Utilization of Fluorogenic Assay for Rapid Detection of *Escherichia coli* in Acidic Fruit Juice

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**ABSTRACT**

This study was undertaken to investigate interference by acids commonly found in fruit juice in *Escherichia coli* assays involving the use of 4-methylumbelliferonyl-β-D-glucuronide (MUG) as a fluorogenic substrate for enzyme reaction. Fluorescence intensity was negatively correlated (\( P < 0.001 \)) with the volume of fresh citrus juice tested by the lauryl tryptose broth (LST)-MUG assay, and the permissible sample sizes were limited to 0.3 and 0.5 ml for fresh citrus juices with pHs of 3.3 and 3.9, respectively. In addition, false-negative results were visually observed under UV light when the E*Colite* assay was used to test large volumes (5 to 10 ml per test) of fresh citrus juice or when the test broth used for the LST-MUG assay was supplemented with citric, malic, or tartaric acid at 2 to 4 g/liter. These results suggest that the size and pH of acidic samples should be controlled in MUG-based fluorogenic assays. The inhibitory effect on fluorescence was due to high acidity, which reduces fluorescence from 4-methylumbelliferone. Buffering improved the assays. When sodium bicarbonate was incorporated in the enrichment broth at 10 g/liter, the permissible sample sizes for fresh grapefruit juice (pH 3.1) increased from 0.3 to 1 ml for the LST-MUG (with 9.9 ml of broth) assay and from 3 to 10 ml for the E*Colite* (with 99 ml of broth) assay.

Rapid assays involving the use of 4-methylumbelliferyl-β-D-glucuronide (MUG) as a fluorogenic substrate for enzyme reaction have been widely used to detect the presence of *Escherichia coli*, an indicator organism of fecal contamination, in water and food 

(7, 18, 19). The enzyme β-glucuronidase, produced almost exclusively by *E. coli*, cleaves the MUG to yield a fluorescent product, 4-methylumbelliferone, that can be detected under UV light. *E. coli*–negative samples can then be identified by a lack of fluorescence in 24 to 48 h. Assays developed on the basis of this principle have gained much acceptance because of the large permissible sample sizes and the relatively short testing time required to confirm the absence of *E. coli*.

Most previous studies involving fluorogenic assays have dealt with the assessment of the quality of drinking water. Information on the compatibility of these assays in detecting *E. coli* in food samples is available, but this information pertains only to non- or low-acid foods (1, 3, 13, 14, 17, 21). Most probable number (MPN) evaluations involving these food samples indicated that the *E. coli* recovery levels for the rapid assays were comparable to those for the standard AOAC method. False-positive fluorescence may occur when food samples possess either β-D-glucuronidase activity or nontarget organisms that are capable of producing β-D-glucuronidase during enrichment (2, 9, 12, 20). Although no false-negative fluorescence has previously been reported in the testing of food samples containing generic *E. coli*, the lack of β-D-glucuronidase activity among some enteropathogenic *E. coli* is well known (6, 9).

New rules and sanitation guidelines have been developed in recent years for the fruit and vegetable juice industry in order to better control the risk of microbial contamination (5). In the fresh citrus juice industry, *E. coli* testing of the final juice product is required as part of the mandatory hazard analysis critical control point program. Verification of the absence of biotype I *E. coli* in one 20-ml sample consisting of two 10-ml subsamples for each 1,000 gallons of juice produced is required by certain processors (5). Many processors have expressed interest in implementing a simple *E. coli* test for on-site verification of processing sanitation and juice safety. The present study was designed to evaluate the suitability of MUG-based fluorogenic assays for the detection of generic *E. coli* in fresh citrus juice. Furthermore, the effect of the incorporation of sodium bicarbonate in the enrichment broth to reduce the acidity of juice was also investigated.

**MATERIALS AND METHODS**

**Juice preparation.** Freshly picked citrus fruits were washed on a commercial packing line with Fruit Cleaner 395 (FMC Co., Lakeland, Fla.), placed in a perforated plastic crate, and completely immersed in water at 80°C for 1 min for surface decontamination (15, 16). The fruits were then squeezed by a sanitized citrus juicer (DeliJuicer, InterCitrus, Brazil). The fresh juice was immediately refrigerated at 4°C in sterile glass bottles and was used for testing within 24 h of storage. The pH of each juice was measured with a pH meter.

**Inoculation.** *E. coli* ATCC 25922, ATCC 8739, and ATCC 11229 cultures were maintained on tryptic soy agar (TSA; Difco
**TABLE 1. Detection of E. coli in fresh citrus juice by the LST-MUG test**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Detection for fresh juice sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Hamlin orange juice (pH 3.9)</td>
<td></td>
</tr>
<tr>
<td>24-h visual fluorescence</td>
<td>+++</td>
</tr>
<tr>
<td>48-h visual fluorescence</td>
<td>+++</td>
</tr>
<tr>
<td>48-h fluorescence (%)</td>
<td>93.7 ± 7.9</td>
</tr>
<tr>
<td>Ruby Red grapefruit juice (pH 3.3)</td>
<td></td>
</tr>
<tr>
<td>24-h visual fluorescence</td>
<td>+++</td>
</tr>
<tr>
<td>48-h visual fluorescence</td>
<td>+++</td>
</tr>
<tr>
<td>48-h fluorescence (%)</td>
<td>95.8 ± 6.8</td>
</tr>
</tbody>
</table>

a Each test tube contained 9.9 ml of LST-MUG broth, and each juice sample contained about 10 CFU of E. coli ATCC 25922. All fermentation tubes except the negative controls showed gas production within 24 h. Triplicate experiments were conducted, and the visual reading for each test tube under UV light against a negative control was recorded as positive (+), weak positive (±), or negative (−).

b Data represent means ± standard deviations for three replications compared with a positive control without juice.

Fluorogenic assays. For lauryl tryptose broth (LST)-MUG tests, 0.01 ml of the E. coli ATCC 25922 inoculum was added to each test tube containing 9.9 ml of LST-MUG broth (Difco), an inverted fermentation tube, and 0.1, 0.3, 0.5, and 1.0 ml of fresh fruit juice. In tests designed to evaluate the effect of acidification, the LST-MUG broth was supplemented with citric, malic, or tartaric acid (at 0.5, 1, 2.5, or 4 g/liter) and then filter sterilized. After inoculation, the tubes were incubated at 35°C for 24 h. Broth tubes with uninoculated fruit juice or acid solution served as negative controls, and tubes containing broth alone with E. coli cells served as positive controls. The visual detection of gas production and fluorescence under long-wave UV light (336 nm) constituted positive results. Fluorescence intensities were measured with a fluorometer (VersaFluor, Bio-Rad, Hercules, Calif.), and the percentage of fluorescence was then calculated by dividing the fluorometric reading for each test result by that for the positive controls.

To evaluate the effect of buffering, LST-MUG broth containing 0.5, 1, 2.5, 5, or 10 g of sodium bicarbonate per liter was filter sterilized prior to inoculation. To further evaluate the effect of buffering at the end of assay, LST-MUG broth containing 0 or 2 g of citric acid per liter was inoculated with E. coli, and sodium bicarbonate and citric acid were introduced into the final broth after incubation at 35°C for 24 h.

For E*Colite tests, each inoculated juice sample (1, 3, 5, or 10 ml; 4°C) was placed in a bottle containing a 99-ml dilution blank (Butterfield’s phosphate buffer, BioPro, Bothell, Wash.) at 35°C. Each juice sample was inoculated with the same number of cells (10 cells per test). Dilution blanks were supplemented with sodium bicarbonate at concentrations of 0.5, 1, 2.5, 5, and 10 g/liter to evaluate the effect of neutralization. The diluted juice sample was then poured into the E*Colite test bag containing dehydrated medium (Charm Sciences Inc., Lawrence, Mass.), shaken briefly, and incubated at 35°C. Test bags containing uninoculated fruit juice served as negative controls, and test bags containing E. coli cells without juice served as positive controls. The visual observation of a blue-green color after 28 h of incubation and the visual observation of fluorescence under long-wave UV light after 48 h of incubation constituted positive results. The same fluorometric method used for the LST-MUG tests was used to detect the fluorescence percentage for each E*Colite test bag.

**TABLE 2. Detection of E. coli in fresh citrus juice by the E*Colite test**

<table>
<thead>
<tr>
<th>Strain</th>
<th>48-h visual fluorescence for fresh juice sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ml</td>
</tr>
<tr>
<td>Valencia orange juice (pH 4.4)</td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>+++</td>
</tr>
<tr>
<td>E. coli ATCC 11229</td>
<td>+++</td>
</tr>
<tr>
<td>E. coli ATCC 8739</td>
<td>+++</td>
</tr>
<tr>
<td>Ruby Red grapefruit juice (pH 3.1)</td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>+++</td>
</tr>
<tr>
<td>E. coli ATCC 11229</td>
<td>+++</td>
</tr>
<tr>
<td>E. coli ATCC 8739</td>
<td>+++</td>
</tr>
</tbody>
</table>

a Fresh juice was inoculated with approximately 10 E. coli cells per sample and diluted in 99 ml of Butterfield’s buffer before it was poured into the test bag. All test bags except the negative controls developed a blue-green color within 28 h. Triplicate experiments were conducted, and the visual reading for each test bag under UV light against a negative control was recorded as positive (+), weak positive (±), or negative (−).
FIGURE 1. Effects of the addition of sodium bicarbonate and citric acid to the final LST-MUG broth. (A) The enhancement of fluorescence (■) with the addition of sodium bicarbonate (10 g/liter) immediately after regular incubation (time 0) and the reversion (●) occurring after the addition of citric acid (10 g/liter) 30 min later to the LST-MUG broth that was inoculated with E. coli ATCC 29522 and acidified with citric acid (5 g/liter). (B) The enhancement of fluorescence (○) with the addition of sodium bicarbonate (10 g/liter) immediately after regular incubation (time 0) and the reversion (●) occurring after the addition of citric acid (10 g/liter) 30 min later to the nonacidified LST-MUG broth that was inoculated with E. coli ATCC 8739.

A conventional (standard) three-tube MPN method for the determination of total coliforms and E. coli was used to evaluate the uninoculated juice samples (10). For each bottle of fresh juice (1 liter per bottle), a 10-ml juice sample was tested by this non-MUG-based assay. To verify E. coli counts in the inoculated juice, sample dilutions were plated with TSA and incubated at 25°C for 2 h. The plates were then covered with a layer of violet red bile agar and MUG (Difco) and incubated for approximately 20 h at 35°C. Purple-red colonies more than 0.5 mm in diameter that were surrounded by a zone of precipitated bile acids were counted after confirmation on the basis of their fluorescent appearance under long-wave UV light.

**Statistical analysis.** Microbial results were based on a minimum of three replications per treatment. The Pearson product-moment correlation and Student’s *t* test for difference were performed with SigmaStat (Jandel Corporation, San Rafael, Calif.) software to determine statistical significance (*P* ≤ 0.05).

**RESULTS**

Detection of *E. coli* in fruit juice. Fresh orange and grapefruit juices extracted from surface-decontaminated fruits were used in this study. Results of the MPN evaluation indicated the absence of coliforms and *E. coli* in these juices (<0.1 MPN/ml) prior to inoculation. After inoculation, two fluorogenic detection methods were used to test for the presence of low levels (an estimated 10 CFU) of *E. coli* ATCC 25922 in the juice samples. Excepting tests for the uninoculated controls, gas production was exhibited in all LST-MUG tests and blue-green color developed in all *E. coli* tests after incubation at 35°C. In the standard 24-h LST-MUG tests, fluorescence was apparent, indicating the presence of *E. coli*, with low volumes of juice (Table 1). However, the *E. coli* did not consistently produce positive fluorescence responses when either 1.0 ml of fresh orange juice or ≥0.5 ml of fresh grapefruit juice was added. Extending the incubation time to 48 h allowed further development of fluorescence responses for visual detection among some weakly fluorescing and nonfluorescing tubes. Fluorometric readings revealed significant negative correlations between the detected percentage of fluorescence and the amount of orange juice (*r* = −0.94, *P* < 0.001) or grapefruit juice (*r* = −0.99, *P* < 0.001) added. Data presented in Table 2 show a similar inhibition pattern (as indicated by visual fluorescence) for the *E. coli* tests when citrus juice was added at relatively high volumes. Positive fluorescence responses were not consistently observed when either 10 ml of fresh orange juice or ≥5 ml of fresh grapefruit juice was tested. Some variations in fluorescence among the three tested strains of *E. coli* were also observed (Table 2).

Interference by common acids. To determine whether the inhibition in fluorescence was caused by juice acidity, citric, malic, and tartaric acids were added to test tubes containing LST-MUG broth to yield acid concentrations of 0.5, 1, 2, and 4 g/liter prior to inoculation. Fluorescence was visually observed after 24 h of incubation at citric acid concentrations of up to 1 g/liter (Table 3). The fluorometer reading was negatively correlated with the concentration of citric acid (*r* = −0.96, *P* < 0.001) and with the reduction in the initial pH of the broth (*r* = −0.64, *P* < 0.001) but not with the size of the final *E. coli* population (*r* = −0.01, *P* > 0.05). In addition, a positive correlation (*r* = 0.73, *P* < 0.01) was found between fluorescence and broth pH after incubation for the growth of *E. coli*. Similar results were noted when either malic or tartaric acid was used for acidification (Table 3).
Prevention of false negatives with sodium bicarbonate. In an attempt to increase the permissible juice sample size, sodium bicarbonate was added to neutralize the acidity of the juice. The data presented in Table 4 illustrate that fluorescence was observed in LST-MUG and E*Colite tests involving fresh grapefruit juice (pH 3.0; inoculated with E. coli ATCC 25922) when sodium bicarbonate was added at 2.5 g/liter. For both tests, the percentage of fluorescence for the juice samples was positively correlated (P < 0.001) with the amount of sodium bicarbonate added. The fluorescence reading either matched or exceeded that for positive controls when sodium bicarbonate was incorporated at 10 g/liter. The data presented in Table 5 demonstrate an improvement in the visual fluorescence reading with the addition of sodium bicarbonate to LST-MUG broth containing citric acid at 2 g/liter. Positive correlations (P < 0.001) were found between the percentage of fluorescence and the concentration of sodium bicarbonate (from 0 to 10 g/liter) in the assay involving either E. coli ATCC 29522 or E. coli ATCC 8739. Again, the fluorescence reading either matched or exceeded that of positive controls when sodium bicarbonate was incorporated at a concentration of 10 g/liter.

Enhancement of fluorescence readings with sodium bicarbonate. To evaluate the effect of buffering at the end of the assay, sodium bicarbonate was added to the final LST-MUG broth prior to the observation of the fluorometer reading. It can be seen in Figure 1A that the incorporation of sodium bicarbonate (at 5 g/liter) in the acidified LST-MUG assay (with citric acid at 2 g/liter) for broth inoculated with E. coli ATCC 29522 increased the fluorescence reading immediately (within 30 s) and doubled the reading within 30 min. The enhanced fluorescence reading was reversible and was immediately reduced with the introduction of additional citric acid to the final test broth. A very similar curve was observed (Fig. 1B) when sodium bicarbonate was added at 5 g/liter to the nonacidified LST-MUG broth inoculated with E. coli ATCC 8739. Again, the fluorescence reading was instantly increased with the addition of sodium bicarbonate and reduced with the further incorporation of citric acid at the end of the assay.

DISCUSSION

The results of this study stress the importance of controlling the initial acidity of enrichment broth systems used for testing acidic fruit juice. The growth of E. coli is required in assays involving the use of MUG as the fluorogenic substrate for enzyme reaction; however, E. coli growth alone does not warrant the success of such assays. One should be concerned about false negatives when testing acidic foods and beverages with MUG-based fluorogenic assays, especially when sample sizes are relatively large. Acids in foods and beverages may reduce the pH of the enrichment broth and could limit the growth of E. coli as well as its production of the enzyme β-glucuronidase. In addition, the acids introduced at the beginning of the assay may influence the pH of the final broth, which in turn could decrease the fluorescence of 4-methylumbelliflorone. It is important to note that the fluorescence of 4-methylumbelliflorone is highly dependent on pH, and the optimal pH for detection has been judged to be 10.3 (6–8). This chemical characteristic explains our observations with regard to the

### TABLE 3. Effects of acids on the fluorogenic detection of E. coli ATCC 25922 by the LST-MUG test a

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for acid concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0 g/liter</td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
</tr>
<tr>
<td>Initial broth pH</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td>Final (24-h) broth pH</td>
<td>5.5 ± 0.0</td>
</tr>
<tr>
<td>Final population (log CFU/ml)</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>24-h fluorescence (%)</td>
<td>100.0 ± 2.3</td>
</tr>
<tr>
<td>24-h visual reading b</td>
<td>+++</td>
</tr>
<tr>
<td>Malic acid</td>
<td></td>
</tr>
<tr>
<td>Initial broth pH</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td>Final (24-h) broth pH</td>
<td>5.5 ± 0.0</td>
</tr>
<tr>
<td>Final population (log CFU/ml)</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>24-h fluorescence (%)</td>
<td>100.0 ± 2.3</td>
</tr>
<tr>
<td>24-h visual reading b</td>
<td>+++</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td></td>
</tr>
<tr>
<td>Initial broth pH</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td>Final (24-h) broth pH</td>
<td>5.5 ± 0.0</td>
</tr>
<tr>
<td>Final population (log CFU/ml)</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>24-h fluorescence (%)</td>
<td>100.0 ± 2.3</td>
</tr>
<tr>
<td>24-h visual reading b</td>
<td>+++</td>
</tr>
</tbody>
</table>

a Mean ± standard deviation for three replications.

b Triplicate experiments were conducted, and the visual reading for each test tube under UV light against a negative control was recorded as positive (+), weak positive (±), or negative (−).
TABLE 4. Effect of sodium bicarbonate on the fluorogenic detection of E. coli ATCC 25922 in fresh grapefruit juice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for sodium bicarbonate concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 g/liter</td>
</tr>
<tr>
<td>LST-MUG assay&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>24-h visual reading&lt;sup&gt;b&lt;/sup&gt;</td>
<td>- - -</td>
</tr>
<tr>
<td>24-h fluorescence (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.6 ± 1.5*</td>
</tr>
<tr>
<td>E*Colite assay&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>48-h visual reading&lt;sup&gt;b&lt;/sup&gt;</td>
<td>- - -</td>
</tr>
<tr>
<td>48-h fluorescence (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 0.4*</td>
</tr>
</tbody>
</table>

<sup>a</sup> One milliliter of fresh grapefruit juice (pH 3.0) was added to 9.9 ml of LST-MUG broth containing sodium bicarbonate. All fermentation tubes except the negative controls showed gas production within 24 h.

<sup>b</sup> Triplicate experiments were conducted, and the visual reading for each test tube or bag under UV light against a negative control was recorded as positive (+), weak positive (±), or negative (−).

<sup>c</sup> Data represent means ± standard deviations for three replications. Values followed by an asterisk (*) are significantly (P < 0.05) lower than the 100% value of the nonacidified positive controls.

<sup>d</sup> Ten milliliters of fresh juice was diluted in 99 ml of Butterfield’s buffer before it was poured into the test bag. All test bags except the negative controls developed a blue-green color within 28 h.

correlation between fluorescence and broth pH as well as the instant alteration of fluorescence with the addition of sodium bicarbonate or citric acid at the end of the assay.

Since the acid level of an orange or grapefruit ranges from 5 to 20 g/liter (4), it was not surprising that the fluorogenic detection of E. coli in citrus juice samples by either the LST-MUG tube method or the E*Colite bag method was hindered when a large volume of orange juice or grapefruit juice was added to the testing broth systems (Tables 1 and 2). Grapefruit juice with higher levels of acid (usually ≥10 g/liter) than those of orange juice tended to limit the permissible juice sample size to a smaller volume. The addition of appropriate amounts of sodium bicarbonate at the beginning of the assay could compensate for the acidity of juice and aid in the avoidance of false negatives. For example, the addition of sodium bicarbonate at 10 g/liter at the beginning of an assay can increase the permissible sample sizes of fresh grapefruit juice from 0.3 to 1 ml for the LST-MUG (with 9.9 ml of broth) assay and from 3 to 10 ml for the E*Colite (with 99 ml of broth) assay, respectively. This finding confirms that the pH of growth media for MUG-based assays should be adjusted to a neutral or slightly alkaline value; alternatively, the pH may be adjusted at the end of the assay to increase the fluorescence intensity of methylumbelliferone (11, 12).

In summary, we have determined that acids commonly found in fruits and fruit juices can interfere with visual E. coli assays that involve the use of MUG as the fluorogenic substrate for enzyme reaction. To avoid possible interference resulting from high acidity, the sizes of acid samples to evaluated unles pH-related false negatives are not further suppressed. Future research should continue to evaluate the maximal permissible sample sizes for juices with different pH ranges and their relationship with buffer concentrations. Sodium bicarbonate may be incorporated in commercial kits for testing acidic juice in industry settings, where simple and prompt detection techniques are highly desirable. Although acid samples may be neutralized by the use of the assay, the added neutralization step may compromise the simplicity of the assay and render the test less practical.

ACKNOWLEDGMENTS

This research was supported by the Florida Agricultural Experiment Station (journal series number R-08452) and the Florida Department of Agriculture and Consumer Services.

TABLE 5. Effect of sodium bicarbonate on the fluorogenic detection of E. coli in LST-MUG broth with citric acid<sup>a</sup> at 2 g/liter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for sodium bicarbonate concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0 g/liter</td>
</tr>
<tr>
<td>E. coli ATCC 29522</td>
<td></td>
</tr>
<tr>
<td>24-h visual reading&lt;sup&gt;b&lt;/sup&gt;</td>
<td>± ± ±</td>
</tr>
<tr>
<td>24-h fluorescence (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.7 ± 0.9*</td>
</tr>
<tr>
<td>E. coli ATCC 8739</td>
<td></td>
</tr>
<tr>
<td>24-h visual reading&lt;sup&gt;b&lt;/sup&gt;</td>
<td>- - -</td>
</tr>
<tr>
<td>24-h fluorescence (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0 ± 0.0*</td>
</tr>
</tbody>
</table>

<sup>a</sup> Citric acid was added to 9.9 ml of LST-MUG broth before inoculation.

<sup>b</sup> Triplicate experiments were conducted, and the visual reading for each test tube under UV light against a negative control was recorded as positive (+), weak positive (±), or negative (−).

<sup>c</sup> Data represent means ± standard deviations for three replications. Values followed by an asterisk (*) are significantly (P < 0.05) lower than the 100% value of the nonacidified positive controls.
Citrus. Research materials provided by Charm Inc. and Ecolab Inc. are appreciated. Technical support from Tony Lopez is also acknowledged.

REFERENCES


