RAPID QUANTITATIVE METHOD FOR DETERMINATION OF ACETALDEHYDE IN LACTIC STARTER CULTURES

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ABSTRACT

The 3-methyl-2-benzothiazolone hydrazone test was adapted for the spectrophotometric determination of acetaldehyde in lactic starter cultures. The method was modified, so that it utilized the same equipment as a recently reported method for determination of diacetyl in starter cultures. Reaction conditions, uses, and limitations of the method are discussed. The procedure was applied in conjunction with diacetyl measurements in studying single- and mixed-strain lactic starter cultures.

In recent years acetaldehyde has been recognized as an important flavor component in certain lactic starter cultures. Whereas low concentrations of acetaldehyde are desirable in normal culture flavor (7) and in yogurt (10), excessive amounts have been shown to be responsible for the green (yogurt) flavor of butter cultures (1, 7). Acetaldehyde has also been implicated in the malty flavor defect of lactic starter cultures (14).

Schulz and Hingst (12) have reported methods for the qualitative detection of acetaldehyde in yogurt cultures. Acetaldehyde was indicated by formation of a blue color in neutralized whey after addition of sodium nitroprusside and either piperidine or hexamethylenimine. Badings and Galesloot (1) utilized these color reactions along with a gas chromatographic procedure in the qualitative examination of mixed-strain butter cultures for the presence of acetaldehyde.

Harvey (3) has described a spectrophotometric method for the quantitative estimation of aldehydes and ketones in cultures of lactic streptococci. The method involves steam distillation of culture samples, reaction of the distillate with 2,4-dinitrophenylhydrazine, and paper chromatographic separation of the resulting 2,4-dinitrophenylhydrazones. Individual chromatographic spots were eluted and concentrations were calculated from spectrophotometric data.

Recently, Sawicki et al. (1) described a very sensitive method for the selective spectrophotometric determination of water-soluble aliphatic aldehydes. The method is based on the reaction of aldehydes with 3-methyl-2-benzothiazolone hydrazone to give a blue cationic dye with a high molar absorptivity in an acidic aqueous system. In earlier work in our laboratory on butter culture flavor (6), acetaldehyde was found to comprise the major portion of the aldehyde fraction in butter cultures. In view of this, it appeared that the 3-methyl-2-benzothiazolone hydrazone method for acetaldehyde measurement could be employed in conjunction with the recovery system described by Pack et al. (9) for rapid diacetyl determination in lactic cultures. This paper relates the adaptation of the 3-methyl-2-benzothiazolone hydrazone method for routine examination of lactic cultures for acetaldehyde.

EXPERIMENTAL PROCEDURES

Cultures. Single-strain cultures were obtained from the culture collection in the Department of Microbiology, Oregon State University. These were Streptococcus lactis strain C,F, Streptococcus lactis var. maltigenes strains M1, 3, 4, and 6, Streptococcus cremoris strain SC, Streptococcus diacetilactis strain 18-16, and Leuconostoc citrovorum strain 91404. Mixed-strain cultures referred to in Table 4 were of commercial origin. All cultures were maintained in a whole milk medium, prepared by adding 0.2% sodium citrate to raw whole milk, then heating for 1 hr in a boiling water bath. The cultures were maintained with an every-other-day transfer, using 2% inoculum. The single-strain cultures were incubated at 30 C for 14 hr and the mixed-strain cultures were incubated at 21 C for 14 hr.

Diacetyl and acetaldehyde determinations. The Owades and Jakovac method, as modified by Pack et al. (9), was used for diacetyl deter-
minations. Twenty-gram samples of culture were used instead of the 20-ml samples described in the original procedure. Acetaldehyde was determined on a separate aliquot of culture at the same time as diacetyl, by a modified 3-methyl-2-benzothiazolone hydrazone procedure. The procedure follows:

Reagents:
(1) Aqueous 0.4% solution of 3-methyl-2-benzothiazolone hydrazone hydrochloride
(2) Dimethyl sulfoxide (DMSO)
(3) 0.2% ferric chloride in 0.1 N HCl
(4) Acetone, reagent grade

Procedure:
Twenty grams (or 5 to 15 g of culture made to 20 g with distilled water) of culture was weighed carefully into a 25- by 250-mm test tube and a small amount of antifoam agent (spray-form) added before unit assembly. The collection trap was similar to that described by Pack et al. (9), except that the 12-nil graduated centrifuge tube was omitted and the collection reagent was added directly to the 50-ml conical centrifuge tube. The collection reagent was prepared in each tube by the combination of 2.5 ml of distilled water, 2.5 ml of 0.4% aqueous 3-methyl-2-benzothiazolone hydrazone hydrochloride, and 0.5 ml of DMSO. The collection system was assembled and the culture samples, along with a control, were placed in a 65°C water bath and purged with 100-125 ml of nitrogen per minute for 1 hr.

Upon completion of purging, and with the nitrogen still flowing, the apparatus was partially disassembled and the purging tips were rinsed with a few drops of distilled water into the collection tubes. After the collection tubes were removed, the initial reaction was allowed to go to completion by holding at room temperature for 25 min. Twelve and one-half milliliters of 0.2% ferric chloride in 0.1 hydrochloric acid was then added, mixed, and allowed to stand for exactly 25 min, at which time 20 ml of acetone was added and mixed immediately, to stop the oxidation reaction. The samples were then transferred to 50-ml volumetric flasks (rinsing with a small volume of acetone) and brought to volume with acetone. The absorbance at 666 m\(\mu\) was determined within 30 min by reading against a similarly handled reagent blank with a Beckman Model DU spectrophotometer. A standard curve was prepared by adding dilutions of acetaldehyde directly to the collection reagent, followed by the regular procedure for color development.

RESULTS AND DISCUSSION
In the development of the acetaldehyde procedure, it was found that the ferric chloride oxidation time was very critical in obtaining reproducible data. In the original procedure of Sawicki et al. (11), the oxidation time was 5 min after addition of ferric chloride, whereas in the present study absorbance values on duplicate samples were not reproducible until after a 25-min oxidation time (Table 1). Further-

<table>
<thead>
<tr>
<th>Oxidation time(min)</th>
<th>Absorbance at 666 m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.405</td>
</tr>
<tr>
<td>10</td>
<td>0.560</td>
</tr>
<tr>
<td>15</td>
<td>0.624</td>
</tr>
<tr>
<td>25</td>
<td>0.630&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All samples were read against appropriate reagent blank.
<sup>b</sup> Obtained using final modified procedure.

more, termination of the reaction after 25 min by immediate addition of acetone is important. The absorption spectrum for the acetaldehyde-dye complex exhibits a distinct maximum at 666 m\(\mu\) and a shoulder at 635 m\(\mu\). Sawicki et al. (11) reported a molar absorptivity of 51,000 for the acetaldehyde-dye complex, whereas we obtained a value of 69,000 by the procedure described herein. The higher sensitivity probably is due to the longer oxidation time employed in the modified procedure.

Data employed for preparation of a standard curve are presented in Table 2. The acetaldehyde-dye complex obeys Beer's Law over the concentration range employed. Since it is not possible to dilute successfully the final dye complex to obtain absorbance readings (Table 3, Trials 13 and 14), dilution of cultures in which acetaldehyde concentrations exceed 25 ppm are necessary. With experience, the technician can ascertain where dilutions are required simply by tasting or smelling the culture.

The average per cent recovery of added acetaldehyde from eight samples of heated milk prepared by adding dilutions of acetaldehyde directly to the collection reagent, followed by the regular procedure for color development.

4 Aldrich Chemical Company, Milwaukee, Wisconsin.
6 Foamkil; Nutritional Biochemical Corporation, Cleveland, Ohio.
TABLE 2
Absorbance readings at 666 nm for the concentrations of acetaldehyde used for preparation of the standard curve

<table>
<thead>
<tr>
<th>µg Acetaldehyde</th>
<th>Corresponding ppm concentration in 20-g sample</th>
<th>Absorbance at 666 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.027</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>0.158</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
<td>0.308</td>
</tr>
<tr>
<td>15</td>
<td>0.75</td>
<td>0.473</td>
</tr>
<tr>
<td>20</td>
<td>1.00</td>
<td>0.628</td>
</tr>
<tr>
<td>30</td>
<td>1.50</td>
<td>0.933</td>
</tr>
<tr>
<td>50</td>
<td>2.50</td>
<td>1.520</td>
</tr>
</tbody>
</table>

a Absorbance was plotted against micrograms acetaldehyde for standard curve as culture sample size was varied.

medium (acidified to pH 4.5) was 80%. Heating the milk medium for 1 hr in a boiling water bath produced small amounts of acetaldehyde (0.10 to 0.15 ppm). The recovery from distilled water was only slightly greater than that from the acidified milk medium.

The influence of a number of variables on the validity of the test is summarized in Table 3. Nitrogen used as the purging gas, antifoam agent, and phosphoric acid did not contribute to the color reaction. Commercial lactic acid contained large quantities of aldehyde as shown in Trial 4 and thus could not be used for adjusting the pH of control milk samples. Phosphoric acid should be used for this purpose. As indicated in Trials 5 and 6, there appeared to be no advantage in purging the sample longer than 1 hr.

The presence of diacetyl in samples, which is transferred to the collection tube by the purging treatment, results in formation of a water-insoluble hydrazone. The precipitate formed caused difficulties in the quantitative transfer of the reaction mixture from the collection tube to the volumetric flask. Initial efforts to overcome the problem involved addition of 0.5 ml of dioxane to the collection reagent. The dioxane was not satisfactory, however, as evidenced by the gross contamination with acetaldehyde or other reactants (Table 3, Trial 7). The addition of 0.5 ml of dimethyl sulfoxide to the collection reagent prevented formation of the diacetyl derivative precipitate and had no adverse effect on the absorbance values (see Trials 8 and 9). Under these conditions, a

TABLE 3
Effect of various conditions and reagents on the modified 3-methyl-2-benzothiazolone hydrazone aldehyde determination

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Sample</th>
<th>Variable</th>
<th>Absorbance a at 666 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 b</td>
<td>20 g Distilled water</td>
<td>Nitrogen and water</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>20 g Distilled water</td>
<td>Antifoam agent</td>
<td>0.000</td>
</tr>
<tr>
<td>3</td>
<td>20 g of 20% Aqueous phosphoric acid</td>
<td>Phosphoric acid</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>20 g of 20% Aqueous lactic acid</td>
<td>Commercial lactic acid</td>
<td>Too dense to read 0.307</td>
</tr>
<tr>
<td>5</td>
<td>20 g pH 4.5 Milk plus 0.01 mg acetaldehyde</td>
<td>1-hr Nitrogen purging</td>
<td>0.310</td>
</tr>
<tr>
<td>6</td>
<td>20 g pH 4.5 Milk plus 0.01 mg acetaldehyde</td>
<td>1.5-hr Nitrogen purging</td>
<td>0.310</td>
</tr>
<tr>
<td>7 c</td>
<td>0.01 mg Acetaldehyde direct to reagent</td>
<td>Dioxane replacing dimethyl sulfoxide</td>
<td>Too dense to read 0.303</td>
</tr>
<tr>
<td>8</td>
<td>0.01 mg Acetaldehyde direct to reagent</td>
<td>Unmodified reagent</td>
<td>0.308</td>
</tr>
<tr>
<td>9</td>
<td>0.01 mg Acetaldehyde direct to reagent</td>
<td>Modified reagent containing dimethyl sulfoxide</td>
<td>0.308</td>
</tr>
<tr>
<td>10</td>
<td>0.24 mg Diacetyl direct to reagent</td>
<td>High diacetyl content</td>
<td>0.000</td>
</tr>
<tr>
<td>11</td>
<td>200 mg Ethanol direct to reagent</td>
<td>High ethanol content</td>
<td>0.063</td>
</tr>
<tr>
<td>12</td>
<td>200 mg Methanol direct to reagent</td>
<td>High methanol content</td>
<td>0.054</td>
</tr>
<tr>
<td>13</td>
<td>0.02 mg Acetaldehyde direct to reagent</td>
<td>Final dye solution (Trial 13) diluted 1:1 with acetone</td>
<td>0.015</td>
</tr>
<tr>
<td>14</td>
<td>0.02 mg Acetaldehyde direct to reagent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Average of two duplicates read against appropriate reagent blank.
b Trials 1-6 utilized nitrogen purging at 100-125 ml per minute.
c Trials 7-14 were direct additions to the collecting reagent.
large excess of diacetyl did not interfere with
the acetaldehyde determination (Trial 10). Ad-
ditional studies showed that sufficient reagent
was available to react with the equivalent of
10 ppm of acetaldehyde in the presence of 10
ppm of diacetyl. Hence, the excess reagent
should be adequate for most applications.

It has been reported that dimethyl sulfoxide
will mediate the oxidation of certain alcohols
to aldehydes (13). Since earlier work (6) had
demonstrated the presence of both methanol
and ethanol in mixed-strain cultures, 200 mg
of each alcohol was added directly to separate
tubes of collection reagent. Trials 11 and 12
in Table 3 show that the high concentrations of
alcohols gave only small absorbance values, and
aldehyde contaminants in the commercial sol-
vents were probably responsible.

Table 4 shows results of analyses of cultures
for diacetyl and acetaldehyde. The flavor,
aroma, and pH of each culture also are listed.
All single-strain lactic streptoccoci cultures
contained concentrations of acetaldehyde well
above the taste threshold of 0.4 ppm reported
by Harvey (3). The L. citrovorum 91404 cul-
ture contained less acetaldehyde than did the
control milk. S. lactis C,F and S. cremoris SC,
produced negligible amounts of diacetyl, but
the S. lactis var. maltigenes strains showed
production of up to 0.5 ppm of diacetyl. This
is consistent with the observations of Gordon
et al. (2), that many strains of S. lactis var.
maltigenes gave positive Voges-Proskauer
reactions.

As indicated in Table 4, all S. lactis var.
maltigenes cultures gave a malty flavor. Zuraw
and Morgan (15) have reported that malty
strains of S. lactis liberate acetaldehyde, and
later work by Jackson and Morgan (4) implica-
ted 3-methylbutanal as the malty flavor com-
ponent. Subsequent work by MacLeod and
Morgan (8) revealed that these organisms are
capable of producing 3-methylbutanal, 2-meth-
ylbutanal, 2-methylpropanal, 3-methylthioprop-
al, and phenylacetaldehyde. Hence, the data
for the malty cultures in Table 4 undoubtedly
include a partial measurement of some of the
aforementioned aldehydes. Sawicki et al. (11)
reported that the molar absorptivity of the 2-
methylpropanal dye complex determined at its
wavelength maximum of 664 nm was only about
one-third that found for acetaldehyde. Other
branched-chain aldehydes, such as 3-methyl-
butanal, appear to behave similarly. Thus,
their contribution to absorbance of the dye
complex would not be equivalent to acetalde-
hyde.

The value of simultaneous determinations of
acetaldehyde and diacetyl in assessing the flavor
of lactic cultures is demonstrated in Table 4. Mixed Culture A exhibited a harsh diacetyl
flavor and aroma. In this case the diacetyl
content was very high and the relative amount
of acetaldehyde is low when compared to the
results for Mixed Culture B, which gave a good
flavor. Mixed Culture C shows the other ex-
treme in which the relative amount of acetalde-
yde is high and the resulting flavor was criti-
cized as being green.

In view of the established significance of
diacetyl in lactic culture flavors and the re-
cently observed desirable modifying effect of
acetaldehyde on such flavors (7), the 3-methyl-
2-benzothiazoline hydrazone method is well-
suited for examination of cultures. The method
is reasonably fast, in that a number of samples

<table>
<thead>
<tr>
<th>Culture</th>
<th>pH</th>
<th>Diacetyl (ppm)</th>
<th>Acetaldehyde (ppm)</th>
<th>Flavor and Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. lactis C,F</td>
<td>4.40</td>
<td>0.10</td>
<td>2.84</td>
<td>Green</td>
</tr>
<tr>
<td>S. lactis var. maltigenes M1</td>
<td>4.20</td>
<td>0.40</td>
<td>3.24</td>
<td>Malty</td>
</tr>
<tr>
<td>S. lactis var. maltigenes M3</td>
<td>4.40</td>
<td>0.30</td>
<td>3.04</td>
<td>Malty</td>
</tr>
<tr>
<td>S. lactis var. maltigenes M4</td>
<td>4.30</td>
<td>0.45</td>
<td>2.60</td>
<td>Malty</td>
</tr>
<tr>
<td>S. lactis var. maltigenes M6</td>
<td>4.40</td>
<td>0.55</td>
<td>3.00</td>
<td>Malty</td>
</tr>
<tr>
<td>S. cremoris SC1</td>
<td>4.60</td>
<td>0.15</td>
<td>2.34</td>
<td>Green</td>
</tr>
<tr>
<td>S. diacetilactis 18-16</td>
<td>4.90</td>
<td>2.80</td>
<td>2.50</td>
<td>Diacetyl, green</td>
</tr>
<tr>
<td>L. citrovorum 91404 b</td>
<td>4.50</td>
<td>3.24</td>
<td>0.08</td>
<td>Diacetyl, not full</td>
</tr>
<tr>
<td>Mixed Culture A</td>
<td>4.60</td>
<td>5.45</td>
<td>0.41</td>
<td>Harsh, diacetyl</td>
</tr>
<tr>
<td>Mixed Culture B</td>
<td>4.50</td>
<td>1.80</td>
<td>0.46</td>
<td>Good</td>
</tr>
<tr>
<td>Mixed Culture C</td>
<td>4.70</td>
<td>1.00</td>
<td>1.12</td>
<td>Green</td>
</tr>
<tr>
<td>Control heated milk b</td>
<td>4.50</td>
<td>0.00</td>
<td>0.15</td>
<td>Strong cooked</td>
</tr>
</tbody>
</table>

*Volatile aldehydes for all strains expressed as acetaldehyde.

b Incubated at 30 C for 12 hr, then acidified with sterile 20% phosphoric acid to pH 4.5
and incubated an additional 6 hr at 30 C.
DETERMINATION OF ACETALDEHYDE IN CULTURES

can be analyzed at the same time. Adaption of the method for use with the same collection system employed for diacetyl measurements (9) allows simultaneous measurements of culture aliquots for both flavor components. Results obtained were accurate and reproducible when reasonable care was taken in performing the determinations.

For duplicate analyses of 26 different cultures, the average deviation of each duplicate analysis from its mean was ± 0.18 µg of acetaldehyde per sample, with a deviation range of ± 0.00 to ± 0.50 µg per sample.

The method is extremely sensitive for acetaldehyde, with the dye complex giving a molar absorptivity of 69,000 as compared to a molar absorptivity of approximately 20,000 (5) for the corresponding 2,4-dinitrophenylhydrazone. Preliminary results also indicate that it is applicable for analysis of acetaldehyde in sour cream and yogurt. The method is being used routinely in this laboratory for examination of lactic cultures.

REFERENCES


