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Effect of water content on sensitivity and stability of the *m*-phenylphenol uronic acid assay

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The influence of climate and hence analytical environment on the uronic acid assay of Blumenkrantz and Asboe-Hansen (*Anal. Biochem.*, **54** (1973) 484–9) is described. Instability of diagnostic chromophore is attributed to variations in the water content of the sulfuric acid–tetraborate reagent used. Water, when added to the sulfuric acid–tetraborate reagent to a final concentration of 1–10% (v/v), caused a significant reduction in color intensity developed by the *m*-phenylphenol reagent. The acid–tetraborate reagent absorbed water from the atmosphere under high relative humidity (RH), owing to its high (85% v/v) sulfuric acid contents. Absorption of water by the reagent increased as RH and temperature increased, and color intensity correspondingly decreased. Exposure of the acid–tetraborate reagent to 25°C and 99% RH for 24 h caused a 30% decrease in chromophore intensity. This phenomenon was prevented by decreasing the aqueous sample size in the assay from 200 to 100 μl. To maximize stability of the reaction, the reagent should be stored under N_2 in a tightly sealed bottle. Also, in particularly hot and humid geographic locations, the aqueous sample size should be reduced by one-half.

INTRODUCTION

The assay developed by Blumenkrantz and Asboe-Hansen (1973) using *m*-hydroxydiphenyl for colorimetric determination of uronic acids is specific and sensitive. The method has therefore been widely used in many applications (Doner, 1986). Personal observations in using this assay have revealed that in some geographic locations the method has worked effectively, whereas in others the chromophore has shown instability. In some cases we have observed that the chromophore, which develops after heating at 100°C, loses intensity immediately or after a few minutes at room temperature. This results in inconsistent and inaccurate absorbance readings at 520 nm. The instability of the reaction was greatest in geographic locations with a high relative humidity (RH), such as parts of Florida and Maryland (Washington, DC), and Costa Rica. In contrast, in geographic locations with a generally low RH, such as parts of California, Israel, and Europe, no problems with instability of the method were encountered. To determine the nature of this phenomenon, effect of water content on chromophore development and stability was evaluated.

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MATERIALS AND METHODS

All chemicals were of reagent grade and were obtained commercially from the following sources: galacturonic acid, sodium hydroxide, and tetraborate were from Sigma Chemical Company (St Louis, MO, USA); *m*-phenylphenol was from Eastman Kodak Company (Rochester, NY, USA); and concentrated sulfuric acid was from Merck & Company, (Rahway, NJ, USA). Water was distilled and deionized (ddH_2O). The procedure for quantitative determination of uronic acid followed the method of Blumenkrantz and Asboe-Hansen (1973), with minor modifications as follows. The *m*-phenylphenol reagent, which contained 0·15% (w/v) *m*-phenylphenol and 0·5% (w/v) NaOH, was prepared fresh every 2–3 days and stored in the dark at 2°C. Tetraborate (12·5 mM) was dissolved overnight in concentrated sulfuric acid under an N_2 atmosphere in a closed bottle at room temperature. The reaction mixture contained sulfuric acid–tetraborate, (1·2 ml) aqueous sample (200 μl) and *m*-phenylphenol reagent. (20 μl).

To examine the effect of ddH_2O content on the reaction, various amounts of ddH_2O were added to the acid–tetraborate reagent to produce final concentrations 1–10% (v/v). Then, ddH_2O (200 μl) containing uronic acid (10 μg) was added. After the addition of *m*-phenylphenol, (20 μl) the absorbance at 520 nm was determined. Three replicates of the sample and blank (0·5% NaOH only) were used in all experiments.

To study the effect of temperature and RH on chromophore intensity, tubes containing acid–tetraborate reagent (1·2 ml) were incubated without stirring for 2–24 h at the following temperature–RH regimes: 25°C–67%, 25°C–99% and 30°C–99%. After incubation, the sample (200 μl) containing uronic acid (100 μg) was added. The chromophore was developed by adding *m*-phenylphenol (20 μl) and incubating at 100°C for 5 min. Changes in absorption of water by the sulfuric acid were determined by placing acid–tetraborate (10 g) in a 100-ml beaker and exposing it to the temperature–RH regimes described above. Increases in the weight of the acid mixture as a result of water absorption were then followed.

The effect of reducing the amount of ddH_2O (aqueous sample size) was studied by using a constant amount of acid–tetraborate and varying the amount of ddH_2O. Each tube received acid–tetraborate (1·2 ml) and ddH_2O (100–200 μl) containing uronic acid (10 μg). Chromophore intensity (absorbance at 520 nm) was determined at zero time and after incubation for 24 h at 25°C and 99% RH.

RESULTS AND DISCUSSION

Increasing the amount of ddH_2O in the acid–tetraborate reagent from 1 to 10% (v/v) caused a corresponding decrease in absorbance at 520 nm (Fig. 1). Color intensity decreased by almost 40% when using acid–tetraborate containing 10% ddH_2O. Incubation of acid–tetraborate at 25°C and 67% or 99% RH, or at 30°C and 99% RH for various times showed that the amount of water absorbed by the sulfuric acid increased as both RH and temperature increased (Fig. 2). Incubation of acid–tetraborate at 25°C for 24 h at 67% and 99% RH caused absorption of 5% and 12% water, respectively. Raising the temperature from 25°C to 30°C resulted in water absorption of about 18%.

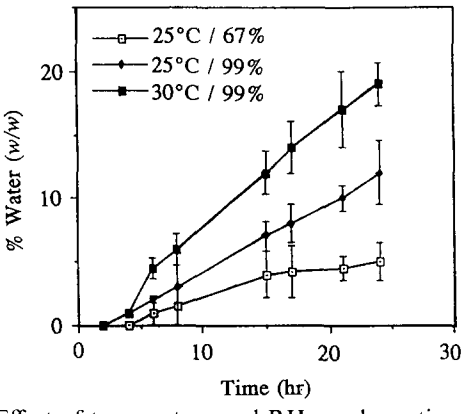

Fig. 2. Effect of temperature and RH on absorption of water by acid–tetraborate.

The effect of RH and temperature on chromophore intensity is shown in Fig. 3. Exposure of acid–tetraborate to 25°C and 67% RH caused an 18% reduction in chromophore intensity after a 24-h incubation. Exposure to 25°C and 99% RH for 24 h caused a reduction of almost 35%, and elevation of the temperature to 30°C at 99% RH increased the loss of chromophore intensity to over 45%.

From these results it is clear that both temperature and RH contribute to decreased color intensity. The primary cause of this phenomenon is the absorbance of water by concentrated sulfuric acid; excess water apparently interferes with formation of and/or leads to instability of the uronic acid–*m*-phenylphenol complex. To alleviate this problem, an experiment was set up in which the amount of ddH_2O in the reaction mixture was reduced from 200 to 100 μl (Fig. 4). When fresh sulfuric acid was used, no change in absorbance at 520 nm was observed as the amount of ddH_2O in the reaction was reduced. Addition of the same amount of ddH_2O to concentrated acid which had been exposed for 24 h at 25°C to 99% RH showed that as the amount of ddH_2O in the reaction mixture decreased, chromophore intensity increased.

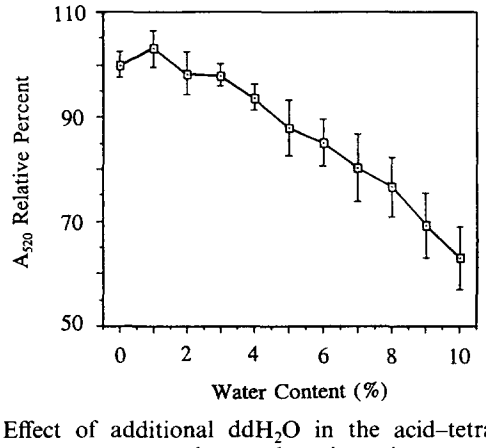

Fig 1. Effect of additional ddH_2O in the acid–tetraborate reagent on chromophore intensity.

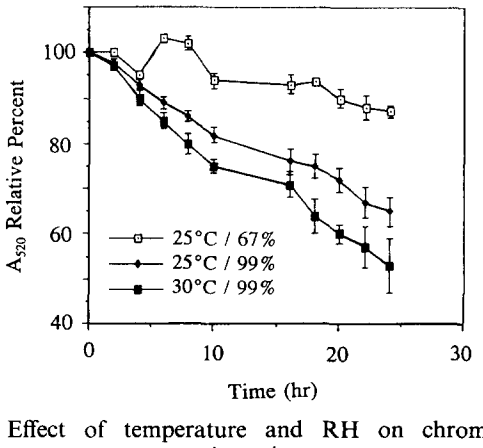

Fig 3. Effect of temperature and RH on chromophore intensity.

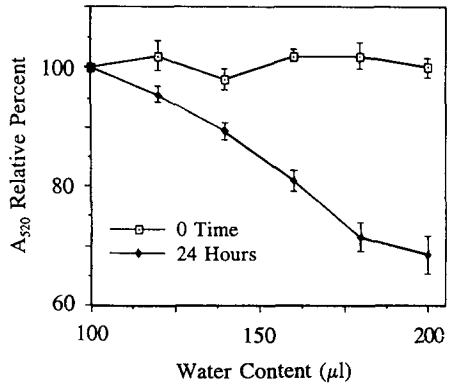

Fig 4. Effect of reducing aqueous sample size by one-half on chromophore intensity.

These results indicate that the *m*-phenylphenol assay for uronic acids is sensitive to excessive water content. For this reason, particular care is necessary in handling the sulfuric acid and acid–tetraborate reagent. From our observations, it appears that sulfuric acid can absorb sufficient water from the atmosphere during the overnight dissolving of tetraborate in the concentrated acid to interfere with the accuracy and sensitivity of the assay. The tetraborate should be dissolved in sulfuric acid only in a closed bottle and under N_2. By keeping the reagent bottle tightly closed, samples have been obtained which are stable for over 3 months. One of the countries in which many problems with color instability have been experienced is Costa Rica. In this tropical country, with high RH and average temperature around 30°C, only by reducing sample water content by 50% could the chromophore be stabilized.

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