A Fluorescent Lectin Test for Mold in Raw Tomato Juice

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ABSTRACT: Fungal (mold) contamination is an important indicator of low quality raw product in the processing tomato industry. A quantitative lectin assay was developed that was less expensive, faster, and more precise than the industry standard Howard mold count. This assay, based on a fluorescent-labeled lectin isolated from wheat germ, had a selective affinity for the chitin in fungal cell walls. Assay values were correlated with mold contamination for 4 fungal species: *Alternaria alternata* ($r^2 = 0.91$), *Cladosporium herbarum* ($r^2 = 0.75$), *Fusarium oxysporum* ($r^2 = 0.97$), and *Stemphylium botryosum* ($r^2 = 0.99$). Combining all 4 species, the lectin assay had a strong correlation ($r^2 = 0.76$) with a linearized Howard mold count.

Key Words: filamentous fungi, biosensor, tomato, lectin, mold detection

Introduction

THE CALIFORNIA TOMATO INDUSTRY MONITORS MOLD LEVELS in raw product at inspection stations and in processed product in quality control laboratories. At the inspection stations, 23 kg of fruit from each 24 metric ton truckload of processing tomatoes are visually inspected for defects. Tomatoes with visible signs of mold are weighed to obtain a percentage of decayed fruit on a mass basis (PTAB 1996). At the processor's quality control laboratories, mold is quantified by the Howard mold count (HMC) method (AOAC 1984), where a small drop of homogenized juice is inspected using a microscope. In the HMC method, 2 slides of 25 fields each are viewed, and the percentage of fields containing mold are recorded. An accurate HMC takes up to 30 min to conduct. Over the last several decades, many attempts have been made to replace manual grading and the Howard mold count with a less subjective and less labor intensive measurement, but no method has been accurate, rapid, and simple enough to use at inspection stations or in quality control laboratories (Jarvis and Williams 1987; Gourama and Bullerman 1995; Cousin 1996). Despite the difficulties and limitations of the HMC (for example, Williams 1968; Jarvis and others 1983), it remains the universal standard for mold assessment almost 90 years after it was first introduced (Howard 1911).

Chitin is an important structural component in fungal cell walls, but absent from plant tissue. The detection of mold based on the chemical isolation and quantification of N-acetyl-D-glucosamine, a breakdown product of chitin, has been proposed as an alternative mold measurement (Ride and Drysdale 1972; Jarvis 1977; Lin and Cousin 1985). Jarvis (1977) found a coefficient of variation (CV) of around 20% for this method. Although the high performance liquid chromatography based isolation method is too slow and labor intensive to be utilized as an industrial replacement for the HMC, a more rapid method that detects chitin could have commercial promise (Cousin 1996).

Lectins are naturally occurring proteins or glycoproteins that bind to specific carbohydrates. They are becoming increasingly valuable as molecular probes, including the labeling of cell-surface components in tissue typing (Lis and Sharon 1986). Hundreds of lectins from microbial, plant, and animal cells have been identified, but most commercially available lectins are isolated from plant seeds. They are available with various enzymatic and fluorescent labels, and their nomenclature derives from the name of their source. There are numerous commercially available lectins that bind polymers of N-acetyl-D-glucosamine. Stoddard and Herbertson (1978) utilized fluorescein labeled lectins to detect human pathogenic fungi. Patel (1992) used fluorescein isothiocyanate (FITC) labeled lectins to observe mold in processed foods. He tested several chitin-binding lectins, and found that a lectin from wheat germ agglutinin (WGA) had the strongest binding to fungal cell walls and the least amount of nonspecific binding to tomato cells. He observed considerable autofluorescent signal, tomato cell tissue that fluoresces at similar wavelengths as the fluorescent probe. Patel and others (1993) used biotinylated lectins and streptavidin labeled magnetic particles to separate and concentrate mold spores and yeasts in fruit juices.

The goal of this research was to develop a rapid method of quantifying mold in raw tomato juice using lectins. Ideally the measurement would have a linear relationship to mold content at levels commonly found in raw tomato juice, have a lower variability than the HMC, and give a similar response for different fungal species. The test should also be simple to conduct and use inexpensive reagents, and give results in the same length of time as the HMC.

Results and Discussion

Tomato tissue autofluorescence

The fluorescence emission spectra maximum from fresh tomato juice occurred at a longer wavelength than the maximum for FITC labeled lectin when excited at 490 nm (Fig. 1). Microscopic observations showed that strongest tomato autofluorescence was observed in the fibrovascular bundles, the stem cells, and the skin cells. The wavelength difference between the emission maximum and the excitation maximum is known as the Stokes' shift, and represents the loss of energy due to molecular dissipation. The Stokes' shift for tomato tissue was consistently around 80 nm for excitation in the visible range. Fortunately, FITC, like most commonly used fluorescent labels, has a considerably smaller Stokes' shift of only 15 to 20 nm. Longpass filters are normally used in fluorometer emission measurements, so the photomultiplier tube would detect the autofluorescent light. However, we used a bandpass filter,

where only a narrow band of light, centered at 520 nm, would be detected by the fluorometer, to eliminate the tomato autofluorescent signal shown in Fig. 1 and also observed by Patel (1992).

Howard mold count results

The Howard Mold Count scores for the juice samples in this study ranged from 0% to 100% for all mold species except *C. herbarum* which had a maximum HMC of 96%. The average (across all dilution levels) amount of mold for each species was 0.75% spoiled tissue by mass. The average (across all dilution levels) HMC scores for each species however, ranged from a low of 37.4% for *C. herbarum* to a high of 64.2% for *A. alternata* (Table 1).

The HMC results were nonlinear with spoiled tissue dilution level (Fig. 2) due to field saturation where additional fungal mycelia in an already positive field did not increase the readings. Considerable variability, particularly at the intermediate spoiled tissue levels, was observed between the HMC scores obtained by the different quality control laboratories (QCL). The variability was lower at both the zero and maximum spoiled tissue levels, since no value can be under 0% or over 100% respectively. The overall average coefficient of variation (CV) between the average HMC scores of all four quality control laboratories was 35%. The HMC scores obtained by the quality control laboratories were well correlated (Table 2), with the best agreement occurring between labs 3 and 4 (r = 0.97).

Lectin assay results

In contrast to the HMC assay, the lectin assay results were linear with spoiled tissue dilution level resulting in the following coefficients of determination: *A. alternata* $r^2 = 0.91$, *C. herbarum* $r^2 = 0.75$, *F. oxysporum* $r^2 = 0.97$, *S. botryosum* $r^2 = 0.99$, all species combined $r^2 = 0.59$, Fig. 3. This linearity is important, both because, unlike HMC, it allows the lectin scores to directly indicate actual mold levels making them easier to compare and because it makes calibration simpler. The nonlinear HMC method was at saturation at 2% spoiled tissue for 3 of the 4 species. A Kruskal-Wallis one way analysis of variance test showed that the precision of the lectin assay was significantly better ($\alpha = 0.02$) than the precision of the HMC assay performed by any of the quality control laboratories (Table 3).

Although the spoiled volume dilution will give accurate relative results (that is, 0.5% level has exactly one quarter the mold as 2% level), it is unlikely that there is the same dry weight of mold mass in the original 2% mold levels for different fungal species. For example, the average HMC at 0.5% spoiled tissue dilution was 94 for *A. alternata* and 40 for *C. herbarum*. Since the HMC gives an indication of fungal biomass, we used these scores to adjust for varying amounts of fungal biomass in the undiluted contaminated juice. Because the HMC is by nature nonlinear with high variability, we developed a linearized HMC score to compare with the lectin assay. The HMC scores of the 2 quality control laboratories (3 and 4)

Table 1—Fungal species variability in average HMC levels for tomato juice with an average spoiled tissue mass of 0.75%

	Howard Mold Count (%)	
Species	Mean*	Standard Deviation
Alternaria alternata	64.2a	18.3
Stemphylium botryosum	53.1ab	16.7
Fusarium oxysporum	46.2bc	14.9
Cladosporium herbarum	37.4c	14.5

* Mean values with the same grouping letter are not significantly different ($\alpha = 0.05$).

Table 2—Correlations between average Howard mold count values obtained by 4 quality control laboratories

	QCL1	QCL2	QCL3	QCL4
QCL1	1.00			
QCL2	0.93	1.00		
QCL3	0.91	0.85	1.00	
QCL4	0.91	0.85	0.97	1.00

Table 3—Average coefficient of variation (CV) values for HMC and WGA lectin mold assay

Evaluator	Ave. CV*
QCL1	50.3%a
QCL2	45.6%a
QCL3	42.0%a
QCL4	21.6%b
WGA Lectin Assay	7.2%c

* CV values with the same grouping letter are not significantly ($\alpha = 0.02$) different.

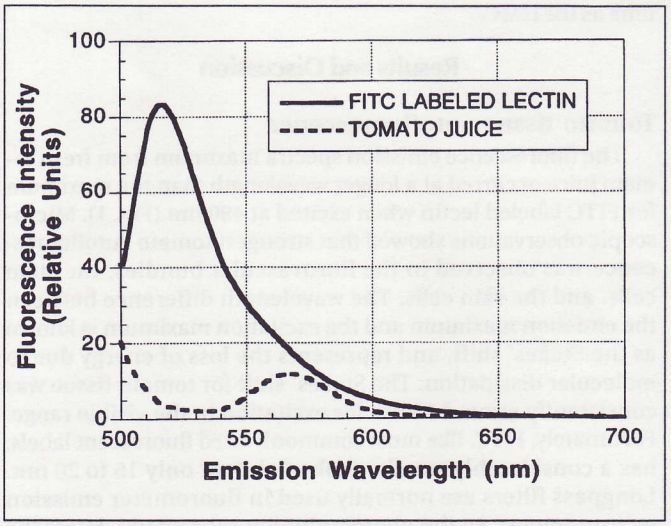

Fig. 1—Tomato tissue autofluorescence and FITC labeled WGA lectin emission spectra for a 490 nm excitation.

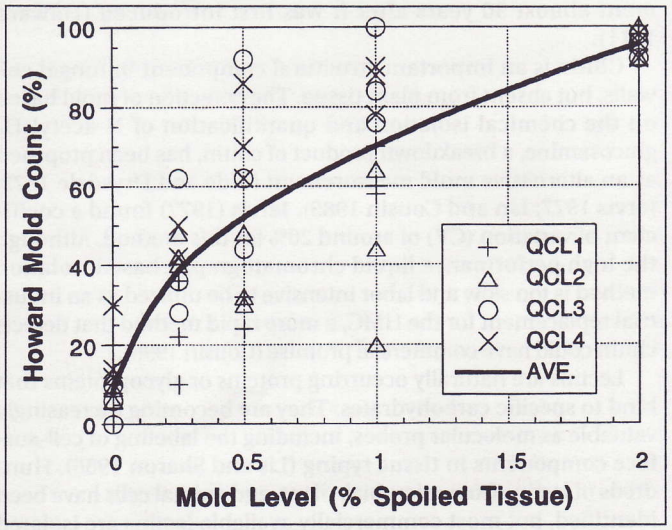

Fig. 2—Howard mold count results for tomato juice infected with Stemphylium botryosum.

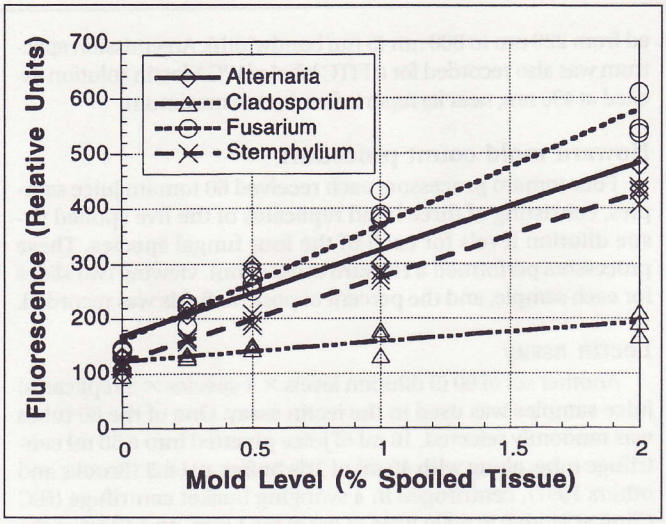

Fig. 3—Lectin assay vs mold dilution level.

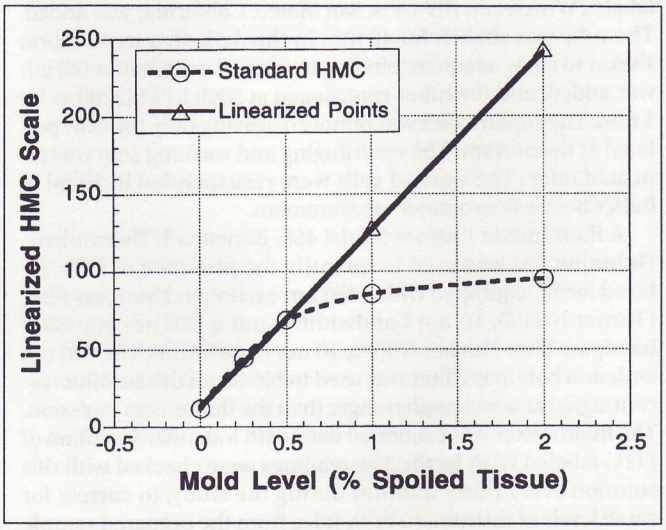

Fig. 4—Linearized Howard mold count for tomato juice infected with Stemphylium botryosum.

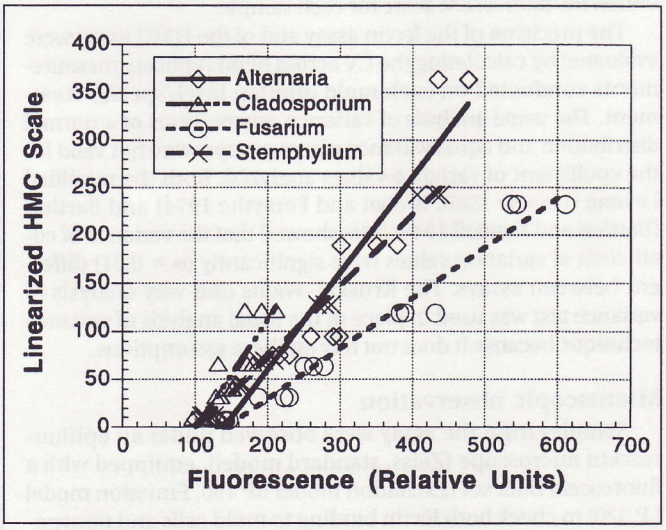

Fig. 5—Lectin assay versus linearized mold count.

which had the best precision among blind replicate measurements and the highest correlation between laboratories were averaged and used as the "true" Howard mold count for mold levels in the study. Four mold levels for *C. Herbarum* (that is, 0% through 1% spoiled tissue) and 3 mold levels for the remaining fungal species (that is, 0% through 0.5% spoiled tissue) were regressed against the spoiled volume to develop linearized HMC models for each species. These models were then used to predict linearized HMC scores above the linear range for each species (Fig. 4). The linearized HMC scores were then regressed against the lectin assay readings resulting in the following coefficients of determination: *A. alternata* r^2 = 0.91, *C. herbarum* r^2 = 0.76, *F. oxysporum* r^2 = 0.97, *S. botryosum* r^2 = 0.99, all species combined r^2 = 0.76 (Fig. 5). These results show that the lectin assay will give generally comparable results to HMC in the linear range for each fungal organism. Differences in response among species are either intrinsic in the lectin assay (that is, species differ in amounts of chitin, or in number of exposed chitin binding sites, Sharma and others 1997; Cousin 1995), may reflect that species differ in ratio of fungal mass to spoiled tissue mass, or HMC scores may differ for similar quantities of different mold species (Table 1). Eisenburg (1952) observed high levels of variability among fungal species when comparing the relationship between HMC and spoiled volume in individual tomatoes. Battilani and others (1996) also observed fungal species variability in both the relationship between HMC and spoiled volume as well as the relationship between HMC and ergosterol content.

The lectin assay required 120 min for a group of 12 samples, or an average of 10 min per sample. This is one-third the time required for an accurate HMC analysis. The total cost of reagents used in the lectin assay were $0.60. The cost of the lectin assay in a commercial application could be reduced further if the reagents were purchased in large quantities.

Microscope observations

WGA lectin bound strongly to the mycelia of all 4 species. Strongest binding occurred at the hyphal tips and in the septa (cross-walls), although there was generally an adequate coating of older wall surfaces. However, it did not bind the asexual conidia of any of the species, with the exception of *F. oxysporum*, which exhibited some lectin binding to macroconidia.

Conclusion

A RAPID AND INEXPENSIVE TEST FOR MOLD WAS DEVELOPED which has the potential to replace the Howard mold count for testing of mold in raw tomato juice. The test is linear, with 3 to 7 times better precision than the Howard mold count for the four fungal species tested. Lectins are inexpensive, and the assay takes around 10 min per sample when analyzed in groups of 12, one-third the time required for an effective official Howard mold count. With binding time reductions, it has the potential to be a much faster assay and can be automated. Correlations with mold count results suggest there is an acceptably low variation across the 4 fungal species tested (*Alternaria alternata, Cladosporium herbarum, Fusarium oxysporum,* and *Stemphylium botryosum*).

Future work will involve a larger number of fungal species and will look more closely at the quantitative differences between species. Work to reduce the binding time from 40 min and to automate the procedure for use in inspection stations and quality control laboratories is also needed.

Materials and Methods

Fungal cultures

Ripe, defect-free processing tomatoes (variety Heinz 8892) were washed and surface disinfected for 10 minutes with a 2% sodium hypochlorite solution. Each of four sets of 20 fruit was placed onto a sterile wire mesh in a sterile container. Cultures of *Alternaria alternata, Cladosporium herbarum, Fusarium oxysporum, and Stemphylium botryosum,* isolated from tomato fields, were grown on potato dextrose agar at 22 °C for up to 21 days. Each fruit was pricked just below the surface with a sterile knife and inoculated with one of four fungal pathogens. The fruit were placed into an incubator at 26 °C for two to five days (depending upon fungal growth rate) with 16 h of light and 8 h of darkness per day. The fruit remained in the incubator until the fungi spoiled approximately 2% (by mass) of the tomato tissue (Fig. 6).

The spoiled volume (Battilani and others 1996) was cut from each fruit in a set and added to unspoiled tissue from additional ripe, defect-free processing tomatoes (variety Heinz 8892) and comminuted for 40 s in a blender (Waring model CB-6, Hartford, CN) to obtain 3.6 kg of juice containing 2% spoiled tissue (by mass). A separate set of 80 defect-free processing tomatoes were also comminuted for 40 s in the blender to obtain 3.6 kg of juice containing no spoiled tissue. The tomato juice with 2% spoiled tissue and the juice with no spoiled tissue were filtered (640 micron pore size) and combined proportionally to obtain five juice samples with spoiled tissue dilution levels of: 0.0%, 0.25%, 0.5%, 1.0%, and 2.0% (by mass). Each dilution level was subdivided into 40 ml replicate subsamples, placed into sealable tubes, autoclaved at 121 °C for 20 min, and then stored at 8 °C for up to three weeks.

Tomato tissue autofluorescence

Clean mold-free juice was diluted and placed into a scanning fluorometer (Hitachi F-2000, San Jose, CA). The excitation wavelength was set at 490 nm and the emission spectrum was recorded from 220 nm to 800 nm (5 nm bandwidth). An emission spectrum was also recorded for a FITC labeled WGA lectin solution excited at 490 nm, near its reported excitation maximum.

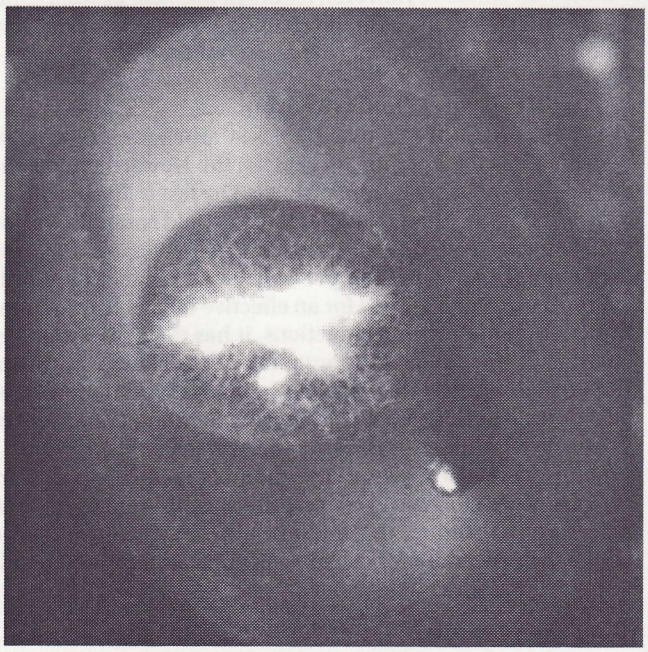

Fig. 6—Infected tomato fruit showing growth of Fusarium oxysporum. The cone-shaped spoiled tissue was cut from each fruit and weighed.

Howard mold count procedure

Four tomato processors each received 60 tomato juice samples, consisting of three blind replicates of the five spoiled tissue dilution levels for each of the four fungal species. These processors performed a Howard mold count, viewing two slides for each sample, and the percent of positive fields was recorded.

Lectin assay

Another set of 60 (5 dilution levels × 4 species × 3 replicates) juice samples was used in the lectin assay. One of the 60 tubes was randomly selected, 10 ml of juice pipetted into a 50 ml centrifuge tube, along with 40 ml of Tris buffer, pH 8.3 (Brooks and others 1997), centrifuged in a swinging bucket centrifuge (IEC Clinical Model) at 5000 RPM (2260 g) for 1 min, and 40 ml of the supernatant were removed. Highly reactive nonspecific binding sites were blocked with 200μl of 30% bovine serum albumin (Sigma Chemical, St. Louis, Missouri), and 50(l of 1mg/ml FITC labeled WGA lectin (EY Labs, San Mateo, California) was added. The tube was shaken for 40 min in the dark on a wrist action shaker to allow adequate binding to occur. Lectin buffer (40 ml) was added, and the tubes centrifuged at 5000 RPM (2260 g) for 1 min. The supernatant was removed, leaving only the cells pelleted at the bottom. The centrifuging and washing step was repeated once. The washed cells were resuspended in 10 ml of buffer before fluorometer measurement.

A fluorometer (Turner Model 450, Barnstead/Thermolyne, Dubuque, IA) was used to quantify the presence of FITC labeled lectin, equipped with a 490 nm excitation bandpass filter (Turner NB490, 10 nm bandwidth) and a 520 nm emission bandpass filter (Turner NB520, 10 nm bandwidth). The 520 nm emission bandpass filter was used to block out the autofluorescent signal at wavelengths longer than the fluorescein emission. The fluorometer was calibrated daily with a standard solution of FITC-labeled WGA lectin. The readings were checked with this solution every thirty minutes during the study, to correct for small levels of instrument drift. Juice from the prepared sample was poured into a 5 ml borosilicate glass circular cuvette (Fisher Scientific, Pittsburgh, PA), and a reading recorded on the fluorometer. The cuvette was emptied and rinsed, and the cuvette refilled with sample. A total of five readings were averaged to obtain the fluorescent score for each sample.

The precision of the lectin assay and of the HMC assay were evaluated by calculating the CV across blind replicate measurements conducted on each mold dilution level/species treatment. The usual analysis of variance assumptions of a normal distribution and equal variance across groups were not valid for the coefficient of variance values analyzed. Both the modified Levene (Levene 1960; Brown and Forsythe 1974) and Bartlett (Bartlett and Kendall 1946) tests showed that the variance of coefficient of variation values were significantly ($\alpha = 0.01$) different between assays. The Kruskal–Wallis one-way analysis of variance test was used in place of the usual analysis of variance technique because it does not rely on these assumptions.

Microscopic observation

Samples from the assay were observed under an epifluorescent microscope (Zeiss, standard model), equipped with a fluorescein filter set (Excitation model BP 490, Emission model LP 520) to check both lectin binding to mold cells and nonspecific binding to tomato tissue.

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