A SIMPLIFIED PHOTOMETRIC METHOD FOR THE DETERMINATION OF CITRIC ACID IN BIOLOGICAL FLUIDS

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Studies on the oxidation and bromination of citric acid to pentabromoacetone were reported as early as 1847 by Cahours (1). This reaction has since formed the basis for many of the procedures for determining citric acid. A qualitative test by Stahre (2), reported in 1897, was adapted for quantitative analysis by Kunz (3). Pucher, Sherman, and Vickery (4) described the first microphotometric method, using the reaction of pentabromoacetone with sodium sulfide for color development. Their procedure, measuring 0.1 and 1.0 mg. of citric acid, was later discarded in favor of a titrimetric method (5). More recent photometric modifications include those of Taussky and Shorr (6), color development with sodium iodide, Wolcott and Boyer (7), potassium iodide, and Natelson, Pincus, and Lugovoy (8), thiourea. Attempts to apply these methods to the measurement of micro quantities of citric acid have prompted search for a technique having greater sensitivity and simplicity.

This report describes a simplification of the procedure for the oxidation and bromination of citric acid to pentabromoacetone. The reaction of trihalogenated hydrocarbons with alkaline pyridine, described by Fujiwara (9), has been adapted to the measurement of the pentabromoacetone. The resulting photometric procedure measures amounts of citric acid in the range of 2 to 40 μg and is accurate to ±5 per cent. The method may be used on a variety of biological materials and requires only common laboratory equipment.

Reagents--

1. Sulfuric acid, 9 N. A dilution of concentrated sulfuric acid 1:4 with water.
2. Metaphosphoric acid, 40 per cent. 80.0 gm. in 200 ml. of solution.
3. Potassium bromide, 2 M. 47.6 gm. in 200 ml. of solution.
4. Potassium permanganate, saturated solution. 13 gm. in 200 ml. of solution.
5. Hydrogen peroxide, 6 per cent, commercial grade.
6. Heptane, practical grade.
DETERMINATION OF CITRIC ACID

7. Potassium hydroxide, 30 per cent. 150 gm. in 500 ml. of solution.

8. Pyridine, analytical grade. This reagent must be redistilled if it does not remain colorless when heated to 80° with strong alkali.

9. Citric acid, stock standard. 100 mg. of anhydrous citric acid, analytical grade, in 100 ml. of 1 N sulfuric acid. A working standard solution is prepared daily by diluting the stock standard 1:200 with water.

Procedure

Filtrates of heparinized whole blood, serum, plasma, or tissue homogenate are prepared by standard tungstic acid technique. Urine is used in an untreated state except when excessive amounts of protein are present; the latter may be removed by acidification and gentle boiling, followed by filtration. The solutions to be tested are adjusted by dilution with water to contain 0.5 to 10.0 γ of citric acid per ml. 4.00 ml. of the working standard solution of citric acid are analyzed concurrently with the test solutions. This is equivalent to a blood level of 50 γ per ml. if a 1:10 dilution of blood is made during deproteinization.

4.00 ml. of filtrate are mixed with 1.00 ml. of sulfuric acid and 0.25 ml. of metaphosphoric acid in a glass-stoppered tube of 15 to 20 ml. capacity. The tube is cooled in an ice water bath to a temperature of 15° or lower. The entire procedure is conducted at this temperature, with the exception of heating for color development. 0.5 ml. of potassium bromide and 1.5 ml. of potassium permanganate are added and stirred. The mixture is allowed to stand for 10 minutes, then decolorized by the dropwise addition of hydrogen peroxide with stirring. Care must be taken to avoid adding an excess of peroxide. 6.00 ml. of heptane are added and the tube is stoppered and shaken for 1 minute.

When separation of the layers is complete, a 5.00 ml. aliquot of the heptane is removed and added to an Evelyn macro cuvette containing 2.00 ml. of potassium hydroxide and 4.00 ml. of pyridine. The cuvette is stoppered with a lead foil-covered cork and agitated vigorously for 30 seconds, avoiding contact of the reaction mixture with the foil-covered stopper. The cuvette is placed in a water bath of 80° for 4 minutes, during which time a red color develops in the pyridine layer. The cuvette is again agitated for a few seconds, then quickly cooled in an ice water bath. The chilled mixture is centrifuged at 2000 r.p.m. for 10 minutes to achieve complete separation of the potassium hydroxide, pyridine, and heptane layers. The color-containing pyridine phase must be free of turbidity. Transmittancy is measured in an Evelyn photometer with a 520 mp filter and a reagent blank at 100 per cent. The "6 ml." slit is used to place the

1 It is advisable to use a burette with a Teflon stop-cock for measuring pyridine.
red-colored pyridine layer in the light path. Approximately 1 hour is required for the determination of citric acid by this procedure.

Results

The spectral absorption curve of the color produced by the reaction of pentabromoacetone with alkaline pyridine is illustrated in Fig. 1. These determinations were made on a Beckman model DU spectrophotometer at 25° with a 1 cm. light path. Maximal absorption occurred at 530 μm. The color slowly increases in intensity over a period of 24 hours if the reaction mixture is allowed to stand at room temperature. Within 16 hours, however, the transmittancy at any given time is directly proportional to the concentration, as illustrated in Fig. 2.

**Fig. 1.** Spectral absorption curve of the reaction product of pentabromoacetone and alkaline pyridine. The pentabromoacetone concentration is equivalent to 5 μg of citric acid per ml. of pyridine.

**Fig. 2.** Relation of absorbancy to concentration of citric acid immediately after completion of the determination and after standing for 16 hours at 25°.
Examples of recovery studies performed in duplicate with citric acid added to whole blood are given in Table I. These and similar data show that good recovery of added citric acid was achieved. As a corollary, pentabromoacetone was prepared in pure form and used as a standard against which known amounts of citric acid were measured. It was found that 92.8 to 98.2 per cent of the citric acid was converted to pentabromoacetone by the described method.

Replicability of the method was demonstrated by performing twenty determinations on a single sample of citric acid. The mean value obtained was 20.0 γ per ml.; the individual values ranged from 18.9 to 20.9 γ per ml.

Twenty-four determinations were done in duplicate on whole blood from twenty normal persons in the fasting state. The mean value was 15.2 γ per ml., with a range of 8.1 to 19.7 γ per ml.

TABLE I
Recovery of Citric Acid Added to Whole Blood

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Added γ</th>
<th>Found γ</th>
<th>Recovered per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.00</td>
<td>10.54</td>
<td>102.7</td>
</tr>
<tr>
<td>2</td>
<td>4.00</td>
<td>10.46</td>
<td>101.6</td>
</tr>
<tr>
<td>3</td>
<td>8.00</td>
<td>11.47</td>
<td>101.1</td>
</tr>
<tr>
<td>4</td>
<td>8.00</td>
<td>10.88</td>
<td>101.1</td>
</tr>
</tbody>
</table>

The following compounds, at concentrations equivalent to blood levels of 500 γ per ml., produced no detectable pentabromoacetone with this method: isocitrate, aconitate, α-ketoglutarate, oxalosuccinate, succinate, fumarate, malate, malonate, oxalacetate, acetate, pyruvate, lactate, glucuronate, glutamate, aspartate, glycine, cystine, methionine, leucine, alanine, heparin, acetone, ethanol, ethyl ether, acetoacetate, and β-hydroxybutyrate. At concentrations equivalent to blood levels greater than 2000 γ per ml., a small but measurable amount of color is produced by acetoacetate and β-hydroxybutyrate. Glucose and fructose, at concentrations equivalent to blood levels of 5000 γ per ml., produced no color. Trihalogenated compounds, such as chloroform, bromoform, chloral hydrate, and trichloroacetic acid, produced positive reactions.

The sensitivity of the pyridine reaction was compared with that of other methods in common use by determination of molar absorbancy indices (10). The final color development of each method was performed on pure pentabromoacetone in strict adherence to the instructions of the respective
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authors. The volumes of the reaction mixtures were the same in all cases. All determinations were performed at the recommended wave-lengths on a Beckman model DU spectrophotometer at 25° with a 1 cm. light path. The results, presented in Table II, show that the pyridine reaction is the most sensitive in the visible light range. If determined in the ultraviolet range as recommended, the sodium iodide method of Taussky and Shorr (6) is most sensitive; at 400 mμ, however, it becomes less sensitive than the pyridine reaction.

<table>
<thead>
<tr>
<th>Method</th>
<th>Wave-length</th>
<th>Molar absorbancy index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>530</td>
<td>15,900</td>
</tr>
<tr>
<td>Sodium sulfide</td>
<td>445</td>
<td>2,500</td>
</tr>
<tr>
<td>Thiourea</td>
<td>450</td>
<td>2,500</td>
</tr>
<tr>
<td>Sodium iodide</td>
<td>400</td>
<td>10,700</td>
</tr>
<tr>
<td>&quot;</td>
<td>360</td>
<td>28,500</td>
</tr>
</tbody>
</table>

DISCUSSION

Hargreaves, Abrahams, and Vickery (11) have contributed much to the development of optimal conditions for the oxidation-bromination of citric acid. They found that metaphosphoric acid prevented the precipitation of manganese dioxide, thus freeing the reaction from many previously rigid controls.

In the method described, initial boiling of the filtrate with sulfuric acid has been eliminated, since it is not necessary for either the oxidation of citric acid or the removal of interfering substances. The amounts of bromide and permanganate in the reaction mixture are critical. It was found that amounts smaller than those recommended decreased the yield of pentabromoacetone when large amounts of citric acid are present. Greater concentrations of bromide and permanganate produce free bromine which reacts with alkaline pyridine.

Potassium hydroxide, in the concentration and volume recommended, was found to separate from pyridine most easily. Heating the final reaction mixture between 3 and 5 minutes at 80° produced maximal color intensity, while continued heating produced a persistent decrease in intensity. Rapid chilling in ice water prevents deterioration of the color and greatly facilitates separation of the phases during centrifugation.

Variations in this procedure may be made to conform to the requirements of the test material or the equipment available. A large volume of test
solution having low citric acid concentration may be reduced by boiling in an oil bath at less than 120°. The absolute amounts of potassium hydroxide and pyridine may be varied to place the pyridine layer in the light path of various types of photometers. Micro cuvettes may be used for an aliquot of the pyridine pipetted out of the 3-phase system after centrifugation. The use of micro cuvettes having extended light paths increases the apparent sensitivity of the reaction.

**SUMMARY**

A photometric method for the determination of citric acid as pentabromoacetone has been presented. A simplified oxidation-bromination procedure has been introduced and the reaction of pentabromoacetone with alkaline pyridine has been developed for color production. The method is suitable for the measurement of 2 to 40 μ of citric acid and possesses a high degree of specificity and accuracy.

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**BIBLIOGRAPHY**

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