Measuring Mold Infestation in Raw Tomato Juice

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ABSTRACT: A modified fluorescent lectin test for molded raw tomato juice was compared with both the visual mold inspection method conducted by the California Processing Tomato Advisory Board and the Howard mold count (HMC) conducted by 4 commercial tomato processors. The assay quantifies fungal contamination by detecting fungal chitin using FITC-labeled lectin that selectively binds to chitin. The mold content of 100 naturally infected raw tomato juice samples was determined using these 3 methods. The coefficient of determination between the lectin assay and the HMC ($r^2 = 0.73$) was better than the coefficient of determination between the California processing tomato visual mold inspection method and the HMC ($r^2 = 0.38$). The coefficient of determination between the fluorescent lectin assay and the HMC ($r^2 = 0.73$) was comparable to the coefficient of determination between different quality control laboratories' HMC values, which ranged from $r^2 = 0.69$ to $r^2 = 0.81$. The fluorescent lectin assay had consistently better precision (average CV = 8%) than the HMC (average CV = 38%).

Keywords: filamentous fungi, tomato, lectin, mold detection, Howard mold count

Introduction

RAW CALIFORNIA PROCESSING TOMATOES are inspected for quality prior to processing at field stations located throughout the state under a program administered by the California Processing Tomato Advisory Board (PTAB). Each 23.6 metric ton load of raw tomatoes is inspected for 7 quality factors, including mold and soluble solids content. PTAB (1996) uses a visual mold inspection method for the raw fruit, in which seasonal inspectors determine the mass percentage of tomatoes containing visibly observable mold contamination. The California processing tomato industry has expressed concerns about the accuracy of this subjective mold assessment method.

A fluorescent lectin test based on the selective binding of a lectin (wheat germ agglutinin) to chitin in the fungal cell wall has been proposed as an alternative method of mold assessment in raw tomato juice (Potts and others 2000). Preliminary research conducted by Potts and others, using 4 fungal species on laboratory-inoculated fruit indicated the potential of this assay. However, the researchers noted that additional assay refinement was required to reduce the time to conduct the test prior to its commercial acceptability, and that it should be evaluated using a wide-range of fungal species with naturally infected fruit in order to determine whether the lectin assay is an appropriate replacement for the current mold assessment method.

The current standard for grading mold in processed food products is the Howard mold count (HMC), a microscopic count conducted at processors' quality control laboratories (Howard 1911; AOAC 1999). Many researchers have noted limitations of the Howard mold count, including poor repeatability, inaccuracy, high labor costs, and dependence on food processing conditions. Howard and Stephenson (1917) noted that a mold count of 40 might be obtained in samples having any amount of rot between 2.2 and 100%. Eisenburg (1952) conducted a limited study comparing fungal species effects on spoiled volume and HMC methods for mold-contaminated tomatoes. He observed, for example, that tomatoes contaminated with *Geotrichum* spp. at a 7% to 17% spoiled volume level had a HMC ranging from 60 to 100, while tomatoes contaminated with *Phytophthora* spp. at the same 7% to 17% spoiled volume level had a HMC ranging from only 20 to 30. Potts and others (2000) studied the repeatability of the HMC on blind triplicate raw tomato juice samples and observed average coefficients of variation (CV) ranging from 22 to 50% depending upon the operator.

Despite its imperfections, the HMC was selected as the method for comparison in this study because it is the only official method of mold assessment, it can be conducted more rapidly than alternative methods (Gourama and Bullerman 1995), and a large number of replicate measurements could be made by 4 commercial tomato processors located in Northern California, improving its reliability.

This research compared the accuracy and precision of an improved fluorescent lectin assay and the current PTAB visual mold inspection method with the Howard mold count, using naturally infected raw tomato samples taken from California processing tomato fields.

Materials and Methods

Sample collection and PTAB visual mold assessment

Over a 6-wk period during the 1999 California tomato processing season, a total of 100 comminuted tomato juice samples were received from California tomato grading stations. The samples were collected during the normal commercial tomato inspection process conducted by PTAB. From each 23.6 metric ton tomato load, PTAB collected 45.5 kg of tomatoes from random locations in the load. The sample was randomly split with 22.7 kg of fruit graded for visibly apparent mold following standard California grading station practices (PTAB 1996). From the remaining sample, a random subsample of 3.6 kg of water-washed tomatoes were comminuted for 40 s under a vacuum following PTAB's standard practice using an industrial blender (Waring model CB-6, Hartford, Conn., U.S.A.). From each comminuted sample, 350 ml of juice was stored in a plastic jar that was then placed in ice. The jars were then transferred to the University's research laboratory within 4 h. At the University laboratory, part of the juice was set aside for the lectin assay, and the rest of the juice poured into a set of 15 ml cen-

trifuge tubes (Falcon), and autoclaved for 15 min at 121 °C for later analysis by the HMC method. The autoclaved samples were stored at 6 °C.

Howard mold count assessment

Three blind replicates of each autoclaved raw juice sample were sent to the quality control laboratory in each of 4 commercial tomato processing facilities in Northern California for HMC analysis (100 samples × 3 replicates = 300 samples per laboratory × 4 laboratories = 1200 total samples). Each commercial laboratory conducted an industry standard HMC (Method 965.41, AOAC 1999) on each 15 ml sample tube, where the number of fields positive for mold was recorded as a percentage of the total fields observed.

Mold assessment using the improved lectin assay

The improved lectin assay was conducted each d on the fresh (unautoclaved) samples as they were received, since we found that autoclaving affected the lectin assay results considerably (Payne 2000). For each sample, 3 ml of juice was poured into each of 12 50-ml centrifuge tubes (Falcon). The lectin assay was conducted on sets of 4 tubes, where the order in which the tubes were analyzed was chosen at random from the total set of tubes collected each d. Five ml of a 10 mg/ml FITC labeled wheat germ agglutinin (WGA) solution in a Tris-HCl solution (pH 10) was added to each tube. Preliminary experiments indicated that this amount of Tris-HCl solution, combined with 3 ml of tomato juice, consistently resulted in an optimal operating pH around 8.0 (Potts 2000). It was also determined that the WGA could be added with the Tris-HCl solution to the juice in a single step, reducing time requirements without reducing performance when compared to Potts and others (2000) original procedure. These tubes were capped and placed on a shaker for 3 min (compared to 40 min in Potts and others original procedure) to allow the WGA to bind to any mold present. After this binding time, 40 ml of distilled water was added to each tube, the tubes shaken to dissolve any unbound WGA, and then centrifuged at 3800 RPM (2250 × g) for 2 min. The supernatant was gently poured off, leaving the pelleted cells at the bottom. An additional 40 ml of distilled water was then added. The tubes were again shaken and centrifuged for 2 min at the same speed, the supernatant removed, and then 15 ml of Tris-HCl solution (pH 10) was added to the sample, which was shaken to resuspend the cells prior to fluorescence measurement.

The quantity of bound FITC-labeled WGA was determined by fluorescence using 490 nm excitation and 520 nm emission filters (Turner model 450, Barnstead-Thermolyne, with NB490 and NB520 10 nm bandwidth bandpass filters). The fluorometer was calibrated and checked for drift using a fluorescent standard of Oregon Green (Molecular Probes, Inc., Eugene, Oreg., U.S.A.). The average of 3 fluorescence readings was recorded for each tube. To measure the autofluorescence in each sample, 4 control tubes were also tested, with the only procedural difference being that no FITC-labeled WGA was included in the assay. The average reading from these 4 autofluorescence controls was subtracted from the fluorescence results. As a check on the binding time required, an additional 4 tubes of each sample were assayed using the same procedure given above, except that the WGA binding time was increased from 3 to 10 min.

The precision of the lectin assay and of the HMC assay were evaluated by calculating the coefficient of variation (CV) across blind triplicate measurements conducted on each juice sample. A Kruskal-Wallis one-way analysis of variance test was used to determine if the CV values for the lectin assay and the HMC assay conducted by the 4 commercial laboratories were significantly different. It was not possible to conduct blind triplicate measurements using the PTAB visual mold method due to economic and logistical constraints.

Total solids and soluble solids content measurement

The total solids content of each of the 100 samples was determined on the autoclaved samples according to AOAC Method 920.151 (AOAC 1999), with the following exceptions: 10 g of material was used instead of 20 g, and the material was dried at 55 °C instead of 70 °C to avoid caramelization of sugars. The soluble solids content of the 100 autoclaved samples was measured using a refractometer (Bellingham + Stanley Inc., Model RFM100, Lawrenceville, Ga., U.S.A.).

Lectin assay of crabshell chitin-spiked tomato juice

Mold-free tomato juice adulterated with crabshell chitin was used to verify the chitin detection accuracy of the improved lectin assay. Purified crabshell chitin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was finely ground in a mortar and pestle. One hundred ml of distilled water was added to 60 mg of the ground chitin. Defect-free processing tomatoes were handpicked from California tomato fields, and comminuted for 40 s in an industrial blender. Chitin solution was added to tomato juice to obtain 10 levels of chitin concentration ranging from 0 to 400 μg dry chitin/ml tomato juice. Six ml of each solution was pipetted into clean, 50 ml centrifuge tubes (Falcon), to obtain 4 replicates for each solution (4 replicates × 10 chitin levels = 40 samples). Four controls (without lectin) at the highest chitin concentration (400 μg chitin/ml tomato juice) and 4 at the lowest chitin concentration (0 μg chitin/ml tomato juice) were also measured using this procedure. The 48 samples were analyzed for chitin content using the 3 min improved lectin assay described previously.

Results and Discussion

Lectin assay of crabshell chitin-spiked tomato juice

The regression of fluorescence reading compared to crabshell chitin concentration shows high linearity and low standard error (Figure 1). Unlike the HMC and chemical assays for quantification of chitin, the lectin assay is linear, with an $r^2 = 0.994$ and a standard error of 11 μg dry chitin / ml tomato juice. This test verified that the lectin assay is linear and quite precise. Any differences in response of the lectin assay to different fungal species is likely to be due to differing amounts of chitin in the mold mass or to variability in chitin accessibility.

Howard mold count results

The individual HMC scores, as determined by the 4 quality control laboratories, for the juice samples ranged from 0 to 90%. The average HMC score for the 100 samples ranged from a low of 17% for quality control laboratory D to a high of 41% for quality control laboratory B, Table 1. While the HMC values between the quality control laboratories were significantly correlated (Table 2), only 2 of the 4 laboratories (B and C) had mean HMC values that were not significantly different from each another (Table 1).

In addition to differences in overall HMC levels, there was considerable variability between the blind triplicate values at each quality control laborato-

ry. The precision of the HMC was defined as the coefficient of variation (CV) of the blind triplicates (Table 2). In calculating the CV for the HMC, a value of 1 was first added to all HMC scores. This addition was needed to allow the CV to be determined for samples with mean HMC scores of zero. The addition of one HMC unit does not greatly affect the CV of nonzero HMC values. 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. A 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. The HMC CV for the blind triplicates ranged from 0 to 156%. The average CV of blind triplicates for the 4 commercial laboratories were not significantly different ($\alpha = 0.01$) with an overall average HMC CV for blind triplicates of 38% for the 4 laboratories.

Table 1—Distribution of HMC values as determined by 4 commercial quality control laboratories for the naturally infected raw tomato juice samples studied

Laboratory	Howard Mold Count[1] (%) Mean[2]	Standard Deviation
B	41 a	26
C	36 a	20
A	25 b	19
D	17 c	14

[1]Calculations based upon 100 samples, each of which was the average of blind triplicate measurements
[2] Means with the same grouping letter are not significantly ($\alpha = .01$) different

Table 2—Average coefficient of variation (CV) of replicates[3] for HMC and lectin assay

Detection Method	Ave. CV[4]
HMC, Lab. A	39.5%a
HMC, Lab. B	35.4%a
HMC, Lab. C	40.9%a
HMC, Lab. D	36.0%a
Lectin assay, 3 minute binding time	8.0%b

[3]Calculations based upon 100 samples. CV determined from blind replicate measurements on each sample
[4]CV values with the same grouping letter are not significantly ($\alpha = 0.01$) different

Table 3—Correlations between HMC, lectin assay, and the PTAB mold scores

Detection method	HMC, Lab. A	HMC, Lab. B	HMC, Lab. C	HMC, Lab. D	AHMC[5]	PTAB Mold Score
HMC, Lab. A	1.0	–	–	–	–	–
HMC, Lab. B	.90	1.0	–	–	–	–
HMC, Lab. C	.87	.88	1.0	–	–	–
HMC, Lab. D	.83	.83	.80	1.0	–	–
PTAB Mold Score	.58	.64	.57	.54	.62	1.0
Lectin assay, 3 min binding time	.79	.85	.79	.78	.85	.67

[5]AHMC is the average HMC value of each of 100 samples for all four commercial laboratories

Comparison of the lectin assay and PTAB mold scores with the HMC

A Kruskal-Wallis one-way analysis of variance test showed that the precision of the lectin assay (3 min binding time) was significantly better ($\alpha = 0.01$) than the precision of the HMC assay performed by any of the quality control laboratories (Table 2). Evaluated as average coefficient of variation across replicates, the precision of the lectin assay is almost 5 times better than that of the HMC.

The PTAB visual mold scores ranged from 0.0 to 13.5%, with an average score of 5.7% and a standard deviation of 3.1%. Correlations between all mold assessment methods are shown in Table 3. As noted earlier, the literature has documented the lack of precision in the HMC. In this study, 20 out of a total 400 HMC blind triplicates had a CV exceeding 150%. In order to reduce the bias caused by the lack of precision in some HMC measurements, HMC readings with blind triplicates CVs over 150% were not included when calculating the average HMC across all 4 commercial laboratories (AHMC) for comparison with the lectin assay or the PTAB method. An important observation is that the correlation between the lectin assay and the AHMC scores is comparable to correlations between HMC results from 1 quality control laboratory to another. Figure 2 shows the regression relationship between the lectin assay and the AHMC, and Figure 3 shows the regression relationship between the PTAB visual mold score and the AHMC. The lectin assay has a much higher correlation with the AHMC than the PTAB visual mold score test does with the AHMC.

It appears that the residual values between the lectin assay and AHMC are reduced at lower AHMC levels (Figure 2). For analysis, these 100 samples were broken into 3 groups by AHMC score, with Group 1 containing samples with AHMC values between 0 and 20%, Group 2 containing samples with AHMC

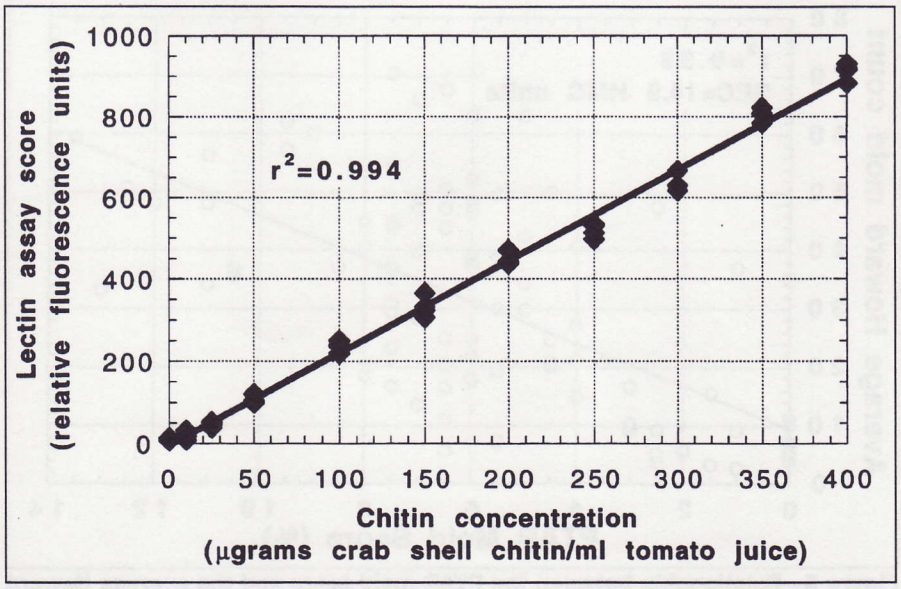

Figure 1—Lectin assay results for tomato juice spiked with crabshell chitin

values between 21 and 40%, and Group 3 containing samples with AHMC values between 41 and 60%. A Kruskal-Wallis one-way analysis of variance test showed that Group 1 had significantly lower residuals than Groups 2 and 3 (α = 0.05). This indicates that the relationship between the lectin assay and the AHMC is better at mold levels between 0 and 20% HMC than above 20 HMC.

It is well known that the HMC becomes nonlinear at higher mold count levels due to field saturation where additional fungal mycelia in an already positive field does not increase the count. Potts and others (2000) observed nonlinearity between mold level and HMC at high HMC levels (that is, above 70 HMC for *Stemphylium botryosum*). Nonlinearity was tested by regressing the lectin assay score with each laboratory's HMC results, and investigating whether the quadratic term was significant. For laboratories A, C, and D, the quadratic term was not significant (α = .05). Only laboratory B had a significant quadratic term, and this was significant at the α = .05 level but not at the α = .01 level. The linearity observed in this study is most likely a result of lower mold levels than that used by Potts and others; the highest average HMC value in this study was 70.4, and 90% of the AHMC values were below 55.

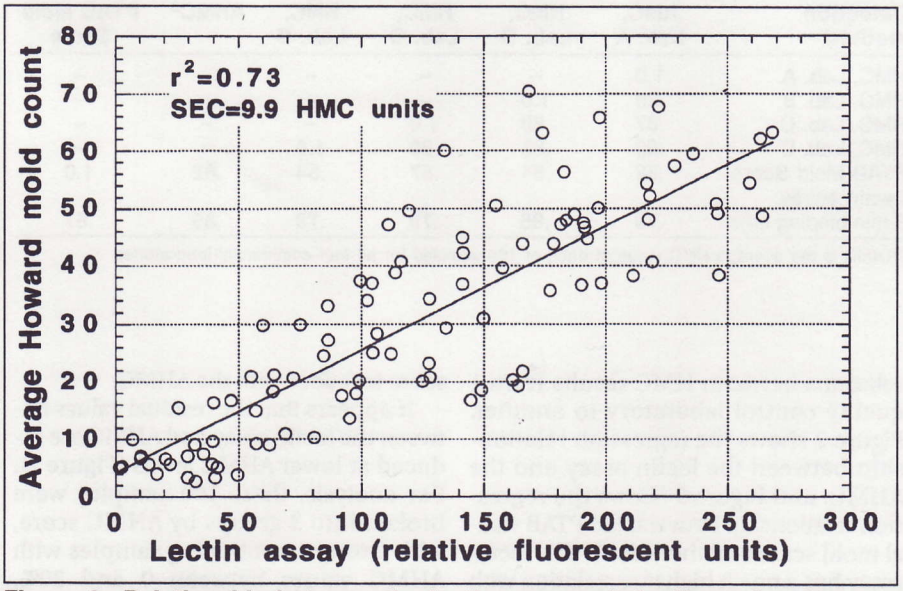

Figure 2—Relationship between the lectin assay and the Howard mold count

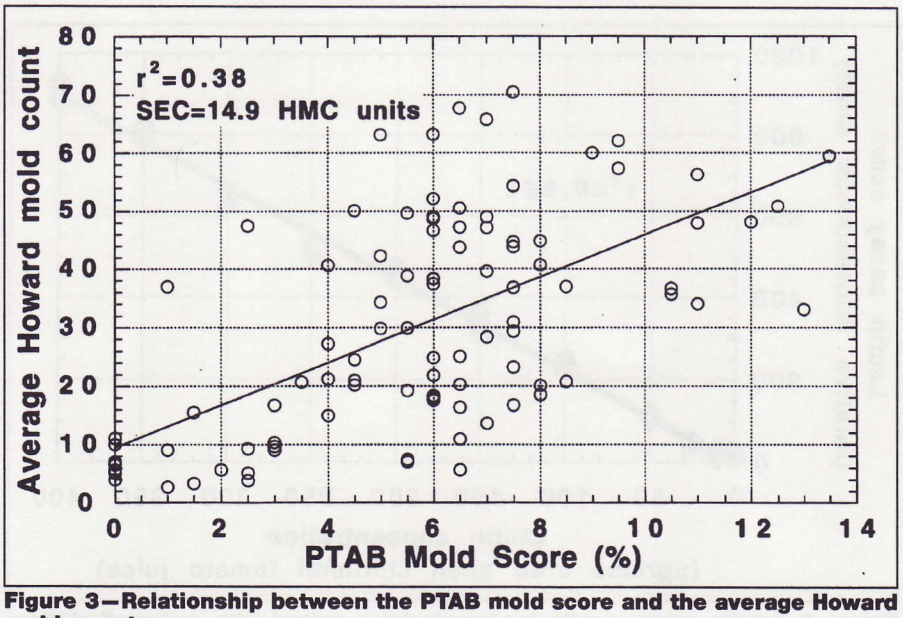

Figure 3—Relationship between the PTAB mold score and the average Howard mold count

Effects of method improvements on the performance of the lectin assay

The lectin assay was conducted using both 3 min and 10 min binding time treatments. The 3 min binding time treatment had a very high degree of correlation with the 10 min binding time treatment (r = 0.97). For the 3 min binding time, the average CV between blind replicates was 8%, with a median value of 7%. For the 10 min binding time, the average coefficient of variation between blind replicates was 11%, with a median value of 9%. The correlations of the 10 min binding time lectin assay and the 3 min binding time lectin assay with both the PTAB visual mold inspection method ($r_{10\ min}$ = 0.69, r_{3min} = 0.67) and with the AHMC ($r_{10\ min}$ = 0.86, r_{3min} = 0.85) were slightly higher for the 10 min treatment than the 3 min treatment. Given the general desire for rapid testing, these results indicate little benefit to the longer treatment.

Three fluorescence readings for each blind replicate were averaged to obtain the results for the correlations in Table 3. While lowering variability, conducting additional fluorometer readings adds time and cost to an industrial assay. To determine whether these replicate readings were beneficial, the AHMC scores were regressed against the 3 min lectin assay using both the average of the 3 fluorometer readings, and individual readings. The standard error of calibration using the average of the fluorometer readings was 9.9 HMC units, while it was only slightly higher for individual readings: 10.0 HMC units for the 1st reading, 10.2 for the 2nd reading, and 10.4 for the 3rd. The extra time required to make multiple fluorometer readings does not appear to be justified given the slight decrease in variability.

The background autofluorescence of the tomato juice ranged from 21 to 76, with an average reading of 43 relative fluorescent units. However, 80% of the autofluorescence values were between 30 and 57 relative fluorescent units, while the WGA-treated samples (3 min binding time) had raw fluorescence scores up to 313. It appears that the variability in autofluorescence is small relative to the change in fluorescence due to mold. To verify this, the 3 min lectin binding scores were regressed against the average HMC with and without the background autofluorescence subtracted. There was no improvement in the correlation to HMC with the au-

tofluorescence subtracted. Since reading a sample with no lectin to correct for autofluorescence did not improve the lectin assay results, it could be dropped from future experiments to save time.

Effects of soluble and total solids

Soluble solids content for the 100 samples ranged between 3.7 and 7.2 °Brix, with a mean of 4.9 °Brix and a standard deviation of 0.7 °Brix. Total solids ranged between 4.3 and 7.8% (wet weight basis), with a mean of 5.3% and a standard deviation of 0.7%. There was a high degree of correlation between the soluble solids content and the total solids content, with an $r^2 = .94$ and a standard error of 0.17% total solids. There was no significant relationship between either total solids or soluble solids and any of the mold measurement methods ($\alpha = .05$).

Conclusion

TESTS USING NATURALLY INFECTED processing tomatoes showed that the lectin assay can be utilized as a replacement for the current PTAB visual mold inspection method and is more precise than the HMC for raw juice samples, providing that careful calibration procedures are followed. The lectin assay is over 4 times more precise than the Howard mold count. It has as good a correlation with the Howard mold count as the correlation of the HMC conducted between different testing sites, and is considerably better correlated with HMC than the current PTAB visual mold inspection method.

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