeneous groups of variables according to a predefined metric (Goyeneche et al., 2014). When applied in feed research, CLA is capable of distinguishing between various processing methods of one variety as well as different varieties of the same feeds (Yu, 2005). A dendrogram (simple tree-like structure) will be created to illustrate the results of CLA. By combining the results of univariate and multivariate spectral analysis, the relationship between structural spectral features and nutrition availability can be revealed (Gamage et al., 2012; Jonker et al., 2012; Yang et al., 2014).

3.4. Applications in plant-based food and feed research

A typical FTIR spectrum in MIR region contains peaks which are sharper and narrower than that of NIR, and

The FTIR technique could generate spectra with higher resolution and better signal-to-noise ratio than NIR (Karoui et al., 2010). As an excellent analytical method, FTIR technique is able to investigate both structural features and compositional characteristics of samples. From microbial material to biological tissues and feedstuffs, a variety of samples could be evaluated by FTIR spectroscopy method (Schmitt and Flemming, 1998; Movasaghi et al., 2008; Yu and Nuez-Ortín, 2010). Among minor and trace constituents, FTIR spectroscopy with chemometric data analysis methods plays an increasing role as a powerful technique to screen for the occurrence and quantities of contaminants (Kos et al., 2016).

In feed studies, the structural characteristics associated with nutrient compositions and biological availabilities have been explored using the FTIR method. For example, the alterations in the structural characteristics of carbohydrates and lipids of distillers dried grains with solubles (DDGS) were studied using FTIR technique (Yu, 2011; Yu et al., 2011). The previous study showed that the changes in nutrient composition and the structural characteristics that caused by silencing *HB12* and *TT8* genes could be revealed by FTIR technique (Li et al., 2015). In another research, FTIR technique was used to study the structural difference of protein in alfalfa harvested at different maturity stages (Yari et al., 2013).

In food research, a recent study applied this technique to identify lard in vegetable oil and other food products, and their results showed that FTIR spectroscopy could be applied for the identification of adulteration of food (Al-Kahtani et al., 2017). An easy-to-use, fast and accurate technique for the detection of oxirane oxygen content as well as changes in iodine value during the epoxidation of vegetable oils was developed by attenuated total reflectance FTIR technique

(Tavassoli Kafrani et al., 2017). In another study, this technique was used to investigate the difference in antimicrobial and antioxidant properties of coffee oil samples (with or without thermal treatment) (Raba et al., 2015). The results of spectroscopy analysis showed that no obvious differences in the oxidative status of crude green and roasted coffee oil were found.

An obvious disadvantage of FTIR technique is that the samples need to be ground before analysis. The destroyed inherent structures may lead to the loss of crucial information with regard to constituent distribution and its spatial origin. Besides, the conventional FTIR technique is unable to reveal the relationship between the digestive characteristics and bioavailability of nutrients and the inherent structural characteristics of food or feed (Yu, 2004).

IV. Synchrotron-based FTIR microspectroscopy (SFTIRM) as a novel tool for mycotoxin detection

4.1. Concept and basic principles of synchrotron-based infrared microspectroscopy

When performing examinations at the cellular level, some shortages of the globar light source could be observed due to the aperture size of equipment and wavelength range of radiation light (Raab and Martin, 2001; Yu, 2004; Miller et al., 2007; Liu and Yu, 2016). Some of them (e.g. unsatisfied signal-to-noise ratio and diffraction) could be diminished by the application of synchrotron light.

The synchrotron is a bright source (100-1000 times brighter than globar source) of infrared photons, and it's the only "white" source which can deliver the whole range of IR wavelength range, from near-IR to far-IR region (Duncan and Williams, 1983; Dumas and Miller, 2003; Yu

et al., 2008). It is noteworthy that the brightness advantage of synchrotron light is not because the synchrotron generates stronger power, but because of the narrow range of emission angles and the small effective source size (Dumas and Miller, 2003). The microscope allows researchers to focus the radiation light onto super small parts of the samples and isolate the histological structures in the sample (Doiron and Yu, 2017). When combining FTIR spectroscopy with microscopy and synchrotron light source, the SFTIRM is created, which could be used to conduct molecular chemistry study at a cellular or subcellular level without destroying the inherent structure of biological tissues (Wetzel et al., 1998; Yu, 2004). The SFTIRM can produce abundant information on the composition, structure, chemistry, and environment of specific tissue simultaneously (Yu, 2004).

The use of SFTIRM has benefited to researchers significantly. However, there have been only limited studies on the relationship between the chemical composition and bio-availability and inherent structural characteristics of feed and food samples. And more notably, no study has been conducted to investigate the application prospect of SFTIRM technique for mycotoxin detection.

4.2. Applications of in feed and food research

As a superior spectroscopy technique, SFTIRM could be applied to a broad range of research area (Yang and Yu, 2017).The high brightness and ideal S/N ratio of SFTIRM are of especially importance to acquire high-quality spectral data on chemical composition. Up to now, the distribution of chemical constitutes of different materials have been localized and characterized at a cellular and subcellular levels by many interdisciplinary studies. For instance, SFTIRM has

been recently used as an excellent technique to explore the C-S stretching band of $^{12}CH_3{}^{32}SH$ (Lees et al., 2016), the coriolis interaction between the $v_8 = 1$ and $v_{10} = 1$ states of ethylene-cis-1, 2-d_2 (Ng et al., 2016), as well as the composition and ultrastructure alterations in single cells (Whelan and Bell, 2015).

The SFTIRM technique has been applied to agricultural research to broaden knowledge of nutritional composition and structural characteristics of feed and food. This advanced technique is capable of exploring the structural information of different botanical parts (Wetzel et al., 2003; Yu, 2007a). Some previous studies used SFTIRM technique to investigate the intrinsic molecular structures of a variety of feeds. For instance, the nutritional information of barley, oat, corn, wheat, and DDGS with regard to spectral and chemical characteristics and biological constituent distribution were evaluated using the SFTIRM technique (Yu, 2004; Damiran and Yu, 2010; Yu and Nuez-Ortín, 2010; Abeysekara et al., 2011; Gamage et al., 2012). The SFTIRM could also be used to explore structural changes induced by feed or food processing. By using this technique, the structural alterations of protein caused by heating were probed within cellular dimension, and the relationship between protein structure and its degradative parameters as well as ruminal and intestinal digestive kinetics in dairy cow was also quantified (Doiron et al., 2009). Besides, results from ultra-structural studies also confirmed that SFTIRM could be used to explore the chemical constitutes and inherent structural information of barley samples (Yu et al., 2004; Yu, 2007b).

Based on the above studies, the SFTIRM technique allows us to nondestructively investigate the structural information of different botanical parts of a sample, and explore the spectroscopic

characteristics, spatial distribution, functional groups or chemical intensity of biological tissues within cellular dimensions. As mentioned before, trace fungal-derived contaminants including mycotoxins might be indirectly assessed by analyzing the IR spectrum for alterations in intrinsic structures of foodstuffs which caused by fungal infection (McMullin et al., 2015). Therefore, more efforts are highly needed in the future to reveal the possibility of using this advanced technique for the determination of trace contaminants such as mycotoxins in feed and food.

V. Recent applications in mycotoxin research with molecular spectroscopy

5.1. Application 1: Rapid identification of mycotoxin-contaminated maize and peanuts by FTIR spectroscopy

Deoxynivalenol and AFB_1 are among the most important toxic fungal metabolites due to their extensive occurrence and toxicity. The rapid identification of them in agricultural commodities is an ongoing concern for traders. This research aims to present classification results for deoxynivalenol in maize and AFB_1 in peanuts at trace levels using FTIR techniques with chemometrics (Kos et al., 2016). Both natural infection (generally yielding low mycotoxin concentrations) and artificial infection (by silk injection or toothpick inoculation for high mycotoxin) were performed to obtain proper contaminated maize samples. For peanuts, 92 samples (naturally infected) were collected from public markets. A portable spectrometer was used to collect FTIR spectra of maize and peanut samples. Classification thresholds of 1750 and 500 μg/kg were selected for deoxynivalenol in maize and 8μg kg^{-1} for AFB_1 in peanuts in agreement with EU legislation. Spectral data preprocessing consisted of baseline correction,

averaging, mean-centring, normalization and calculation of first derivative. Spectral data were classified using a bagged decision tree method, evaluating the protein and carbohydrate absorption regions of the spectrum. Results showed that the bagged decision tree classification method was capable of classifying maize samples at the 1750 and 500μg kg^{-1} thresholds for deoxynivalenol with an accuracy of 79% and 85%, respectively, and peanut samples at the 8μg kg^{-1} threshold for AFB_1 with an accuracy of 77%. The bagged decision tree classification method showed better performance than the established PCA classification method, which consistently showed overlapping clusters.

5.2. Application 2: An empirical evaluation of Raman, FT-NIR, and FTIR techniques for determination of aflatoxins in maize

Raman, FT-NIR, and FTIR are commercially available vibrational spectroscopic techniques. The objective of this research is to compare the use of different vibrational spectroscopic methods in association with chemometric tools for the determination of aflatoxins in maize (Lee et al., 2015). A total of 232 maize samples naturally contaminated or artificially inoculated with aflatoxins (0–1206 μg/kg) were selected and analyzed by both biochemical and spectroscopic techniques. Raman spectra (200–3500 cm^{-1}), FT-NIR spectra (4000–9999 cm^{-1}), and FTIR spectra (450-4000 cm^{-1}) of ground maize samples were acquired and preprocessed (baseline correction, normalization, first and second derivative calculation). Classification and quantification models were developed and validated with preprocessed spectra. Results showed that all the three spectroscopic methods obtained similar classification accuracy in separating contaminated samples from non-contaminated samples. However, FTIR and Raman techniques

surely generated multivariate regression models with higher regression coefficients and lower prediction error than FT-NIR method.

5.3. Application 3: Determination of AFM_1 in milk using spectroscopy and multivariate analyses

The existing wet chemical analytical techniques for detecting AFM_1 are too expensive and time consuming. This research aims to test the possibility of developing a mathematical model for detecting and quantifying the AFM_1 in milk by FTIR spectroscopy with chemometrics (Jaiswal et al., 2016). AFM_1 were dissolved in double distilled water to obtain a solution of 1 μg/ml. This solution was further diluted with milk to obtain AFM_1 spiked milk (0, 0.02, 0.04, 0.06, 0.08 and 0.1μg/ml). FTIR spectra (4000-500 cm^{-1}) of milk samples were collected and the Unscrambler software (version 10.2; CAMO ASA, Norway) was used for data processing. The soft independent modeling of class analogy (SIMCA) and partial least square regression (PLSR) methods were applied to develop the classification and regression models. Using the SIMCA approach, the models could correctly classify > 86% of the samples into their respective class. The concentration of AFM_1 was best predicted in wavenumber range of 1800-650 cm^{-1} with a calibration coefficient of 0.99 and validation coefficient of 0.98, using PLSR method. This study suggested that reasonably accurate prediction of AFM_1 could be done in milk by FTIR spectroscopy combined with chemometric methods.

5.4. Application 4: Test the possibility of using NIR and FTIR spectroscopy techniques to determine ergot alkaloids concentration in barley and wheat

To date, no published study has examined whether we can develop spectroscopy models for the rapid detection of ergot alkaloids concentration in cereals. Therefore, a project aims to examine the possibility of using NIR and MIR spectroscopic techniques for the determination of ergot alkaloids content in barley and wheat was launched by the University of Saskatchewan in 2016 (unpublished data). A total of 80 wheat and 42 barley samples were collected and detected for six major ergot alkaloids concentration by LC-MS/MS. The NIR (680-2500 nm) and MIR (4000-700 cm^{-1}) spectra of all the samples were collected with the NIR and FTIR spectrometers. Spectral data preprocessing and chemometric models development were performed using the Unscrambler software (version 10.4, CAMO ASA, Norway). Both the raw spectra and preprocessed spectra (10 different transformation methods) were used to construct calibration models using PLSR method. Descriptive statistics results showed that the ergot alkaloids concentration of contaminated wheat (N = 56) and barley (N = 30) were rather large (e.g., total ergot alkaloids of wheat samples ranges from 0.03 to 21970 ppb), and the frequency distribution of contaminated samples were unsatisfactory for calibration purpose (Total ergot alkaloids concentration of 45% samples is lower than 10 ppb). According to the preliminary regression results, no satisfactory calibration model was obtained by NIR or MIR techniques. Next step, more efforts will be made to improve the quality of sample sets. The possibility of whether we can develop satisfactory regression models for wheat and barley ergot alkaloids detection by NIR and MIR spectroscopy techniques is expected to be revealed in the future.

VI. Conclusion

Conventional wet chemical techniques are well known due to their precise and accurate detection of mycotoxin in foods and feedstuffs. However, they usually require labour-intensive sample preparation, expensive equipment, skilled operators, and long detection time. Growing market competition forces producers to seek cheaper and faster detection methods to shorten the delivery time of their goods. To meet the requirement of the increasing number of mycotoxins of interest and sample matrices, simple, rapid, less expensive, and non-destructive analytical methods are highly expected. Advanced molecular spectroscopy techniques are interesting and promising tools for detection of mycotoxins in feed and food, which need minimum sample preparation and yield result in minutes. Up to the present, the shortage of proper calibration materials is the substantial restriction. More efforts are highly needed to work on calibration development and verify the true potential of advanced molecular spectroscopy techniques in the field of mycotoxins monitoring.

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Table 1. Recent applications of LC-MS/MS techniques in food and feed research[1]

Samples	Mycotoxins	Detection	LOD/LOQ (μg/kg)	Reference
Wheat, rye, maize, rice, barley and oats	Aflatoxin B1 and ST	ESI, tandem MS, MRM	LOD: 0.03; 0.02 LOQ: 0.5	Zhao et al. (2017)
Rice, barley, oats, and mixed grain	Aflatoxins, OTA, fumonisins, ZEN, deoxynivalenol, T-2 toxin, and HT-2 toxin	ESI, tandem MS, dynamic MRM	LOD: 0.1-10 LOQ: 0.1-50	Al-Taher et al. (2017)
Apple, sweet cherry, tomato and orange	Alternaria toxins, patulin, OTA, citrinin	ESI, tandem MS, MRM	LOQ:1-5	Wang et al. (2016)
Complex feed samples	Aflatoxins, ZEN, OTA, T-2 toxin, ST	ESI, tandem MS, MRM	LOD: 0.006-0.12 LOQ: 0.06-0.75	Hu et al. (2016)
Corn meal	Aflatoxins, fumonisin B1, and ZEN	ESI, tandem MS, MRM	LOD: 0.24-5.23 LOQ: 0.8-32.16	Paschoal et al. (2016)

Maize, wheat, pasta, and rice	Beauvericin and enniatins (A, A1, B, B1)	ESI, tandem MS, MRM	LOD: 0.1-1.0 LOQ: 0.3-2.9	Decleer et al. (2016)
Durum wheat	Trichothecenes, fumonisins, ST, OTA, aflatoxins, ZEN, alternaria toxins, and five other mycotoxins	ESI, tandem MS, MRM	LOD: 2-70 LOQ: 3.5-80	Juan et al. (2016)

[1]LOD, limits of detection; LOQ, limits of quantification; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; MS, mass spectrometry; OTA, ochratoxin A; MRM, multiple reactions monitoring mode; ZEN, zearalenone; ST, sterigmatocystin.

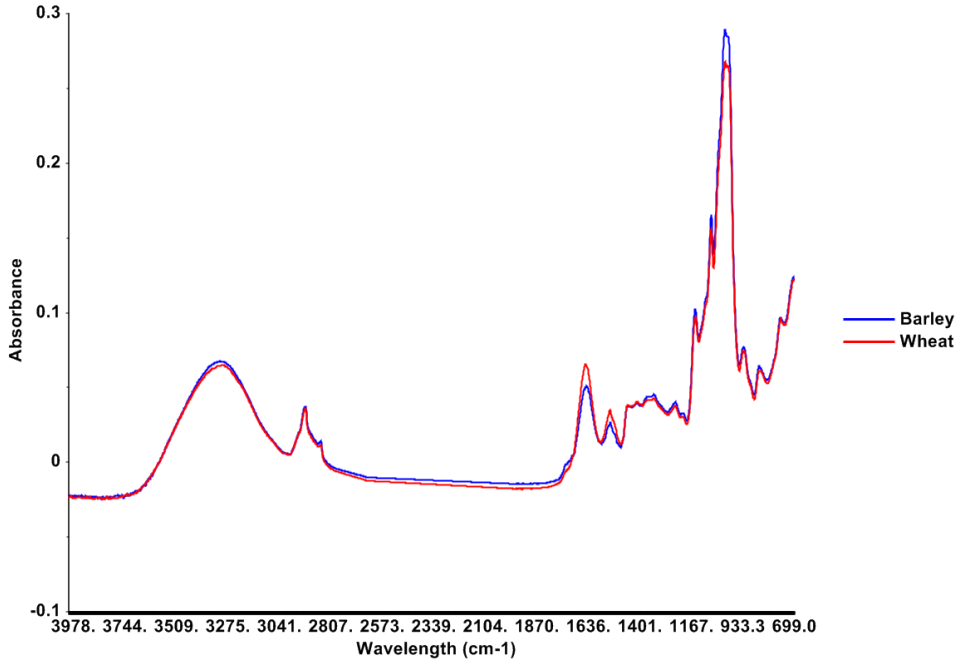

Figure 1. Typical Fourier transform infrared (FTIR) spectra of barley and wheat (ca. 4000-700 cm^{-1})

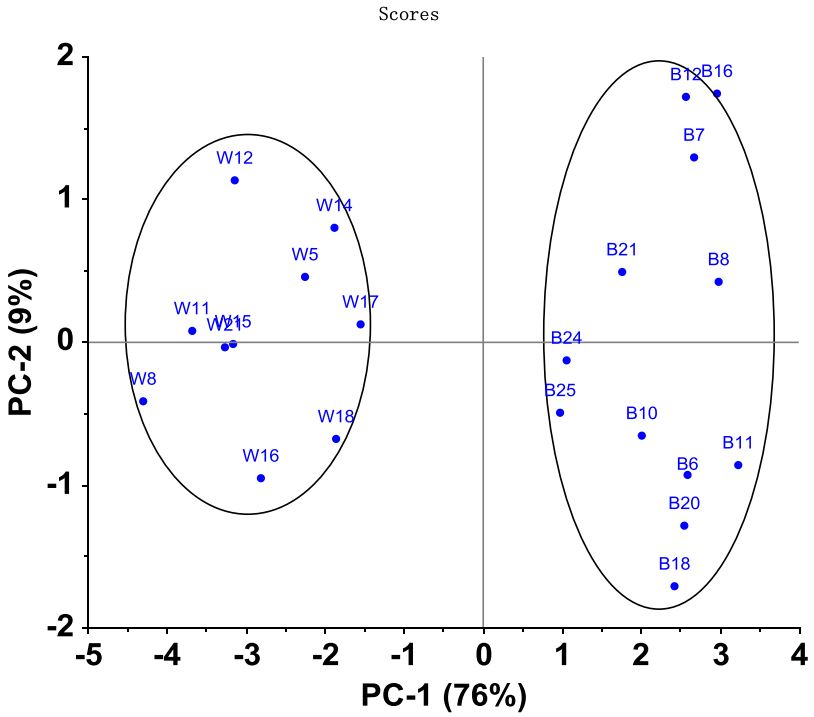

Figure 2. Score/score plot of FTIR spectra data (4000-700 cm^{-1}) of barley (B) and wheat samples (W). Spectra data was preprocessed by standard normal variate transformation, followed by PCA.