A Modified Method for the Microdetermination of Citric Acid

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An investigation of the different steps in the method of Weil-Malherbe & Bone (1949), as modified by Taylor (1953), has led to an altered procedure for citric acid determination, with increased sensitivity and accuracy. The main modifications involved are the use of (i) a higher concentration of sulphuric acid during the formation of pentabromoacetone, and (ii) a thiourea-borax-sodium sulphide solution in place of 2% sodium sulphide.

METHOD

Reagents

Sulphuric acid. 33N- H_2SO_4 (A.R.).

Bromide-bromate-vanadate solution. This contains 19.836 g. KBr, 5.44 g. KBrO_3 and 12.00 g. NaVO_3 in 1 l.

Ferrous sulphate solution. 22% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (A.R.) in $\text{N-H}_2\text{SO}_4$. It is important that this solution is freshly made up every 4-5 days or complete removal of the free bromine may not take place. Taylor (1953) has found storage under hydrogen satisfactory.

Thiourea-borax-sulphide solution. This solution should be made up freshly each day but should not be used until 30 min. has elapsed after the addition of Na_2S . Thiourea (4 g.) is dissolved without heating in 100 ml. 2% solution of sodium borate (A.R.) and 2 ml. of a freshly made up 10% (w/v) solution of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (A.R.) are added. The Na_2S crystals if at all moist are liable to become contaminated with sodium thiosulphate, giving low and irregular results. Washing and drying has usually sufficed to remove the impurities but on one occasion recrystallization was required.

Sodium sulphate (A.R.). Anhydrous.

Light petroleum, b.p. 80-100°. A.R. aromatic-free grade was found satisfactory.

Citric acid standard. 1.0935 g. of the crystalline acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) (A.R.)/l. in $\text{N-H}_2\text{SO}_4$. This solution contains 1 mg. anhydrous citric acid/ml.

Preparation for analysis. Proteins, if present, are removed by precipitation with 10% (w/v) trichloroacetic acid (TCA). Blood, plasma and cerebrospinal fluid are added in the proportion of 1 ml. to 6-5 ml. TCA, but estimations on cerebrospinal fluid can be carried out satisfactorily on 0.5 ml. added to 6 ml. TCA. Urine is diluted in the proportion of 1 ml. to 40 ml. of TCA if the specific gravity is between 1.01 and 1.02; if the specific gravity is above 1.02 it is diluted 1 to 60 ml., or if it is below this value, diluted 1 to 20 ml. Salicylic acid and certain other aromatic substances may interfere (Weil-Malherbe & Bone, 1949). If their presence is suspected, preliminary bromination should be carried out and the bromination product removed by extraction with light petroleum.

Procedure

5 ml. 33N- H_2SO_4 are run from a burette into 5 ml. of the solution to be analysed (preferably containing not more than 80 μg . citric acid) contained in a test tube (150 $\times$ 24 mm.) fitted with a ground glass stopper, and the contents mixed thoroughly. After 10 min., 5 ml. of the bromide-bromate-vanadate solution are added from a burette and the tube placed in water at 30° for 20 min. It is convenient to make up the FeSO_4 and the thiourea-borax-sulphide solutions at this stage. The tube is removed from the bath, 3 ml. FeSO_4 added, the contents mixed and the tube left for at least 5 min. The tube is then inverted to remove the last traces of bromine from the air space, and the stopper and neck of the tube are wiped with filter paper to remove any solution that might have been trapped and not have come in contact with FeSO_4 . Light petroleum (6.5 ml.) is added, the tube stoppered and well shaken for 2-2.5 min. The lower aqueous layer is sucked off as completely as possible with a Pasteur pipette attached to a water pump. About 15 ml. of distilled water are then added, washing down the sides of the tube, and the tube shaken for 15-30 sec. The aqueous layer is sucked off as before, the last drops of water being removed if necessary with a Pasteur pipette. This step, suggested by Dr El Hawary, ensures the complete removal of any residual acid. A small amount of anhydrous Na_2SO_4 is added to remove the last traces of water, and 5 ml. of the washed light petroleum extract pipetted into another test tube. 4 ml. of thiourea-borax-sulphide solution are added and mixed by bubbling a steady stream of oxygen, air or N_2 through the solutions for 1-5 min. If the tube is shaken by hand, an occasional slight cloudiness occurs which has never been noticed when a stream of oxygen is used. The yellow aqueous layer is pipetted into a cuvette having a 2 cm. light path and the absorption read in a photoelectric absorptiometer using an Ilford 'Spectrum Violet' filter, no. 601, with maximum transmission at about 440 $\text{m}\mu$. In a spectrophotometer the colour is read at 435 $\text{m}\mu$. If glucose or acetoacetic acid are present in high concentration, it is better to determine the intensity of the colour using an Ilford 'Spectrum Green' filter, no. 603 (maximum transmission at about 500 $\text{m}\mu$).

Effect of sulphuric acid concentration. The effects of varying the concentration of H_2SO_4 in the mixture before the addition of the bromide-bromate-vanadate are shown in Fig. 1. Greatest sensitivity is achieved by increasing the concentration to 16.5N. Above 17N the sensitivity declines. In the methods of Weil-Malherbe & Bone (1949) and Taylor (1953) the concentration of sulphuric acid at this stage is 13.5N, but 16.5N is clearly preferable.

Colour development and stability. Sodium sulphide (2%) has been commonly employed for the extraction of the pentabromoacetone from the light petroleum, but Natelson,

Lugovoy & Pincus (1948) discarded this in favour of a 4% thiourea-2% borax solution, which they thought gave increased sensitivity. Weil-Malherbe & Bone (1949), on the other hand, preferred to use 2% Na_2S . The complex interaction of pentabromoacetone with thiourea and sodium sulphide was re-examined. Pentabromoacetone extracted by a 2% soln. of Na_2S gives a lemon-yellow colour with a peak absorption in the Beckman spectrophotometer at 445-450 $\text{m}\mu$. With a 4% thiourea-2% borax solution (pH 9.2) a yellow colour of much the same intensity is obtained but with a maximum absorption at 440 $\text{m}\mu$.

Fig. 1. Effect of varying the concentration of sulphuric acid in the mixture before addition of the bromide-bromate-vanadate. Results of two experiments are given, and the values are expressed as percentages of the highest reading found in that experiment. For normalities higher than 18N, part of the H_2SO_4 was included in the citric acid sample.

Thiourea in neutral solution, after prolonged shaking, gives a colour which is more pink, with maximum absorption at 510 $\text{m}\mu$. Weil-Malherbe & Bone (1949) found that if the neutral thiourea solution contained 0.06% of Na_2S the pink colour developed very much more rapidly. Na_2S (0.2%), in a 4% thiourea-2% borax solution shaken with pentabromoacetone, gives an orange-yellow colour with maximum absorption at 435 $\text{m}\mu$, having a 20-30% higher extinction (using an Ilford violet filter, no. 601) than the colours given either by Na_2S or thiourea-borax. 0.06% Na_2S in thiourea-borax gives the same colour but a slightly lower extinction. The absorption spectrum of the pentabromoacetone complex in thiourea-borax-sulphide is similar to that described by Natelson *et al.* (1948) for thiourea-borax but differs (a) in that the peak transmission is at 435 $\text{m}\mu$, as opposed to 440 $\text{m}\mu$, (b) instead of the absorption decreasing progressively to reach negligible proportions about 560 $\text{m}\mu$, it falls off rather more steeply at first and then develops a secondary peak between 460 and 500 $\text{m}\mu$, thereafter declining progressively.

The absorption of the colour developed in 4% thiourea-2% borax-0.2% sodium sulphide has been found stable

over a period of 3 hr. There is a very slight fading whilst the absorption is being measured in a photoelectric absorptiometer of a type which concentrates the light beam in a sharp focus on the solution. The colour intensity returns within 2 min. following removal from the light path. This fading has not been sufficient to give rise to any difficulty, in contrast to the pentabromoacetone complex in 2% Na_2S . The thiourea-borax-sulphide reagent differs also from 2% Na_2S in being unaffected by temperature over the range 15-30°.

Specificity. As in the method of Weil-Malherbe & Bone (1949), acetoacetic acid when present in high concentration gives rise to some interference. Using the Ilford violet filter no. 601, 1 mg./ml. of acetoacetic acid gives the same extinction as 30 $\mu\text{g.}/\text{ml.}$ of citric acid. The absorption spectrum of the final complex of acetoacetic acid in thiourea-borax-sulphide has its maximum absorption in the visible range at 435 $\text{m}\mu$, but has negligible absorption at 500 $\text{m}\mu$. Citric acid can be determined therefore in the presence of large quantities of acetoacetic acid, though at some sacrifice in sensitivity, by using an Ilford 'Spectrum Green' filter, no. 603 (maximum transmission about 500 $\text{m}\mu$). With this filter, 1 mg./ml. of acetoacetic acid gives the same extinction as 0.65 $\mu\text{g.}/\text{ml.}$ of citric acid.

Glucose, when present in very high concentration, was found (using the violet filter having a maximum transmission about 440 $\text{m}\mu$) to interfere slightly, a solution containing 8 mg./ml. giving the same extinction as 0.6 $\mu\text{g.}/\text{ml.}$ of citric acid. With the green filter (maximum transmission about 500 $\text{m}\mu$) the interference was negligible. The presence of salicylic acid in concentrations of 60 $\mu\text{g.}/\text{ml.}$ or above was found to give rise to sufficient interference to warrant preliminary bromination for its removal. Lower concentrations were not tested.

Accuracy. The calibration curve with 5-800 $\mu\text{g.}$ of citric acid is linear, although determination of the larger amounts of pentabromoacetone required appropriate dilution with light petroleum. The straight line does not always cut the origin and, as the slope may vary slightly from batch to batch, two or more standards should be included in each batch of determinations. The accuracy achieved in a consecutive series of thirty analyses of citric acid solutions is given in Table 1. The recovery of known quantities of

Table 1. Analysis of pure citric acid solutions

No. of determinations	Citric acid ($\mu\text{g.}$)		Coefficient of variation (%)
	Added	Found	
30	10	8.7-10.9	± 3.9
30	40	38.7-41.3	± 1.65
30	80	77.0-82.0	± 1.66

Table 2. Recoveries of pure citric acid added to 4 ml. of deproteinized serum filtrate containing either 16.0 or 18.5 $\mu\text{g.}$ citric acid

No. of determinations	Citric acid added ($\mu\text{g.}$)	Recovery (%)	
		Mean	Range
3	10	98.3	93-101.0
3	20	99.8	99-100.5
3	30	99.1	98.7-99.5
3	40	99.9	97.5-101.3

citric acid added to deproteinized serum filtrate has been studied, and the results of a series of three consecutive determinations are given in Table 2.

SUMMARY

A modified colorimetric procedure is described for the estimation of 10–80 μ g. of citrate in biological fluids. The final colour is developed in a 4% thiourea–2% borax solution containing 0.2% sodium sulphide.

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Studies in Carotenogenesis

15. THE ROLE OF CARBOXYLIC ACIDS IN THE BIOSYNTHESIS OF β -CAROTENE BY *PHYCOMYCES BLAKESLEEANUS**

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Schopfer & Grob (1950) found that a medium containing lactate as the sole carbon source supported only meagre growth of *Phycomyces blakesleeanus* which synthesized no β -carotene. Addition of acetate to this medium stimulated growth and initiated carotenogenesis; pyruvate, butyrate, palmitate, tartrate and citrate behaved similarly to acetate whilst glycerol, acetone and an unspecified ester of β -methylcrotonic acid were ineffective. Later Schopfer & Grob (1952) demonstrated that lactate was not necessary and that growth and carotenogenesis occurred when acetate was the only carbon source.

Grob, Poretti, von Muralt & Schopfer (1951) and Grob, Schopfer & Poretti (1952), using labelled acetate, found that the methyl and carboxyl carbon atoms were incorporated equally into the β -carotene molecule and, recently, Grob & Bütler (1954*a, b*) found that the lateral methyls of β -carotene arise from the methyl of acetate and the carbon atoms adjoining these methyl groups from the carboxyl of acetate. Glover, Goodwin & Lijinsky (1951), using methyl-labelled acetate, could not demonstrate specific incorporation of acetate into β -carotene either in growing cells of *P. blakesleeanus* in the presence of glucose or in vegetative mats placed on a medium containing only acetate and phosphate buffer.

Because of the importance of acetate as the possible primary precursor of β -carotene, further investigations into the role of this and other carboxylic acids in carotenogenesis in *P. blakes-*

leeanus have been undertaken. Some of the results presented here have already been briefly recorded (Friend, Goodwin & Griffiths, 1954).

EXPERIMENTAL

Cultures. *Phycomyces blakesleeanus* (–strain), obtained from the Centraalbureau voor Schimmelcultures, Baarn, was used throughout. Cultures were grown either in 15 ml. of medium in 8 oz. medicine bottles (Garton, Goodwin & Lijinsky, 1951) or in 100 ml. of medium in penicillin flasks. The latter were used with media not containing glucose, because of the smaller growth obtained. Incubation was carried out at 25° in a glass incubator (Garton *et al.* 1951).

The glucose media were those described by Garton *et al.* (1951). Five non-glucose media were used: (A) containing per 100 ml.: ammonium lactate 0.2 g.; magnesium lactate, 3H₂O, 0.2 g.; KH₂PO₄, 0.15 g.; MgSO₄.7H₂O, 0.05 g.; MnSO₄, 0.01 g. and thiamine 2 μ g. (Grob *et al.* 1951); (B) medium A + 1.0% (w/v) sodium acetate anhyd.; (C) medium A with ammonium lactate and magnesium lactate replaced with equivalent (carbon) amounts of the corresponding acetates; (D) medium B with ammonium lactate replaced by 0.12% (w/v) NH₄NO₃ and (E) medium A + 1% (w/v) glucose.

Dry weight, lipid and carotenoid determinations. With mycelia grown on glucose media, these determinations were carried out by the methods of Garton *et al.* (1951). On non-glucose media the pH increases during growth of the mycelium from 6.5–7.0 to about 8.5. This results in a considerable amount of precipitated inorganic phosphate accompanying the mycelium when it is filtered from the medium. In order to free the mycelia from this precipitate they are washed with about 5–10 ml. of 2N-HCl. Table 1 shows that washing reduces the dry weight very considerably but this is not surprising from visual examination of the crucibles in which can be seen large crystals. Washing

* Part 14: Goodwin, Land & Osman (1955).