

[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIO-CHEMISTRY OF THE UNIVERSITY OF MINNESOTA.]

MODIFICATION OF THE PRATT METHOD FOR THE DETERMINATION OF CITRIC ACID.

By J. J. WILLAMAN.

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In working with Pratt's method¹ for the determination of citric acid, considerable difficulty was encountered in getting duplicate determinations to agree. This difficulty was mentioned in the 1912 report of the Association of Official Agricultural Chemists,² but no suggestions were made for remedying it. It is believed that the modifications described herein will give very satisfactory results.

Discussion of Process.

The primary operations in this method are the fractional oxidation of the citric acid to acetone by means of potassium permanganate, the removal of the acetone as fast as formed by distillation, the formation of an insoluble mercury-acetone complex by boiling with mercuric sulfate, and the weighing of this precipitate. A thorough study has been made of each step of the process, in the attempt to discover all the influencing factors and the combination of procedures that would give the most satisfactory results. A discussion of the various investigations follows:

1. Fractional Oxidation.—The process is purely empirical, the results varying with each change of conditions. The potassium permanganate attacks any organic substance that may be present along with the citric acid, distributing itself among them according to the ease with which each is oxidized. Among the substances likely to be present in working with plant products are other acids brought down by the barium acetate, and sugars and coloring matter occluded by the barium precipitate. These cannot be washed out completely, hence are always present in greater or less amount and capable of consuming permanganate. Acetone itself, the desired end-product of the oxidation of the citric acid, can of course be further oxidized; hence the necessity of removing it as fast as formed. It was found that the more dilute the solution of citric acid is, the less the acetone obtained. This is undoubtedly due to the fact that if only a small amount of citric acid is present at the point of contact between the drop of permanganate and the boiling liquid, the oxidation will proceed beyond the acetone stage before the acetone can escape and before the drop of permanganate is consumed. Several factors, then, would tend to give larger yields of acetone; *viz.*, (1) more

¹ David S. Pratt, "Determination of Citric Acid," U. S. D. A., Bur. of Chem., *Circ.* 88 (1912).

² "Proceedings of the Twenty-Ninth Annual Convention of the Association of Official Agricultural Chemists," 1912; U. S. D. A., Bur. of Chem., *Bull.* 162, 60-62.

concentrated solution of citric acid, (2) less concentrated solution of permanganate, (3) the presence of substances other than citric acid to partially consume the permanganate, (4) rapid boiling and slow addition of permanganate.

In Table I are presented the results of a few of the experiments which were made to determine the effect of various changes of conditions on the yield of acetone. The several groups of determinations illustrate the four points enumerated above.

TABLE I.
Effect of Various Changes of Conditions of Oxidation on the Yield of Acetone.

Factor under observation.	Citric acid present. Mg.	Vol. of soln. start. Cc.	Strength of KMnO ₄ soln., g. per liter.	Added subs. other than citric acid.	Citric acid recovered.	
					Mg.	%.
Vol. of soln. of citric acid at start.....	25	400	0.50	17.7	70.8
	25	200	0.50	18.9	76.0
	25	100	0.50	21.9	87.6
Conc. of citric acid.....	25	400	0.35	18.6	74.4
	50	400	0.35	85.3	85.3
	150	400	0.35	133.7	89.1
Strength of KMnO ₄ soln.....	50	100	0.20	46.9	93.8
	50	100	0.50	45.2	90.4
	50	400	0.35	43.8	87.6
	50	400	0.70	42.0	84.0
Rate of oxidn. of citric acid						
(11 min.).....	50	400	0.70	42.0	84.0
(16 min.).....	50	400	0.70	44.6	89.2
Presence of substances other than citric acid.....	50	100	0.50	50 mg. tartaric acid ¹	45.4	90.8
	75	100	0.50	50 mg. tartaric acid	75.2	100.2
	50	100	0.20	10 mg. sucrose	48.9	97.8

It is evident that if constant results are to be obtained, a certain set of conditions must be adopted and then rigidly adhered to. This has been done in the proposed modified procedure. A volume of citric acid solution of 100 cc. at the beginning of the operation was chosen as the most convenient; ordinarily with 100 mg. citric acid present and no other oxidizable material, and the permanganate solution of 0.5 g. per liter dropping in at the rate of 120 to 150 drops per minute, the reaction will be complete with a residual volume of 15 to 25 cc. in the flask. Thus the rate of distillation must be a little faster than the rate of flow of the permanganate. The volume of solution and a slightly increased rate of permanganate flow are the only items changed from Pratt's original specifications. Although the presence of substances other than citric acid does tend toward slightly higher results, the effect has been found to be inconsiderable.

¹ Blank determinations were made with these substances, and no mercury precipitate was obtained.

2. **The concentration of Denigès' Solution**, in which the acetone distillate is boiled, has a very marked influence on the amount of mercury-acetone precipitate obtained; the stronger the solution the less the precipitate obtained. This is shown by the data in Table II and may be due either to increased solubility of the compound, or a change in its composition. Whatever the cause, it can be avoided by always having the same concentration of mercuric sulfate. Pratt's directions call for 30 to 40 cc. of Denigès' solution in the distillate, but do not specify the volume of that distillate. Since the smaller the amount of oxidizable material, the less is the time required for the operation, the smaller is the distillate, and the more concentrated is the mercuric sulfate. The writer has adopted, therefore, a concentration of 15 cc. of Denigès' solution for each 100 cc. of distillate. The reaction is then proportional with all amounts of citric acid, at least those from 25 to 150 mg. Great accuracy in measuring the distillate is not required; a 700 or 800 cc. Erlenmeyer marked at 300 cc., 400 cc., and 500 cc. does very well.

TABLE II.

The Effect of the Concentration of Denigès' Solution on the Yield of the Acetone-Mercury Compound. 50 Mg. Citric Acid Taken.

Vol. of distillate containing 40 cc. Denigès' soln., cc.....	200	250	300	350	400	500
Recovery of citric acid, %.....	96.4	96.8	99.3	102.0	100.3	100.7

3. **Amount of the Mercury-Acetone Precipitate Formed.**—This was determined by Pratt by weighing on a Gooch. This has been found less easy and trustworthy than the process of determining the mercury in the precipitate by titration. As the precipitate has a tendency to adhere to the sides of the Erlenmeyer flask in which it is formed, it is difficult to remove it completely to a Gooch. It is, however, readily dissolved by hot 5% hydrochloric acid. The resultant mercuric chloride may be titrated against standard potassium iodide solution according to Perronne's method.¹ This procedure was used in all the present experiments, and proved to be rapid and accurate.

4. **The flow of Permanganate** into the oxidation flask in the writer's apparatus, is controlled by a screw pinch-cock on a piece of soft rubber tubing, which replaces the glass dropping funnel in Pratt's apparatus. One end of the rubber tubing is connected to a bent glass tube passing through the stopper of the oxidation flask and ending in a fine nozzle; the other end of the tubing connects with a two liter supply reservoir of permanganate. This arrangement gives better control of the flow of permanganate than the glass-stoppered dropping funnel.

5. **Oxidation Flask.**—A 200 cc. Kjeldahl flask with all but three centimeters of the neck removed is very satisfactory. A 200 cc. side-arm distilling flask is equally good.

¹ *Compt. rend.*, 56, 63; also Sutton's "Volumetric Analysis," 10th edition, 1911, 264.

6. **Removing the Citric Acid** from a plant juice with barium acetate. The writer has adopted a medium of 30% alcohol instead of 50%. It throws out all the citric acid, and only a little of the other acids, as pointed out by Jørgensen.¹ The precipitate is also more granular in this case, and thus more easily filtered and washed.

7. **Quantitative Relation.**—In order to determine the quantitative relation between the citric acid and the potassium iodide used to titrate the mercury of the final product, a series of analyses was made with varying amounts of pure citric acid, using the exact procedure as given below. The potassium iodide solution used contained 26.70 g. KI per liter. The results are given in Table III. In order to compare the accuracy of these figures with those obtained by Pratt in ascertaining the factor to be used with his gravimetric method, an excerpt is given of his table (the last column was computed by the writer).

TABLE III.

Showing the Determination of the Factor for Potassium Iodide, in Comparison with the Factor in Pratt's Gravimetric Method.

Present method.				Pratt's method.			
Gram citric acid.	KI soln. Cc.	KI soln. per 1 g. citric acid. Cc.	Recovery using value of 0.001906. Per cent.	Gram of citric acid used.	Wt. of precipitate.	Calc. factor.	Recovery using factor 0.220. Per cent.
0.025	12.90	516	98.3	0.020	0.104	0.187	114.4
0.025	12.90	516	98.3	0.049	0.234	0.208	105.1
0.025	13.05	522	99.5	0.052	0.227	0.224	96.0
0.050	26.40	528	100.6	0.080	0.343	0.231	94.3
0.050	26.90	538	102.5	0.097	0.453	0.214	102.7
0.100	52.80	528	100.6	0.104	0.430	0.240	91.0
0.100	52.00	520	99.1	0.104	0.451	0.229	95.4
0.150	78.80	525	100.8	0.104	0.445	0.232	94.1
0.150	79.40	529	100.9	0.104	0.442	0.234	93.5
				0.107	0.510	0.210	104.9
				0.128	0.559	0.229	96.1
				0.128	0.556	0.230	95.6
				0.146	0.679	0.215	102.3
				0.246	1.181	0.208	105.6
				0.491	2.364	0.208	106.0
Average,			524.7 or	Average,		0.220	
			1 cc. \equiv 0.001906				

According to this procedure, then, 1.0 cc. of a solution of potassium iodide containing 26.70 g. of the salt per liter is equivalent to 1.906 mg. citric acid. For convenience, a solution of potassium iodide of 28.0218 has been adopted, 1 cc. of which is equivalent to 2.0 mg. citric acid.

¹ Jørgensen, "Über die Bestimmung einiger der in den Pflanzen vorkommenden Säuren," *Z. Nahr. Genussm.*, 13, 241 (1907).

The Modified Method in Detail.

The following solutions are required:

1. Hydrochloric acid, 5% (approximately).
2. Sodium hydroxide, 10%.
3. Phosphoric acid, 6%.
4. Denigès' solution, made as follows: Add about 500 cc. of water to 50 g. of mercuric oxide; then add 200 cc. of concentrated sulfuric acid with constant stirring; make up to a liter, heat on steam bath an hour or two, and filter till clear.
5. Potassium iodide; contains 28.0218 g. pure KI per liter. One cc. of this is equivalent to 2 mg. citric acid under the conditions of the experiment.
6. Mercuric chloride; contains 10.8038 g. HgCl_2 and 25 g. NaCl (to assist in solution) per liter. It is equivalent to the potassium iodide solution volume for volume. It is best made up by weighing out a slight excess of the salt, then standardizing against the potassium iodide solution and diluting to the proper strength.
7. Potassium permanganate, 0.5 g. per liter.
8. Barium acetate, 10%, in 30% alcohol by volume.

The complete method of analysis follows. The directions are the same as given by Pratt, except where the present modifications enter in.

Throw out the pectins by adding twice the volume of 50% alcohol. When the precipitate has settled, filter through paper on a Büchner funnel, and wash twice with 65% alcohol, sucking the gelatinous precipitate as dry as possible. Dilute the filtrate with water to give approximately a 30% alcohol content by volume and add slowly 5 cc. of the barium acetate solution. When the barium citrate has settled to the bottom, filter through asbestos in a Gooch, wash once with 30% alcohol, and dry in a water oven. The barium citrate is next dissolved with hot 6% phosphoric acid solution, using three portions of 20 cc. each, and followed by hot water. The filtrate and washings should be about 100 cc. They are transferred to the oxidation flask and the latter connected with a spiral condenser and with the device for adding potassium permanganate. To prevent bumping, introduce into the flask a piece of glass tubing sealed at the upper end, and long enough to remain upright. Sometimes a few glass beads are also necessary. The adapter of the condenser dips into 40 cc. of Denigès' solution contained in a 500 cc. Erlenmeyer flask. Heat the citrate solution over a naked flame, then allow the permanganate to drop into the briskly boiling solution at the rate of 20 to 25 drops in 10 seconds. When a deep pink color has persisted for a couple of minutes the reaction is complete. If the volume of liquid in the flask becomes too small, successive portions of 20 cc. of water may be added, care being taken to shut off the flow of per-

manganate a few moments before the distillation is stopped and the system is opened. The distillate is made up to 300 cc., put under a reflux condenser and boiled gently for 45 minutes. If the distillate is over 300 cc., more Denigès' solution should be added at the rate of 15 cc. for each 100 cc. of distillate in excess of 300 cc.

The precipitate is filtered hot through paper, washed by decantation twice with hot water, then the filter paper washed thoroughly. The precipitate in the Erlenmeyer is dissolved in two or three small portions of 5% HCl by heating, each portion being poured through the filter containing the rest of the precipitate and collected in a 100 cc. flask. The acid solution is returned through the filter till all precipitate is dissolved. After thoroughly washing the filter and flask with hot water, the filtrate is cooled, very nearly neutralized with 10% sodium hydroxide, and made up to 100 cc. The mercury can now be titrated by either one of the two following methods: (a) The whole or an aliquot of the mercury solution can be poured into an excess of the potassium iodide solution, and the excess potassium iodide titrated back with the standard mercuric chloride solution. The amount of potassium iodide solution necessary for the whole sample can then be calculated, together with the citric acid equivalent. (b) The mercuric chloride solution under examination can be placed in a buret and titrated directly against say, 10 or 15 cc. of the standard potassium iodide, and the amount of citric acid in the whole sample computed. For amounts of citric acid of 100 mg. or over, (b) is the preferable procedure. If (a) is used, the aliquot used should not be less than one-fourth.

Malic, tartaric (except quantities of 0.5 g. or over), oxalic, and aconitic acids do not interfere with this determination. The presence of much sugar, or other substances capable of reducing permanganate, tends to give slightly high results, but the difference is inconsiderable.

TABLE IV.

The Analysis of Certain Plant Products for Citric Acid.

The author with modified method.		A. O. A. C. collaborators with Pratt's method, using orange juice titrating 0.83 % citric acid.			
Substance analyzed.	Citric acid.	Collaborator.	Citric acid.	Collaborator.	Citric acid.
Sorghum syrup.....	{ 0.599%	J. M. J.....	{ 0.79%	P. B. D.....	{ 0.72
	{ 0.586		{ 0.89		{ 0.73
Lemon juice, titrating	{ 6.69		{ 0.79		{ 0.63
6.64% citric acid...	{ 6.64		{ 0.76		{ 0.72
			{ 0.84		{ 0.69
Grapefruit juice, titrat-	{ 0.976	P. A. Y.....	{ 0.824	H. C. G....	{ 0.81
ing 0.90% citric acid	{ 0.993		{ 0.836		{ 0.80
			{ 0.787		{ 0.73
			{ 0.840		{ 0.87
					{ 0.80
					{ 0.84

In order to show the accuracy of duplicate determinations by the modified method, several products were analyzed for citric acid. The results are shown in Table IV. For the sake of comparison, the table of results given in the 1912 report of the A. O. A. C. is also reproduced.

These results seem to warrant the conclusion that the modified method, if followed rigidly, will give much more satisfactory results than the original one. It is hoped that other workers will give the proposed method a trial, as a trustworthy method for determining citric acid in plant products is needed.

DIVISION OF AGRICULTURAL BIO-CHEMISTRY
UNIVERSITY OF MINNESOTA.

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AN EXAMINATION OF CERTAIN METHODS FOR THE STUDY OF PROTEOLYTIC ACTION.

BY H. C. SHERMAN AND DORA E. NEUN.

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There is no lack of methods for the study of proteolytic action but, in general, each method has been devised to meet the needs of a particular line of investigation, or to determine whether or not a commercial pepsin or trypsin complies with the standard arbitrarily established for it. As a preliminary to research upon the proteases, it seemed desirable to study some of these methods with reference to their accuracy and delicacy and their adaptability to proteases of more than one type. The methods studied were: The determination of total digested nitrogen, the increase of amino nitrogen as measured by the Van Slyke method, the acidimetric titration of digestion products, the change in electrical conductivity, and in rotation of polarized light, the Mett method, and the biuret and ninhydrin reactions. The work of Long and Barton¹ published soon after the present investigation was begun, made it unnecessary for us to include certain other well-known methods in our comparison. We have used commercial pepsins and trypsins of good quality and have arranged our experiments with a view to ascertaining to what extent the various methods will permit comparisons of these two types with each other. In the following pages typical results obtained by each of the methods tested are presented very briefly, the work on the ninhydrin reaction being further abbreviated in view of the investigations of Van Slyke and his associates² and of Harding and MacLean,³ the results of which have become available while our studies were in progress.

¹ THIS JOURNAL, 36, 2151 (1914).

² *Proc. Soc. Expt. Biol. Med.*, 11, 154 (1914); *J. Am. Med. Assoc.*, 65, 945 (1915); Harvey Society Lectures, 1915-16.

³ *J. Biol. Chem.*, 20, 217 (1915); 24, 503 (1916); 25, 319, 337 (1916).