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Infrared Spectroscopy Applied to Identification and Detection of Microorganisms and Their Metabolites on Cereals (Corn, Wheat, and Barley)

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1. Introduction

Over the last several years, mycotoxins, which are metabolites secreted by fungi, have been the subject of numerous studies. These eukaryotes play a major ecological role in the life cycle of plants. Indeed, for some fungi, the role of saprophyte places them at the heart of ecosystem dynamics [Alexopoulous et al., 1996].

Some 350 mold species produce a large range of secondary metabolites (over 300, of which ~30 are toxic) [Fremy et al., 2009] and represent a potential danger for animal and human health and cause significant losses for the cereals industry [Le Bars et al., 1996]. The effects of mold are well illustrated by decreases in crop and livestock yields, public health problems, or write-offs on the international cereal market [Le Bars, et al., 1996]. The United Nations Food and Agriculture Organization estimates annual global losses from mycotoxins at 1 billion tons of foodstuffs [Fao, 2001]. The primary organisms impacted by mycotoxins are plants. Currently, about 25% of agricultural crops worldwide are contaminated by these metabolites [Charmley et al., 2006].

In response to these significant economic and health risks, global non-tariff barriers (i.e., specific food-safety standards imposed on imported products) were erected to control commercial trade based on the mycotoxic quality of foodstuffs. These measures generate significant economic and material losses for countries that export contaminated foodstuffs, either because their cargo is refused or because of a reduction in prices. To limit these consequences, farmers and the food industry strive to reduce the presence of mycotoxins in their products. Therefore, producers and processors are searching for alternative analytical methods to determine, in a quick, simple, and inexpensive manner, the risk of their products containing fungi or mycotoxins. The use of infrared spectroscopy—a mature technology—to monitor foodstuffs could respond to this need.

In this chapter, we focus on mycotoxins found mainly in wheat, barley, and corn and that have been studied in the international literature; namely, deoxynivalenol, fumonisins, and aflatoxin B1.

2. Advantages of using infrared spectroscopy to manage fungal and mycotoxic risk for wheat, barley, and corn

Fungus can be detected by microbiological methods involving visual, microscopy, and microbial-cultural methods.

Conventional methods of mold detection are based on direct observation by eye or by microscope of thalli, contaminated foodstuffs, or microbial cultures. These methods are time consuming and require viable samples and a good deal of expertise. Counting methods are difficult to apply to fungi because, during their reproduction, a spore generates a mycelium that can in turn divide itself into tens of individuals. Furthermore, a fungal contamination may not be visible at the surface of grains [Hirano, et al., 1998, Pearson, et al., 2001].

Other methods are based on molecular biology or on the detection of antigens specific to given molds. Organisms, either dead or alive, can be detected by the polymerase chain reaction (PCR) by copying a large number of DNA sequences that are originally present in small quantities (with a multiplicative factor on the order of 10^9). By amplifying certain genes of toxigenic strains, PCR serves as a tool to determine the risk. Various researchers have tested PCR to detect *Fusarium* contamination in corn [Jurado et al., 2006; Jurado et al., 2005; Nicolaisen et al., 2009]. These methods are rapid, sensitive, and can be automated. They are good qualitative methods (e.g., good selectivity) but offer only average precision in quantitative terms (they are called "semiquantitative"). These techniques are thus very reliable, provided the fungal strain to be detected is known beforehand, and so are used as referential methods. With such methods, a grain is deemed of suitable microbiological quality if less than 10 000 germs of the storage flora per gram of grain are detected.

New approaches are based on detecting constituents and fungal metabolites. Such approaches exploit the fact that molds have specific characteristics that distinguish them from other eukaryotes. These characteristics include the regulation of certain enzymes, the synthesis of lysine amino acid by a particular metabolic route, extremely structural characteristics (e.g., the Golgi apparatus), and genetic characteristics (e.g., haploid). From among these attributes, two types of compounds can be used as indicators of a fungal contamination.

The secreted compounds are synthesized compounds such as soluble carbohydrates (e.g., disaccharide trehalose and polyhydric alcohols such as mannitol or arabitol) or products of the metabolization of complex carbons such as volatile aldehydes, alcohols, ketones, spores, primary metabolites, secondary metabolites (i.e., volatile compounds). The last item gives rise to the characteristic fungal odor and is often detected by an electronic nose. For nonvolatile compounds, other tools such as infrared spectroscopy seem better suited.

The structural compounds of mold can also be used for their detection. The main polysaccharides of the cell wall of mold are the α et β (1-3) glucans, as well as chitin. Ergosterol is a component of fungal cell membranes.

Chitin may absorb infrared light, making it useful for infrared spectroscopy [Nilsson, et al., 1994; Roberts et al., 1991]. The main inconvenience in using this component as an indicator of fungal contamination is that chitin is not limited to fungi; it is found in insects, diatoms, arachnids, nematodes, crustaceans, and several other living organisms [Muzzarelli, 1977]. In addition, it may take different forms, each of which requires a specific detection technique. Roberts et al. [Roberts, et al., 1991] estimates the quantity of mold on barley by detecting this molecule but also detects glucans by near-infrared spectroscopy.

Ergosterol, however, is more specific to mold. This molecule, which may still be called provitamin D2, is a C24-methylated sterol (and is part of the subgroup of organic compounds that are soluble in lipids) and is found in the cell membranes of yeasts and filamentous fungi. This molecule is not found in animal cells [Verscheure et al., 2002] and is in the minority among the sterols found in higher plants [Pitt et al., 1997] and insects [Weete, 1980]. Griffiths et al. [Griffiths et al., 2003] demonstrated that ergosterol is the primary sterol found in molds: it represents 95% of the total sterols, with the remaining 5% being ergosterol precursors from *Leptosphaeria maculans*. This specificity makes this molecule a potential tracer of fungal activity. It is generally agreed that the ergosterol content of grains must be less than a given threshold; the limit for corn is 8 µg/g.

3. Infrared spectroscopy to detect fungal and mycotoxic contamination of wheat, barley, and corn

3.1 Background and methods

The first application of infrared spectroscopy to detect microorganisms dates from the 1950s [Miguel Gomez et al., 2003]. In these applications, the spectrometers were calibrated depending on the method of dosing the fungi or mycotoxins. In the 1980s, Fraenkel *et al.* [Fraenkel et al., 1980] and Davies *et al.* [Davies et al., 1987] published their first works on the detection by infrared spectroscopy of fungal contamination (*Botrytis cinerea* and *Alternaria tenuissima*), but the application of this tool to detecting mold really grew in the 1990s. This growth was due to the fact the existing agronomic models required collecting a significant amount of data in the field, making this approach unsuitable for routine use. In addition, industry required nondestructive techniques to assess the health safety of crops. Therefore, several research teams used infrared spectroscopy to detect mold and mycotoxins on cereals, which could be done concomitantly with the quantification of other parameters such as protein content, humidity, etc.

One method proposed to determine the fungal or mycotoxin content is to quantify the total fungal biomass. Toward this end, ergosterol is used as a fungus marker [Castro et al., 2002; Saxena et al., 2001; Seitz et al., 1977; Seitz et al., 1979]. Very often, this type of study is coupled with a study of the mycotoxin content and fungal units (colony-forming units or CFU). Indeed, the quantity of fungi is not proportional to the quantity of mycotoxins; it is possible to have small quantities of fungi but large quantities of mycotoxins, and vice versa. Indeed, fungi may disappear after secreting its toxins, either because of the evolution of the mycoflora or because of the application of chemical treatments. In addition, certain strains are more toxic than others. Two conclusions exist from the work on this subject: some researchers find a correlation between the mycotoxin content, the ergosterol content, and/or the fungal units [Lamper et al., 2000; Le Bouquin et al., 2007; Miedaner et al., 2000; Seitz et al., 1977; Wanyoike et al., 2002; Zill et al., 1988] whereas the others find no correlation or cannot make categoric conclusions [Beyer et al., 2007; Diener et al., 1982; Gilbert et al., 2002; Nowicki 2007; Penteado Moretzsohn De Castro et al., 2002; Perkowski,et al., 1995].

Covering the last 20 years, we count over 20 articles dealing with the use of infrared spectroscopy (primarily near-infrared) to detect molds and mycotoxins in wheat, barley, and corn. Because some of the work in infrared spectroscopy deals both with the detection and the identification of mycotoxins, we separate the articles into three groups. Table 1 is for molds, Table 2 compiles the trials dealing with deoxynivalenol (DON), fumonisins (FUMs), and B1 aflatoxins (AF1). Finally, Table 3 contains articles in which the authors worked

Molds	Matrix	Infrared Specificities	Performances	Characteristic wavenumbers and wave...
F. verticillioides and *A. flavus*	corn	FTIR-photoacoustic and FTIR-diffuse reflectance 400-4000 cm^{-1}		* Amide I [1650 cm^{-1}] and amide II [1550 cm^{-1}] = increase in pr... content * Peak [3400 cm^{-1}]: shift = increase of NH absorptions wit... * [2855 and 2925 cm^{-1}] = increase of lipid... * [400 to 1500 cm^{-1}] = change in carbohydrate... * Conclusion: the best for establishing a regression is t...
A. flavus	112 corn	FTIR-photoacoustic 4000-600 cm^{-1}	Classification between uninfected and infected corn: - training: 100% of correct classification - test: 94% of correct classification	On infected grains: CO_2 evolution [2366 cm^{-1}] Ester peak shift [1256 to 1240 cm... COOH band elevation [3200 to 2200... CH_2 peak ratio [2853 cm^{-1}/2923 c... OH, NH_2 peak shift [3400 to 3360 ... Protein (amide II) increase [1550 c... NH_2, COOH peak shift [3400 to 330... Carbonyl shoulder height [1725 c... FTIR-PAS measures the level of fungal infection, rather than the... mycotoxin concentrations of interest and concern are too low to b... data.
A. flavus	20 corn	Transient infrared 2000-600 cm^{-1}	Correct classification rate between healthy and infected corn: 85% to 95%	* Decrease in absorption of carbonyl group = consumpti... * Increase in absorption of amide II group and changes in absor... fungal proteins and carbohydrates. This may also be the manifes... molds: they expose the grain interior to the infrared, where the... differs from that of the grain surfa... * Conclusion: the damages suffered by corn seem more significa... itself.
Identification of species *Penicillium, Memnoniella, Fusarium*		FTIR microscopy 4000-600 cm^{-1}		* [1655 and 1546 cm^{-1}]: proteins: amide I a... * Shoulder [1750 cm^{-1}]: C=O of lip... * [1465 cm^{-1}]: CH2 of cell lipids... * [1450 cm^{-1}]: CH3 of end ethyl groups o... * [1396 cm^{-1}]: C=0 of lipids * [1377 cm^{-1}]: CH of CH2 * [peaks at 1237 and 1082 cm^{-1}]: PO2- and ph... * [1064 cm^{-1}]: carbohydrates
Aspergillus and *Penicillium*: **identification of 10 different species**		FTIR 4000-500 cm^{-1}		Best results obtained using : [1765-1590 cm^{-1}] [1470-1275 cm^{-1}] [1170-1000 cm^{-1}] [930-715 cm^{-1}] corresponding to amide I and amide II, polysaccharides and...
Different species of fungi	corn	Reflectance 500-1700 nm	Correct classification rate between fungal infected or undamaged kernels: above 96.6%	Use of two wavelengths: 715 and 965nm => fungal damaged ker... modifications in oil content.
F. graminearum, F. proliferatum, F. subglutinans, F. verticillioides	PDA	400–2500 nm	Correct classification rate awaiting external validation: 98.8%	Significant spectral differences : [500–950 nm], [1500–1850 nm]... *subglutinans,* and *F. proliferatum* and *F. verticillioides*), and [4... (between *F. graminearum* and the 3 c...

Table 1. Infrared spectroscopy applied to identification of mold species and gena (FTIR spectroscopy... spectroscopy).

Mycotoxins	Matrix /number of samples	Spectral Range	Content Range	Performance	Characteristic wavelength
Deoxyni-valenol	188 barley	NIR 400–2500 nm	0.3 to 50.8 ppm	R² between 0.933 and 0.805 SEP between 3.097 and 5.461 ppm	
	14 corn	FTIR with attenuated total reflection (ATR) 4500-650 cm⁻¹		Correct classification rate between blank and contaminated samples: 79 to 100%	* Choice of spectral area: 1800–80 * Sieving the samples increases the perce classification
	340 wheat	NIR	70–11000 ppb	0.33< R² <0.801 421 ppb<SECV<1900 ppb	
	197 wheat (durum + common)	FTIR-NIR 10000-4000 cm⁻¹	0-3000 µg/kg	* Correct classification rate between blank and contaminated wheat (limit at 300 µg/kg DON): 69% * Quantification Training: R²=0.71 and SEC: 386 µg/kg	
	wheat	diffuse reflectance 350–2500 nm	0-100.75 mg/kg	SECV=3.61 mg/kg R²=0.84	* [1400–1900nm]: highest differences betw damaged kernels: changes of kernel con pigmentation * The SEC is too high in comparison with t mg/kg)
Fumonisin	corn (330 kernels)	NIR transmittance 500–1050nm and NIR reflectance 400-1700 nm	0.5 – 610 ppm	Correct classification rate (distinction between corn containing high *>100 ppm* and low *<10 ppm* levels of fumonisin, classified as positive or negative): from 80 to 91% (use of entire spectra or ratios of two or four wavelengths)	* Models are relying on the damages done the kernel (changes in color, vitreousness, p or oil content) as fumonisin present at the not absorb detectable amounts of NI * Transmittance: 650 nm, 710 nm, 935 * Reflectance: 590 nm, 995 nm, 1200 n
Aflatoxin	corn (500 kernels)	NIR transmittance 500–950nm and NIR reflectance 550-1700 nm	1 to >1000 ppb	Correct classification rate (distinction between corn containing high *>100 ppb* and low *<10 ppb* levels of aflatoxins, classified as positive or negative): >95%	* The differences in spectra of contam uncontaminated kernels can be explained b fungi in the kernel. * The use of ratio of 2 absorbencies gives be the use of full spectra - Transmittance: 720/780 nm or 710/760 a - Reflectance: 735/1005 nm or 1075/1135 n nm
	76 corn 76 barley	NIR grating 400–2500 nm FT-NIR: 9000-4000 cm⁻¹	0–50 ppb	* Correct classification rate for samples classified as positive (aflatoxin B1>20 ppb) and negative (aflatoxin B1 <20ppb) : 100% for corn and barley * Quantification - Corn # NIR grating: R²=0.80 and SECV=0.211 ppb # FTNIR: R²=0.82 and SECV=0.2 ppb - Barley # NIR grating: R²=0.85 and SECV=0.176 ppb # FTNIR R²=0.84 and SECV=0.183 ppb	* Indirect detection of aflatoxins because small concentrations. Specific bands can be infection and fungal cellular comp - [480–600nm]: changes in color of fungal - [870–1200nm], [1750–1800nm] and [20 deteriorative alterations of ker

Table 2. Infrared spectroscopy applied to quantification of levels of deoxynivalenol (DON), fumonis cereals (NIR=Near Infrared Spectroscopy / FTIR=Fourier-transform infrared spectroscopy)

Mycotoxin or mold	Matrix /number of samples	Spectral Range	Content Range	Performance	Cha
Deoxynivalenol and ergosterol	wheat (114 for DON and 46 for ergosterol)	NIR 400-1700 nm	deoxynivalenol: 0-789 ppm ergosterol: 0-1497 ppm	* deoxynivalenol (>5 ppm) R²=0.64 and SEC=44 ppm R²=0.66 and SEP=52 ppm * ergosterol (>50 ppm) R²=0.64 and SEC=108 ppm	* [750 * [1200 * [1
Fusarium, **ergosterol and deoxynivalenol**	52 corn	FTIR-ATR 4000-650 cm⁻¹	ergosterol : 0.79-947 mg/kg deoxynivalenol: 0.13-2.59 mg/kg	Discrimination of blank and contaminated samples : at least 75% correct classification when concentrations of ergosterol and deoxynivalenol are respectively greater than 8.23 mg/kg and 0.13 mg/kg	
F. graminearum, **ergosterol and deoxynivalenol**	14 corn	FTIR-ATR 4500-650 cm⁻¹	ergosterol : 880-3600 µg/kg deoxynivalenol: 310-2596 µg/kg	* Classification : up to 100% * Quantification : - deoxynivalenol : R² = 0.66 and SECV: 494.5 µg/kg - ergosterol :R² = 0.60 and SECV = 833.7 µg/kg	* Choice o * Changes in prot
Deoxynivalenol and *Fusarium*	50 wheat	NIR 570–1100 nm		R²=0.98 and SECV = 404 µg/kg	* Some of these fa and some from o deoxynivalen
Fusarium, **deoxynivalenol and ergosterol**	21 corn	FTIR-ATR and mid-infrared diffuse reflection	ergosterol : 880-3600 µg/kg deoxynivalenol : 310-2600 µg/kg	* Correct classification rate between blank and contaminated samples : - ATR : 100% - Diffuse reflection: 79% * Quantification - deoxynivalenol-ATR: R² = 0.94 and SEC = 178.8 µg/kg R² = 0.65 and SECV = 438.7 µg/kg - deoxynivalenol-diffuse reflection: R² = 0.87 and SEC = 297.3 µg/kg R² = 0.51 and SECV = 597.5 µg/kg - ergosterol-ATR : R²=0.80 and SEC = 593 µg/kg R²=0.60 and SECV = 864.7 µg/kg - ergosterol- diffuse reflection : R²=0.72 and SEC = 708.4 µg/kg R²= 0.36 and SECV = 1102 µg/kg	* Choice o
Ergosterol, fumonisin B1, *F. verticillioides*	corn fungi infection (220); *F. verticillioides* (217); ergosterol (160); fumonisin B1 (180)	NIR 400–2500 nm	fungal infection: 28-100% *F. verticillioides*: 2-100% ergosterol: 0.78-41.52 mg/kg fumonisin B1: 0.01-19.6 mg/kg	* fungal infection: R²=0.80, SEP=6.34 % * *F. verticillioides* R²=0.78, SEP=9.64% * ergosterol: R²=0.81, SEP=1.74 mg/kg * fumonisin B1: R²=0.78, SEP=1.33 mg/kg	* Berardo rep relationship for ergos
Fusarium **and deoxynivalenol**	wheat 4800 grains	410–865 nm and 1032–1674 nm		* Correct classification rates of damaged kernels: - only VIS: 94% - only NIR: 97% - NIR+VIS: 86%	* Selec - on - only - NIR
Deoxynivalenol and fumonisin	539 corn for deoxynivalenol 198 corn for fumonisins	1100–2500 nm	deoxynivalenol: 1.04–8.68 ln mg/kg fumonisin: 0.14–6.43 ln mg/kg	* Deoxynivalenol: R²=0.90 and SEC=0.44 ln mg/kg R²=0.88 and SECV=0.50 ln mg/kg * Fumonisin: R²=0.68 and SEC=0.80 ln mg/kg R²=0.46 and SECV=1.04 ln mg/kg	* Better perform * Due to the fact t low concentratio mycotoxins migh disease

Table 3. Infrared spectroscopy applied to fungal and mycotoxic quantification in cereals (NIR=Near FTIR=Fourier-transform infrared spectroscopy / ATR=Attenuated Total Reflection)

simultaneously on the fungal and mycotoxic aspects. Each table lists the matrix studied (wheat, barley, corn), the apparatus, the content ranges, the performance of the models, the principle conclusions, and the characteristic wavelengths.

3.2 Principal conclusions

3.2.1 Identification and quantification of fungi

For identification of fungi, the performances, given in terms of percentage of correct classification, are very satisfactory because they exceed 77%. Each study identifies the peaks or spectral zones related to the growth of fungi or to the damage inflicted on the grains by fungi.

Moreover, the performance of the quantification of ergosterol always gives enticing results.

3.2.2 Quantification of mycotoxins deoxynivalenol, fumonisins, and aflatoxins

Mycotoxins are present in quantities too small (in the order of parts per million) for direct detection. Their detection is thus associated with a complex ensemble of information related to the growth of the fungus on the cereal; notably with modifications of the protein or carbohydrate level (starch, cellulose, etc.).

Regarding the capabilities of infrared spectroscopy to quantify mycotoxins, the conclusions differ from one author to another. In general, when dealing with deoxynivalenol, the performance is higher than when dealing with fumonisins. Yet despite this, even if the quantification of mycotoxins appears possible, it is not sufficiently precise to be used in the field. Indeed, the standard error of prediction (SEP) is too large with respect to the regulatory limits—notably European limits. This could be explained by a magnification of the non-negligible standard errors of the chemical benchmarks from which they are developed. Moreover, to work under conditions of realistic of toxin levels, the main avenues for improvement of these studies may be the following:

- Increasing the number of samples;
- Increasing the annual variability (samples often come from a single harvest);
- Allowing a natural contamination of the grains (artificial contamination does not account for all the natural parameters of contamination, notably so for multicontaminations);
- Accepting a range of mycotoxin levels more adapted to the reality in the field (the ranges are often very narrow);
- Acquiring spectra not grain-by-grain, but from entire lots of grain. Indeed, it is more difficult to assign a global mycotoxin level to a lot, because the distribution of mycotoxins is very heterogeneous, as is the case for the molds that synthesize the mycotoxins;
- Using a set of test samples (the proposed performances are often cross validated and are thus better than they would be for an external test);
- Displaying the ratio of standard error of prediction to sample standard deviation (RPD).

Thus, instead of quantification, several studies propose a classification of cereals samples as a function of the mycotoxin level. This qualitative approach works better and, at least until the quantitative models are improved, seems the most conclusive for applications in realistic conditions. Note also that, even if the SECs (Standard Error of Calibration), SECVs (Standard Error of Cross Validation), and SEPs (Standard Error of Prediction), are improved for the quantifications, these models are developed based on chemical benchmarks that themselves have non-negligible standard errors.

4. Conclusion

Infrared spectroscopy offers multiple advantages. This tool requires no preparation and does not use toxic products. It enables nondestructive analysis of the samples. Moreover, it is a rapid, low-cost technique that can be used online. The sample cell is resistant and inexpensive (glass or quartz). Finally, infrared spectroscopy enables multiparametric analyses and a large range of robust devices are available.

Models to classify cereals samples as a function of the type of fungus present or its level, or the presence of mycotoxins, represent an attractive tool to determine the fungal and mycotoxic risk in the food industry; for example, in the field or in the silo. Regarding the quantification of mycotoxins, chemometrics, which is a field in constant progression, may one day deliver performances that meet agricultural and industrial needs.

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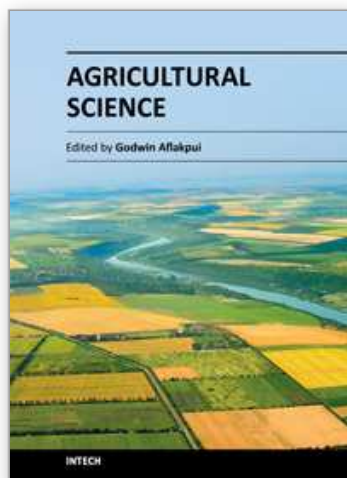
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