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A Simple Colorimetric Assay for Muramic Acid and Lactic Acid

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ABSTRACT

The Barker and Summerson method of assaying lactic acid colorimetrically is modified to provide a simple and fast method of measuring lactic acid and other compounds such as muramic acid and glyceraldehyde that will release acetaldehyde on incubation in hot sulfuric acid. The assay can be done with open tubes and no more complicated equipment than a spectrophotometer. A further modification allows a relatively specific determination of formaldehyde.

Index Entries: Muramic acid; lactic acid; glyceraldehyde; acetaldehyde; formaldehyde; colorimetric assay.

INTRODUCTION

The measurement of lactic acid has been used to monitor bacterial spoilage in preserved foods, the preservation of silage, and human medical disorders such as acidosis (*1*) and acidosis in ruminants (*2*). Lactic acid also has been measured during the characterization of grapes for wines (*3*) and the specific fermentation of the acid itself (*4*).

Several methods of measurement have been developed using chemiluminescence (*5*), fluorometry (*6*), HPLC (*3*), GC (*7*), and colorimetry (*1,8*). By far the most common method is the colorimetric assay developed in 1941 by Barker and Summerson (*8*). This method is still being used in the agricultural sciences, especially for large scale screening of silage (*9,10*) and rumen (*4*) samples. In this method, acetaldehyde is released from lactic acid by hot sulfuric acid. The acetaldehyde is reacted with copper and *p*-phenylphenol (pPP) to yield a chromagen which absorbs at 570 nm.

Muramic acid has been used as a bacterial marker, being present in almost all procaryots except archaebacteria in amounts from 3 to 10 μg per mg dry bacteria (*11*). Muramic acid contains a lactic acid moiety, which can be cleaved by alkaline or acid treatment. The lactic acid so derived has been quantitated enzymatically (*12,13*). It is also possible to continue the acid treatment and go from muramic acid to lactic acid, and then to acetaldehyde. Millar and Casida (*14*) investigated bacterial biomass in soil using the Barker and Summerson method as modified by Markus (*15*). This procedure was analyzed by Hadzija (*16*), and his modifications have been used in subsequent studies of muramic acid as a peptidoglycan marker (*17,18*). King and White (*17*) determined that the colorimetric assay was more accurate than the enzymatic assay owing to a lack of specificity in lactic dehydrogenase.

In our laboratory, we use the colorimetric lactic acid assay on a routine basis to characterize the preservation of silage. We are also interested in the estimation of rumen bacteria biomass. A study of the Barker and Summerson assay was undertaken with the aim of making it as simple and sensitive as possible for the estimation of both lactic acid and muramic acid.

MATERIALS AND METHODS

Chemicals Used

p-phenylphenol (pPP) was from Eastman Kodak (Rochester, NY) and from Sigma (Mississauga, Ontario). All other chemicals were from Fisher Scientific (Toronto, Ontario) or from Sigma.

Reagents

1. concentrated H_2SO_4 (96%).
2. 4% $CuSO_4 \cdot 5H_20$ in ddH_20.
3. 1.5% *p*-phenylphenol in 95% ethanol.

Standard Method and Curve

The following is the optimized method for lactic acid determination as developed in this paper. For a standard curve, add 0–30 μg lactic acid to 16 × 150 mm borosilicate tubes. The curve should be in 5-μg increments or less. Bring the volume in the tubes up to 0.5 mL with ddH_2O. Add concentrated H_2SO_4 (3 mL), and mix on a vortex mixer. This quantity of acid is defined here as 82% acid. Incubate at 95–100°C for 10 min (in a steam water bath). Keep extraneous water out of tubes. Cool to room temperature using a water bath. Add $CuSo_4$ reagent (50 μL) and then pPP reagent (100 μL), and mix well on vortex mixer keeping the tube at room temperature. Leave the tubes at room temperature for at least 30 min and then read absorbance at 570 nm. Blanks will show values of 0.2–0.5 compared to water.

Muramic acid must be held at 95–100°C for at least 30 min before cooling and adding copper and pPP reagents.

The optimum conditions for determining formaldehyde are described in the Results section.

EXPERIMENTAL

Acid Strength for Maximum Absorbance

Varying amounts of acid were used in the assay while keeping all other reagents constant. Volume was maintained by addition or removal of water.

Boiling Time for Maximum Absorbance

Using the optimum acid concentration, tubes containing different substrates were held at 95–100°C for various times to determine the maximum absorbance obtained.

Volatility of Acetaldehyde

Using the optimized method, an analysis of the reacting substrates, acetaldehyde (15.76 μg, 358 n*M*), glyceraldehyde (15 μg, 167 n*M*), muramic acid (15 μg, 60 n*M*), formaldehyde (7.4 μg, 246 n*M*), and lactic acid (15 μg, 167 n*M*) was performed. Open and closed tubes were boiled for 10 and 30 min and compared to each other and to tubes that were not subjected to boiling at all.

Stability of Chromagen

Open and closed tubes containing different amounts of lactic acid and acetaldehyde were boiled for 10 and 30 min. The absorbance was measured and the tubes were allowed to sit at room temperature overnight before being read again. After reading, the tubes were boiled again for 15 min and read once more.

Source of Assay Variation

The order of reagent addition and boiling was investigated in order to isolate problems with assay variation.

Absorbance Maxima of the Chromagens

The absorbance curves of the chromagens produced by various substrates in the optimized assay were investigated.

Other Reacting Compounds

Several substances that may be found in samples to be analyzed were used in the optimized assay. The absorbances of these substances alone

and in combination with 5 μg of lactate were recorded. The substrate absorbance was compared with that from an equivalent weight of lactate, and the assays with added lactate were used to determine recovery values.

Comparison to Previous Method

The optimized method was compared to the Hadzija method to determine the effect of interfering substances on each, and the response slopes of the substrates of interest.

Determination of Formaldehyde

The optimum acid concentration to detect formaldehyde was determined by varying the concentration of acid while holding all other reagents, volumes, and procedures constant.

RESULTS AND DISCUSSION

We have, for several years, been using the Barker and Summerson (*8*) method as modified by Hadzija (*16*) to measure lactic acid. The typical assay involved 1 mL of sample in water placed in a screw-capped tube. Five mL of concentrated H_2SO_4 was added and the tubes boiled for 10 min. Upon cooling, 100 μL $CuSo_4$ reagent, and 200 μL pPP reagent were added and the tubes incubated at 30°C until all traces of precipitate were dissolved. This concentration of sulfuric acid is defined as 79% acid in this paper.

Occasionally, we have had problems with the assay in terms of erratic response in the standard curves and an effort to isolate the problem was undertaken.

Acid Strength for Maximum Absorbance

Figure 1 shows the effects of various acid concentrations on the assay. Acid percent refers to the amount of concentrated sulfuric acid in the mixture. For all three substrates (lactic acid, acetaldehyde, and glyceraldehyde) a marked precipitation was seen on addition of pPP at acid concentrations of 77% or less. All of these substrates showed maximum absorbance at 82% acid. If the lactic acid and glyceraldehyde curves are extrapolated, a value of 80.5% acid might give a higher absorbance, but the 82% level was accepted for ease of use and measurement.

The 82% acid mixtures showed immediate, complete, and stable dissolution of the pPP reagent at room temperature, eliminating the need to incubate the tubes at 30°C. If the tubes are cooled below 20°C, some precipitation is seen again but this dissolves immediately on return to room temperature.

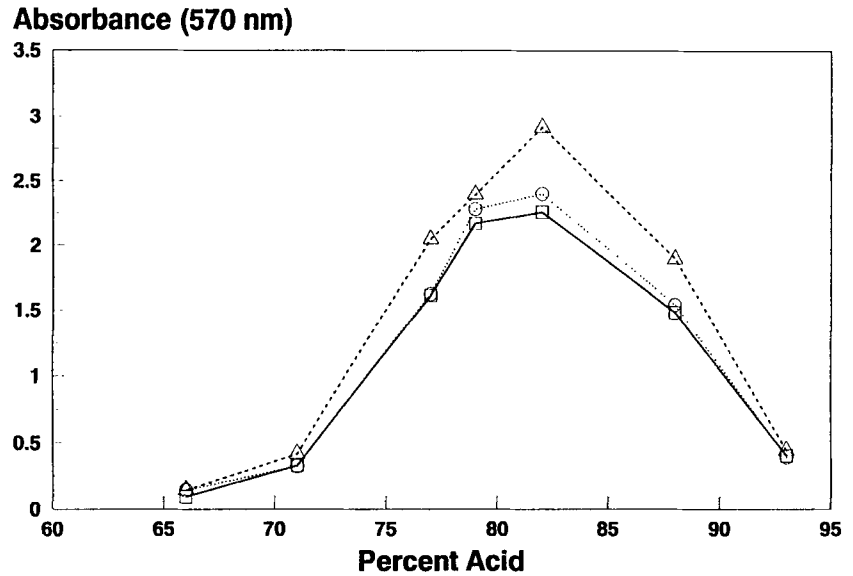

Fig. 1. The effect of acid concentration on absorbance. Tubes were boiled 10 min and cooled; copper and pPP reagents were added and, after 3.5 h at 30°C, tubes with a precipitate were boiled to dissolve it. Tubes were read at A570 nm against water. 167 n*M* (15 μg) of lactic acid, 358 n*M* (15.8 μg) acetaldehyde, and 167 n*M* (15 μg) of glyceraldehyde were used. —□—, lactic acid; ---△---, acetaldehyde; ---○---, glyceraldehyde.

Formaldehyde 493 n*M* (14.8 μg) was also investigated under the same conditions and was found to have a maximum absorbance of 88% acid, with a precipitate appearing at an acid concentration of 79% and total precipitation of chromagen at lesser concentrations.

Boiling Time for Maximum Absorbance

Using an acid concentration of 82% as outlined in the methods section, the boiling times for maximum absorbance were investigated. Acetaldehyde gave maximum absorbance with no boiling time required; formaldehyde, lactate, and glyceraldehyde reached maximums at 10 min and muramic acid reached maximum at 30 min. Time to maximum absorbance was defined as that time where the absorbance reached 90% of the final levels. The boiling times determined here are similar to those determined by Hadzija (*16*).

Acetaldehyde needs no heating to react with the pPP, which would indicate that this is the substance that is being measured in this assay. Lactic acid and glyceraldehyde (both 90.08 mol wt) show maximum absorbances at 10 min heating time, and further heating has no other effects. Muramic acid is a slightly more complex molecule from which lactic acid may be cleaved (*16,*) or from which acetaldehyde may be cleaved directly (*6*) in acid.

Formaldehyde, in 82% acid, benefits from some heating, which probably reflects some insolubility of the chromagen.

Volatility of Acetaldehyde

One possible source of experimental variation mentioned by Hadzija (*16*) is the volatility of acetaldehyde, although Barker and Summerson (*8*) mention that they had no problems when using an open tube to boil the lactic acid.

In this experiment comparing open and closed tubes, no 30°C incubation was needed. Acetaldehyde, boiled in acid for 30 min, showed an absorbance value of 93% that of a closed tube boiled for a similar period and 98% of the value of an open tube that was not boiled. Acetaldehyde, made to a stock solution of about 1 mg/mL in water, showed losses over several months when stored in the refrigerator, but it seems relatively stable when boiled in acid. Samples boiled in acid and then left at room temperature overnight in closed tubes showed losses of over 70% of the absorbance when the copper and pPP reagents were added the next morning. If, however, the chromagen was formed and then left overnight, the absorbance was stable.

Stability of Chromagen

In this experiment, no differences were seen between the open and closed tubes. No loss of absorbance was seen in any of the tubes left to stand overnight, although a color change from blue to purple was noted. When the overnight tubes were boiled for 15 min, the color changed from purple back to blue but the absorbance levels dropped by 50% or more. This absorbance drop after boiling the developed chromagen is of interest since Barker and Summerson (*8*) suggested boiling the samples briefly (90 s) to dissolve the precipitate formed when the copper and pPP reagents are added and to stabilize the color by destroying excess reagent after the 30°C incubation. With the present modification of the method, neither of these problems is encountered and this boiling is not necessary.

Source of Assay Variation

It is this temperature lability of the pPP that has lead to the variations we have encountered with the assay. Even when no boiling step is used, as in an acetaldehyde assay, the heat developed when the ethanol and water of the pPP and copper reagents mixes with the acid is enough to cause some destruction of the pPP. All absorbance can be destroyed in an acetaldehyde assay by adding all the reagents and then boiling for 5 min, before the chromagen has been formed.

Investigations were performed on the order of adding the reagents and the make-up water from the samples. pPP cannot be added before the acetaldehyde sample, it cannot be added with the make-up water (450 μL), nor can the acid be added last. There is also a problem when the copper reagent is added after the pPP reagent. The only acceptable method of mixing is to add the pPP reagent last, with the tubes at room temperature.

As was noted by Barker and Summerson (*8*), some color is generated with pPP alone but the copper reagent is needed for best color development.

With careful temperature control to avoid heating the pPP, the chromagen is blue, turning to purple with time. Rao and Pattabiraman (*19,20*) have suggested that hot sulfuric acid will sulfonate phenol and cause a reduction in the absorbance of the phenol sulfuric acid sugar assay. A similar mechanism may be operating here.

Absorbance Maxima of the Chromagens

Utilizing the currently proposed assay, the absorbance maxima were determined for the reacting substances. Acetaldehyde and glyceraldehyde both showed a maximum absorbance at 569 nm, whereas muramic acid and lactic acid showed a maximum at 570 nm. It is likely that all four of these compounds generate the same chromagen.

Depending on reaction conditions, samples may appear blue or purple, but the maximum absorbance for both colors was the same for acetaldehyde.

In contrast, formaldehyde generates a chromagen with a maximum at 607 nm.

Other Reacting Compounds

Table 1 shows the reactions of various compounds in the modified assay, and the recovery of added lactic acid. Some of the more reactive sugars (rhamnose, arabinose, and fucose) show some absorbance in the assay. Mannosamine, methionine, and tryptamine show some interference in recovery of added lactic acid. Formic acid at high levels will cause a reduction in color formation but seems to have little effect at the quantity tested. Overall the method is quite specific.

Comparison to Previous Method

Table 2 shows the reaction of various aldehydes in the proposed method as compared to the method of Hadzija. Most interfering compounds show less absorbance as the concentration of acid is raised in the assay. When the reacting substances are compared on a molar basis in standard curves using both assays, the new method is at least twice as sensitive as that of Hadzija. Neither method showed identical slopes for lactic acid, acetaldehyde, and muramic acid, even when compared on moles of acetaldehyde evolved. Standard curves for each substrate are recommended.

The current method will quantitate lactic acid, muramic acid, acetaldehyde, and glyceraldehyde from 0 to 150 n*M*. In the case of lactic acid, this is a value of 15 μg or less per assay tube. As mentioned, the assay is about twice as sensitive as the method of Hadzija (*16*). Figenschou and Marais (*1*) proposed a colorimetric assay of lactic acid using oxalyldihydrazide. They compared their method to the original Barker and Summerson method. The method proposed here is an order of magnitude more sensitive than either assay.

Table 1
Reactivity of Other Substances and Recovery of Added Lactic Acid

Sugar	μg	%w/w apparent lactate[a]	%Recovery added lactate[b]
D-Arabinose	20	5.6	120
D-Fructose	20	0	91
L-Fucose	20	2.5	132
	100	4.1	66
D-Galactose	200	0.4	88
D-Glucose	20	0.5	84
	100	0.5	94
D-Mannose	20	0.9	105
L-Rhamnose	20	12	119
	100	9.5	99
D-Ribose	20	0	94
D-Ribulose	100	0	86
L-Sorbose	20	0	102
D-Xylose	10	0	110
D-Xylulose	100	0	120
Amygdalin	20	0	128
D-Cellibiose	20	0	102
Lactose	200	0.3	105
Maltose	200	0.3	98
Melibiose	20	0	119
Rutin	20	0	108
Sucrose	20	1.2	78
Heparin	20	0	122
Inulin			
dahlia	20	0	121
artichoke	20	0	97
Mannan	20	0	106
Pectin	20	0	98
Poly-galacturonate	20	0	98
Raffinose	20	0	103
Stachyose	20	0	93
D-Sorbitol	200	0	87
D-Arabitol	200	0	101
Dulcitol	200	0	90
Mannitol	20	0	85
myoInositol	20	0	104
D-Mannosamine	200	0.8	47
	1000	0.1	18
Galactosamine	200	1.2	83
D-Glucosamine	200	0.7	92

(*continued*)

Table 1 (Continued)

Sugar	mg	%w/w apparent lactate[a]	%Recovery added lactate[b]
D-Galactonic acid	200	0	111
D-Galacturonic acid	20	0.5	93
D-Glucuronic acid	20	0	88
Phytic acid	20	0	127
D-Gluconic acid	200	0	106
D-Saccharic acid	200	0	84
6-Phosphogluconate	20	0	111
Formic acid	50	0	93
Bovine serum albumin	1000	0.4	81
Methionine	1000	0	0
Tryptamine	196	0.2	40
KIO_3	500		44

[a]The percent absorbance this substance gives compared to the absorbance of an equivalent weight of lactate.

[b]5 μg of lactate were added and percent recovery compared to 5 μg lactate alone was reported.

Table 2
Interference by Other Substances in the Assay

	Hadzija method		Modified assay	
Other substances	μg	abs lactate, %[a]	μg	lactate, %
Glutaraldehyde	100	.085 1	200	0.3
Propionaldehyde	81	.943 16	162	1.4
Hexanal	83	.642 11	166	1.0
Octanal	82	.352 6	164	1.9
Benzaldehyde	105	.214 3	210	0.3
2-Heptanone	82	.148 2		
Lactose	100	.144 2	200	0.3

[a]The percent absorbance this substance gives compared to the absorbance of an equivalent weight of lactate.

The fluorometric method of Asabe et al. (*6*) and the chemiluminescence method of Sato and Tanaka (*5*) are both slightly more sensitive than this colorimetric method but both require specialized detectors and offer no more specificity to any of the substrates than the current method.

Asabe et al. (*6*) used sodium nitrite to accelerate the reaction of acetaldehyde with *o*-phenylphenol (not pPP). The same was tried for this assay (using pPP) with the result an almost complete depression of color.

Determination of Formaldehyde

As mentioned earlier, the chromagen created with formaldehyde has a maximum absorbance at 607 nm, and is created without the need for heating. Using this information, the best method of assaying for formaldehyde using this assay system was investigated.

By increasing the acid concentration in the assay beyond 90% (0.2 mL sample and 3.3 mL acid), the color owing to acetaldehyde decreases while the color owing to formaldehyde remains quite high. At acid concentrations of 90 and 90.7% (0.2 mL sample, 3.4 mL acid), the color reactions of acetaldehyde, lactic acid, and muramic acid drop below 10% on a molar basis compared to formadehyde. Glyceraldehyde reacts at about 20% under the same conditions. At 93% acid the standard curve deviates from linearity and thus a concentration of 90–91% is recommended. Formaldehyde reacts in the same way as does acetaldehyde, and the comments on order of reagent addition and need for copper apply here as well.

CONCLUSION

The assay proposed here provides a fast, simple way to quantitate acetaldehyde and those substances that will release acetaldehyde on heating in acid. The chromagen produced is stable, and the standard curves are linear. The method has proven easy to use in trials for the analysis of lactic acid in silage and in rumen contents.

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