Chemical and Spectral Quantification of Mold in Contaminated Barley[1]

C. A. ROBERTS,[2] R. R. MARQUARDT,[3] A. A. FROHLICH,[3] R. L. McGRAW,[2]
R. G. ROTTER,[3] and J. C. HENNING[2]

ABSTRACT

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This work was conducted to estimate mold in barley by *N*-acetyl-D-glucosamine determined by ion-exchange chromatography and near-infrared reflectance spectroscopy (NIRS). In one study, barley fungal contamination was assessed visually using a scale of 0 (no mycelia or spores) to 5 (prolific mycelial growth or spore production). The contaminated barley was then analyzed for glucosamine using ion-exchange chromatography. Glucosamine correlated highly ($P < 0.01$) with mycelia and spores; correlation coefficients were 0.86 and 0.85, respectively. Glucosamine was quantified by NIRS; coefficients of determination for calibration and performance exceeded 0.92. In a second study, a population of artificially contaminated barley was created by mixing mycelial dry matter with noncontaminated barley. Mycelial tissue in these samples was quantified by NIRS; the performance coefficient of determination was 0.94. Based on results with naturally incubated samples, we concluded that glucosamine determined by ion-exchange chromatography represented fungal contamination in barley and that it could be determined by NIRS. Based on the successful artificial calibration, we concluded that mycelia at naturally occurring levels could be quantified by NIRS and that NIR spectrophotometers can detect mycelia directly, despite the empirical nature of NIRS technology.

The ability to quantify fungal contamination in forage and grain tissue is important to livestock and consumers. Fungal contamination can reduce nutritional value (Jones et al 1955), cause respiratory problems (Hintz and Lowe 1977), and facilitate mycotoxin synthesis (Abramson and Mills 1985). Many commercial cereals are analyzed routinely for quality components (Williams et al 1984, 1985) but not for fungal contamination.

Common procedures for mold estimation include visual assessment (Goering and Gordon 1973, Jeffers et al 1982), spore counts (Lacey and Dutkiewicz 1976), and determination of loss of dry matter (DM) (Dawson and Musgrave 1949). Studies have shown that chemical procedures for estimating fungal contamination offer an efficient alternative to nonchemical methods (Seitz et al 1979, Cousin et al 1984, Roberts et al 1987). One chemical procedure is the determination of chitin, a fungal cell wall polymer quantified as the monomer, *N*-acetyl-D-glucosamine (Ride and Drysdale 1971). Chitin is generally quantified by alkaline hydrolysis, followed by celite precipitation of chitosan and colorimetric determination of glucosamine. Although error can be introduced by repeatedly rinsing the chitosan pellet and because of the number of steps required in the assay, this procedure has been used successfully to quantify mycelia in forages (Jones et al 1985, Roberts et al 1987), grains, (Donald and Mirocha 1977, Nandi 1978, Seitz and Pomeranz 1983), and consumer food products (Bishop et al 1982, Cousin et al 1984).

The chitin procedure has been criticized because glucosamine in insect exoskeletons and seed glycoproteins can interfere with fungal glucosamine (Pusztai 1964, Sharma et al 1977). Despite this potential interference, chitin is a reliable estimate of relative mycelia because glucosamine from nonfungal sources does not interfere with fungal glucosamine. Nonfungal glucosamine concentrations can be ignored if they are low or constant (Donald and Mirocha 1977, Cousin et al 1984, Lin and Cousin 1985, Roberts et al 1987).

Recently developed chromatographic procedures may contribute to analysis of fungal contamination in forage and grain tissue. Rotter et al (1989) proposed using acid hydrolysis of chitin and ion-exchange chromatographic separation of amino sugars to estimate fungal mycelia in barley. If this procedure can accurately estimate mycelial contamination, the data could be used with near-infrared reflectance spectroscopy (NIRS) to develop a prediction equation for fungal contamination in barley and other cereals.

Several NIRS equations have been developed for grain and forages; one has been used to estimate mold in alfalfa (*Medicago sativa* L.) (Axell et al 1981, Asher et al 1982, Marten and Martin 1985, Roberts et al 1987). The alfalfa mold equation produced chitin data that correlated with visible mold scores, in spite of minute concentrations of glucosamine and inherent error in the colorimetric reference method. Since barley contains higher glucosamine concentrations, a fungal calibration based on chromatographic data is certainly feasible.

The primary objectives of this study were to relate the presence of glucosamine to visible mold scores in contaminated barley and to develop an equation for barley glucosamine using NIRS. A further objective was to investigate the possibility that spectral detection was related to fungal mycelia per se and not limited to basic grain constituents.

MATERIALS AND METHODS

Barley seed and fungal mycelia were used to create two populations; these populations and their analyses are summarized in Figure 1. The first population was created after incubation of moisturized barley that had been naturally inoculated with fungal spores. The second population, referred to as artificial (Coleman et al 1985), was created by mixing mold-free barley with various quantities of fungal mycelia.

Barley and Fungal Tissues

Barley samples were collected from storage bins throughout Manitoba from 1985 to 1987. Cultivars included Argyle, Bedford, Bonanza, Conquest, Heartland, and Samson. Spores of

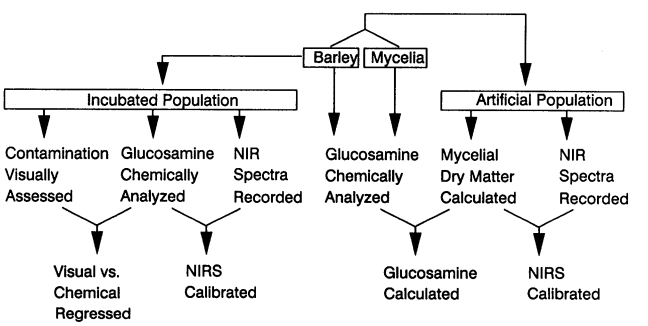

Fig. 1. Scheme of methods followed to develop naturally incubated and artificially contaminated barley populations and to quantify mold using chemical and spectral procedures.

[1]From the University of Missouri Agricultural Experimental Station. Journal Series No. 10,779. Mention of specific products does not imply preferential endorsement.
[2]Department of Agronomy, 214 Waters Hall, University of Missouri, Columbia, MO 65211.
[3]Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2.

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Aspergillus ochraceus Wilhelm (NRRL3174) were provided by the Northern Regional Research Laboratory (Peoria, IL). In addition, *A. ochraceus* and *Penicillium aurantiogriseum* were isolated from a barley sample obtained at the Glenlea Research Station (Manitoba). *A. ochraceus* and *P. aurantiogriseum* were grown on liquid media as described by Rotter et al (1989). Mycelia from the liquid media were harvested after 10 days, washed with distilled water, freeze-dried, and passed through a 1-mm sieve. Mycelial dry matter was analyzed chemically and spectrally according to procedures outlined in the next section.

Incubated Samples

Barley samples from the storage bins were incubated in the laboratory to create a population totaling 116 samples containing a wide range of mycelia. Duplicate 60-g samples of barley were placed into sterile 500-ml flasks, moisturized to a 25% moisture content, and incubated for 14 days. Incubation was terminated when samples were dried at 21°C to a moisture content of 10%. Although all samples naturally contained fungal spores indigenous to Manitoba barley, varying degrees of deterioration were produced only after moistening and incubating.

Glucosamine in incubated barley and mycelia was determined by acid hydrolysis of chitin and ion-exchange chromatography as described by Rotter et al (1989). Before chromatographic procedures were performed, the sample was filtered through an MSI 0.22-μm nylon 66 membrane filter (Fisher Scientific, Pittsburgh, PA).

In addition to chemical analysis, incubated samples were visually evaluated for mycelial growth of fungal mass and spores. In the first visual procedure, extent of mycelia present on whole barley was assessed on a scale of 0–5 (0 = no visible mycelia, 5 = prolific mycelial growth). In the second procedure, the presence of gray or green spore formation was assessed using a similar scale (0 = no spores, 5 = extremely high level of gray or green spores). In both procedures, visual evaluations were performed independently by two technicians, and data were averaged. The mycelial and spore data were regressed against chemical glucosamine using standard multiple regression techniques (Draper and Smith 1981).

Chemical glucosamine data were used to develop an NIRS prediction equation according to the procedure of Shenk et al (1979). Reflectance spectra were recorded between 1,100 and 2,500 nm on a scanning monochromator (Pacific Scientific 6250, NIRSystems, Silver Spring, MD) with software developed by Infrasoft International (Port Matilda, PA). Second-derivative mathematical transformations were performed on spectra, and derived spectra were regressed against glucosamine. To test the performance of the regression equation, 15–20 random samples not used in equation development were scanned with NIR radiation. Laboratory data were predicted with these samples based on their spectral data, and predicted glucosamine was regressed against actual laboratory data. Optimum equations were selected on the basis of high coefficients of determination (R^2) for calibration and performance, low standard errors of calibration and performance, significance of coefficients in equation development, and slope between predicted and actual glucosamine calculated in equation testing.

Artificial Samples

To investigate characteristics of spectral detection, NIR spectral equations were developed with mold-free barley containing different amounts of added mycelia. An artificial population (Coleman et al 1985) comprising 75 samples was created by mixing the two species of fungi (*P. aurantiogriseum* or *A. ochraceus*) with the barley cultivars Argyle, Bedford, Bonanza, Conquest, Heartland, and Samson. Before mixing, mycelia and barley were ground to pass through a 1-mm screen in a cyclone-type grinder. Glucosamine concentration for the mixed sample was calculated on the basis of values determined for mold-free barley and cultured mycelia. Glucosamine concentration in the artificial population was used as an indicator to ensure that the level of mycelia in mixed samples was similar to that in the incubated population. Mixed samples were then analyzed by NIRS using procedures described previously (Shenk et al 1979), and prediction equations were derived for mycelial DM.

To study wavelengths represented in regression, second derivative reflectance spectra were recorded for barley and fungal tissues. These spectra were compared with wavelengths selected from calibration and with wavelengths previously reported (Roberts et al 1987) that showed mycelia-related effects.

RESULTS AND DISCUSSION

Barley and Fungal Tissues

Glucosamine in healthy barley remained relatively constant among cultivars; concentrations ranged from 0.54 to 0.70 g/kg of DM and averaged 0.61 g/kg of DM. These levels can be expected in healthy small grain seed (Nandi 1978). Pusztai (1964) found glycoprotein-bound hexosamines ranging from 0.13 g/kg of DM in oats (*Avena sativa* L.) to 1.90 g/kg of DM in wheat (*Triticum aestivum* L.). Seitz and Pomeranz (1983) found that glucosamine in ergot-free barley reached 0.63 g/kg of DM. Although extremely low levels of amino sugars in small grain seed have been reported (Golubchuk et al 1960, Pusztai 1964), these levels were measured with early colorimetric procedures; hexosamine observations are influenced by both substrate and methodology (Jarvis 1977, Roberts et al 1987).

Glucosamine averaged 147.4 and 78.2 g/kg of mycelial DM, respectively, in *P. aurantiogriseum* and *A. ochraceus* isolated from local grains and 93.7 g/kg of DM in *A. ochraceus* provided by the Northern Regional Research Laboratory. These concentrations were typical for fungal tissues, although lower glucosamine concentrations have been reported in other fungal species. Jarvis (1977) reported *Aspergillus versicolor* and *A. terreus* that contained, respectively, 43 and 15 g/kg of mycelial DM. The difference in glucosamine between two sources of *A. ochraceus* can be expected also because chitin concentrations fluctuate in vitro (Swift 1973, Cheng and Boat 1978, Lin and Cousin 1985). Plassard et al (1982) reported two cultures of *Suillus luteus* that contained 44.8 and 69.8 g/kg of DM.

Incubated Samples

Glucosamine concentrations in infected barley were higher than those reported for infected corn and soybean, yet similar to those in other tissues. Concentrations ranged from 0.55 to 6.74 g/kg of DM (Fig. 2). Donald and Mirocha (1977) reported that glucosamine reached 0.98 g/kg of DM in corn infected with *Aspergillus* spp., *Penicillium* sp., and *Chaetomium* sp. and 0.26 g/kg of DM in soybean infected with *Aspergillus* spp., *Alternaria* sp., *Penicillium* sp., and *Phoma* sp. Concentration in contaminated wheat, however, varies among studies. It reached only 0.39 g/kg of DM when wheat was inoculated with *Penicillium* sp. (Golubchuk et al 1960) but approached 1 and 3 g/kg of DM when inoculated with *Penicillium* sp. and *Aspergillus candidus,* respectively (Nandi 1978).

The relationship between chemical and visual estimations can be seen in Figure 2. Glucosamine was correlated with both mycelia and spores in incubated barley, and the respective correlation coefficients were 0.86 and 0.85 ($P < 0.01$). A similar relationship was reported between chitin and visual mold scores in moldy alfalfa (Roberts et al 1987). Barley with high levels of mycelia and spores contained high concentrations of glucosamine; only two samples contained high levels of glucosamine with no visible spores, yet these samples had high concentrations of mycelia. This relationship demonstrates that glucosamine derived from ion-exchange chromatography is a representative chemical marker for fungal contamination, and it should reduce apprehension among practitioners regarding the reliability of chemical procedures for predicting mold in grains.

Spectral calibrations for glucosamine in incubated barley revealed that the R^2 of calibration and performance were greater than 0.90, and the regression equation provided an adequate explanation of variability (Table I). Means and standard errors were 1.25 and 0.31 g/kg of DM for calibration and 1.17 and 0.22 g/kg of DM for performance, respectively.

Artificial Samples

Mycelia in the artificial population was accurately predicted by NIRS (Table II). It cannot be said that the calibration was successful simply because it was based on extremely high levels of mycelia—levels that could be detected by the spectrophotometer and would not occur naturally. The glucosamine concentration of the artificial population ranged from 0 to 9.30 g/kg of DM, based on calculations involving glucosamine in mold-free barley and pure mycelia. This was comparable to levels found in the incubated population, which ranged from 0.55 to 6.74 g of glucosamine per kilogram of DM (Fig. 2). In addition, one incubated sample, although not represented in Figure 2 because it was not visually evaluated, contained 8.47 g of glucosamine per kilogram of DM.

Although the artificial population exhibited acceptable regression statistics, the equation was not developed for practical use because the data are not from field samples. The samples would therefore be considered chemically and spectrally different from naturally incubated barley. However, this equation can be used to explain analytical aspects of NIRS. This is important, as NIRS technology is empirical and often does not directly quantitate the compound of interest. Without this equation, it could be argued that spectral quantification of fungal contamination is unrelated to the actual content of fungal mass in incubated grain. The high correlation coefficients (Table II) partially resolve this uncertainty, demonstrating that NIRS can detect the presence of mycelia in a spiked sample of grain, even at low levels that can occur naturally.

TABLE I
Calibration and Performance Statistics for Spectral Determination of Glucosamine in Infected Barley

	n	R^2	Mean	Standard Error (g/kg)	Slope[a]
Calibration	96	0.94[b]	1.25	0.31	...
Performance	20	0.92[c]	1.17	0.22	0.95

[a]Slope derived in regression of predicted data against chemical data.
[b]Coefficient of determination derived in multiple regression of spectral data against chemical data.
[c]Coefficient of determination derived in linear regression of predicted data against chemical data.

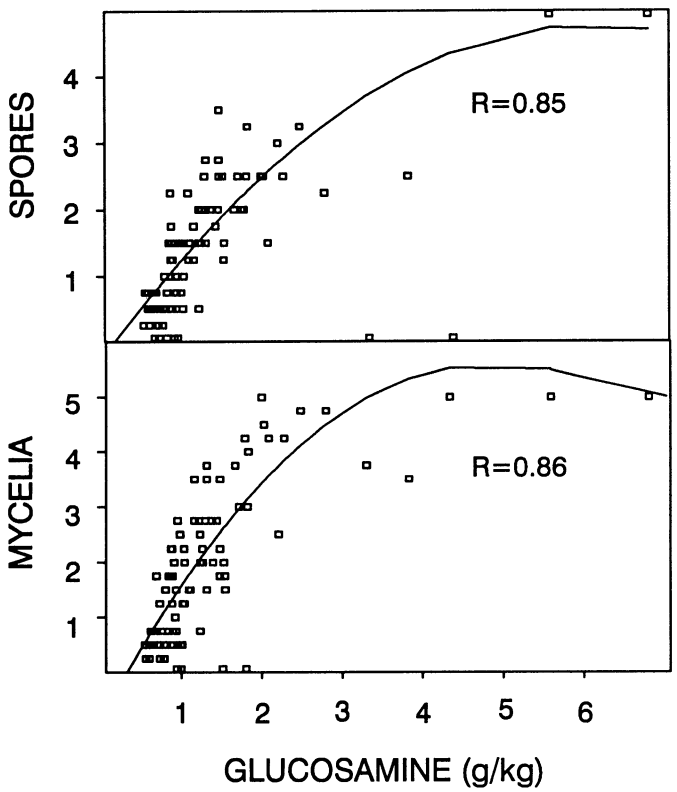

Fig. 2. Glucosamine versus visible spores and mycelial level in contaminated barley. Visual data ranged from 0 (no visible mycelia or spores) to 5 (prolific mycelial growth or spore production).

Barley and Fungal Spectra

Because NIRS is empirical, analytical criteria for wavelength selection are not definitely established. However, apparent trends in wavelength selection often warrant further investigation. These trends may eventually assist in the understanding of relationships between functional groups and absorption regions. In this study, several wavelengths were repeatedly selected for the natural and artificial populations. These wavelengths included 1,610, 1,742, 2,094, 2,156, and 2,356 nm. Of these, 2,094 and 2,356 nm were used to quantify mold in alfalfa (Roberts et al 1987). The selection of these wavelengths was particularly interesting, since 2,094 and 2,356 nm represent peaks in second-derivative spectra of glucosamine (Roberts et al 1987).

Figure 3 illustrates the difference between spectra of mold-free barley and *A. ochraceus* mycelia. Although this figure is not intended to solicit quantitative inferences, it demonstrates a noticeable difference between the two tissues at 2,356 nm. Much larger differences occurred at other wavelengths, evident in the downward peaks that result from second-derivative transformations. These larger peaks, however, were not correlated with glucosamine. For example, large differences in absorption occurred at 1,900 nm; this wavelength is representative of water and was therefore excluded from the glucosamine calibrations.

CONCLUSIONS

We concluded that chitin, as quantified by ion-exchange determination of glucosamine, is representative of fungal contamina-

TABLE II
Calibration and Performance Statistics for Spectral Determination of Mycelial Dry Matter in Mold-Free Barley Mixed with Mycelia

	n	R^2	Mean	Standard Error (g/kg)	Slope[a]
Calibration	60	0.90[b]	40.43	8.23	...
Performance	15	0.94[c]	33.64	5.25	1.08

[a]Slope derived in regression of predicted data against chemical data.
[b]Coefficient of determination derived in multiple regression of spectral data against chemical data.
[c]Coefficient of determination derived in linear regression of predicted data against chemical data.

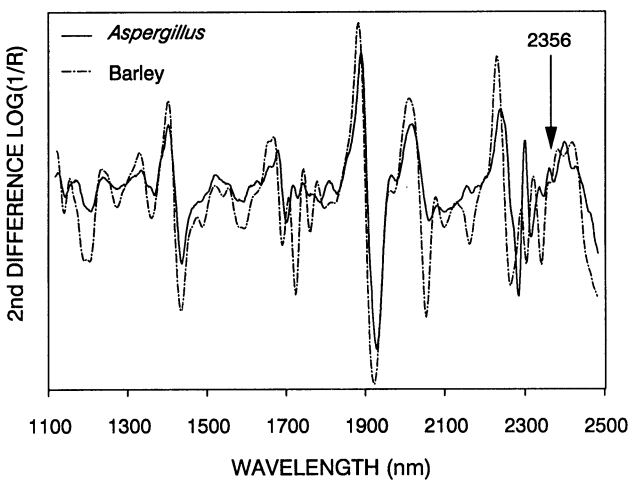

Fig. 3. Near-infrared spectra of *Aspergillus ochraceus* and mold-free barley seed. Spectra are second-derivative transformations of log 1/reflectance. A peak at 2,356 nm was consistently selected in glucosamine calibrations.

tion in barley. Glucosamine concentrations were similar to those reported in other tissues and with other procedures. More importantly, glucosamine was correlated with mycelial growth and spore production. We also concluded that NIRS could successfully quantify glucosamine in contaminated barley. Calibrations produced acceptable statistics and evidence that spectral detection was not necessarily limited to seed products. Because NIRS is rapid and objective, spectral analysis of barley mold offers a valid alternative to cumbersome or subjective methods.

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