XXXII. OBSERVATIONS ON THE CHEMICAL METHOD FOR THE ESTIMATION OF VITAMIN C.

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Since Svirbely and Szent-Györgyi [1932] showed experimentally that vitamin C was identical with hexuronic or ascorbic acid, the reducing property of the latter has been utilised for the estimation of vitamin C by a chemical method. Tillmans and his colleagues using the indicator 2 : 6-dichlorophenolindophenol estimated the reducing value of certain foods and correlated it with their vitamin C content. Following the same technique, Birch *et al.* [1933] have developed a simple method for the vitamin C assay of natural foodstuffs. The method consists in preparing a trichloroacetic acid extract of the material and rapidly titrating it with 2 : 6-dichlorophenolindophenol. These authors have put forward substantial evidence to show that under the conditions of the test naturally occurring substances other than ascorbic acid, which are likely to reduce the indicator, do not interfere with the estimation, and the values so obtained for a large number of fruits and vegetables agree with their antiscorbutic values as estimated by the biological method.

This simple method has obvious advantages over the long and tedious biological technique. More recent work, however, appears to indicate the existence of unknown reducing substances which may interfere with the test [Svirbely, 1933; Harris, 1933]. At the same time ascorbic acid is known to undergo a reversible oxidative change which does not detract from its value as an antiscorbutic, but in that state ascorbic acid will not react with the indicator dichlorophenolindophenol. Recently Guha and Ghosh [1934] have pointed out that trichloroacetic acid by itself would reduce the indicator even in a considerably dilute solution. Further, trichloroacetic acid can by no means be expected to extract quantitatively the ascorbic acid present in the material, and the proportion extracted may vary from material to material depending on the state in which it is held within the tissues.

These observations appear to show that the chemical method may be liable to errors of a rather disturbing magnitude. Opportunity was afforded to study this method during examination of the nutritional values of Indian foodstuffs which is being undertaken in this laboratory. The results of these studies are reported in this paper.

Experimental.

(1) *The reaction of trichloroacetic and ascorbic acids with the indicator*[1].

We have confirmed the observation of Guha and Ghosh [1934] that trichloroacetic acid reduces the indicator (Table I). The reaction takes place slowly, more

[1] The indicator solution referred to in this paper was of the strength

1 ml. indicator = 1 mg. ascorbic acid.

It was standardised against pure ascorbic acid solution which was in turn standardised with 0·01 *N* iodine solution.

Table I. *Reaction of trichloroacetic acid with 2 : 6-dichlorophenolindophenol.*

Concentration of trichloroacetic acid %	Volume of trichloroacetic acid added to 0·1 ml. of indicator ml.	Time of complete reduction mins.
50·0	0·5	0·75– 1·0
20·0	0·5	0·75– 1·0
10·0	0·5	1·0 – 1·25
5·0	0·5	2·0 – 2·25
1·0	0·5	13·0
1·0	1·0	8·0
1·0	5·0	6·0
0·5	0·5	20·0
0·5	1·0	15·0
0·5	5·0	10·0
0·1	1·0	40·0
0·1	5·0	30·0
0·1	10·0	25·0

so in lower concentrations when the time taken to reduce 0·1 ml. of the indicator is considerably prolonged. On the other hand the reaction of ascorbic acid solution with the indicator is quicker, being in fact almost instantaneous (Table II).

Table II. *Reaction of ascorbic acid with 2 : 6-dichlorophenolindophenol.*

Concentration of ascorbic acid mg./100 ml.	Volume of indicator solution used ml.	Volume of ascorbic acid solution required to reduce the indicator ml.	Time for completion of reaction mins.
100·0	1·00	1·05	Instantaneous
50·0	1·00	2·10	,,
10·0	0·50	5·25	,,
5·0	0·50	10·50	0·75–1·0
1·0	0·10	10·50	2·0 –2·5
0·5	0·10	21·00	3·0
0·1	0·02	21·00	5·0

When, however, a mixture of trichloroacetic and ascorbic acids is titrated against the indicator, the reaction depends upon three factors: (i) the concentration of trichloroacetic acid, (ii) the concentration of ascorbic acid, and (iii) the time taken to complete the titration.

When the concentration of ascorbic acid is not below a certain limit and the concentration of trichloroacetic acid is not very high, and the titration is finished reasonably quickly, the estimation of ascorbic acid is not interfered with by the presence of trichloroacetic acid. If, however, the concentration of ascorbic acid is low, the interaction of trichloroacetic acid with the indicator introduces a significant error in the estimation. These observations will be clear from a study of Table III.

These results show that when the concentration of ascorbic acid in the solution is over 2 mg./100 ml., even 10 % of trichloroacetic acid will not interfere with the titration of ascorbic acid with the indicator. Birch *et al.* [1933] have recommended a concentration of 5 % trichloroacetic acid in the vegetable extracts at the time of titration. Therefore it seems that this concentration is safe even for slightly higher dilutions than 2 mg./100 ml. of ascorbic acid.

Table III. *Reaction of a mixture of trichloroacetic and ascorbic acids with the indicator.*

Concentration of trichloroacetic acid %	Concentration of ascorbic acid mg./100 ml.	Actual volume of mixture required to reduce 0·2 ml. of indicator ml.	Volume predicted from the concentration of ascorbic acid ml.
0·0	10·80	1·90	1·85
10·0	5·40	3·70	3·70
10·0	2·16	9·25	9·25
10·0	1·08	14·00	18·50
5·0	5·40	3·70	3·70
5·0	2·16	9·25	9·25
5·0	1·08	18·30	18·50
2·5	5·40	3·70	3·70
2·5	2·16	9·25	9·25
2·5	1·08	18·50	18·50

When ascorbic acid solutions are allowed to stand in the laboratory under ordinary conditions, even after a short time their titration value with the indicator falls, apparently owing to oxidation. But when the solutions are acidified with a drop of trichloroacetic or even acetic acid at the time of titration the value is found to be higher than when titrated without the addition of the acid (Table IV).

Table IV. *Reaction of the partly oxidised solutions of ascorbic acid with the indicator.*

Time the solution was allowed to stand	Volume of solution required to reduce 0·2 ml. indicator ml.	Volume of solution required to reduce 0·2 ml. indicator after adding 1 drop of glacial acetic acid ml.
0	1·85	1·85
10 mins.	1·90	1·85
30 ,,	2·10	1·85
3 hrs.	2·20	1·85
24 ,,	2·40	2·00
72 ,,	3·60	3·25

It appears from these observations (Table IV) that even 10 minutes after the preparation of ascorbic acid solution the titration value is lowered, showing an appreciable oxidation of ascorbic acid. The oxidation continues and at the end of 3 hours the apparent loss is about 16 %. Up to this time one drop of glacial acetic acid added to the titration flask at the time of titration would bring the reading back to normal. As more time passes the reading does not come back to normal on acidifying though it always gives a higher value for ascorbic acid than when titrated in the unacidified form. It appears that a part of the oxidised ascorbic acid, and presumably the reversibly oxidised form, returns to the reduced state on the addition of the acid.

(2) *The titration of natural fruit juices with the indicator.*

For extracts of natural fruit juices the results of the estimation of ascorbic acid are less likely to be vitiated by the presence of trichloroacetic acid than those for pure solutions. Different dilutions of orange and lemon juices were titrated with the indicator in the presence of 5 % trichloroacetic acid. The readings were

theoretically correct when the titrations were finished within 1–2 minutes. On the other hand, when the titration was delayed to 3–5 minutes, high values for ascorbic acid were obtained (Tables V and VI). Lemon juice mixed with known quantities of ascorbic acid solution also gave theoretical values.

Table V. *The titration of orange juice with the indicator.*

Concentration of juice %	Concentration of ascorbic acid in juice mg./100 ml.	Concentration of trichloro-acetic acid %	For 0·5 ml. indicator	
			Predicted reading	Actual reading
100	31·25	0	—	1·60
25	7·81	5	6·4	6·4
20	6·25	5	8·0	8·0
25*	7·81	5	6·4	6·2
25†	7·81	5	6·4	6·0

* Titration delayed to 3 minutes.
† Titration delayed to 5 minutes.
In all other cases the titration was finished within 1–2 minutes.

Table VI. *The titration of lemon juice with the indicator.*

Concentration of juice %	Concentration of ascorbic acid in the juice mg./100 ml.	Concentration of trichloro-acetic acid %	For 0·5 ml. indicator	
			Predicted reading	Actual reading
100	25·8	0	—	0·425
100	25·8	5	0·425	0·425
10	2·58	5	4·25	4·25
4	1·03	5	10·65	10·65
2	0·515	5	21·5	21·5

(3) *The extraction of ascorbic acid from fruits and vegetables by trichloroacetic acid.*

Birch *et al.* [1933] have recommended the use of trichloroacetic acid for the extraction of vitamin C from natural food materials. Trichloroacetic acid may precipitate protein matter and help to break up the cell-walls, thus facilitating the extraction of ascorbic acid, and may stabilise the vitamin so extracted by inhibiting the action of oxidising enzymes. But it can not be stated what proportion of the vitamin actually present in the tissues comes out in the extract. To investigate this question properly it would be necessary to test the various fractions of extracts and residues by animal feeding experiments. We, however, attempted to obtain maximum yields of ascorbic acid by modifying the method and time of extraction, varying the concentration of trichloroacetic acid used, repeating the extractions, and by the application of heat *etc.* The results of these experiments are summarised in Tables VII and VIII.

It appears from the results summarised in Table VII that 20–25 % is the optimum concentration of trichloroacetic acid to extract the maximum quantity of ascorbic acid. Boiling the vegetable with water or trichloroacetic acid did not increase the amount of vitamin C in the extract. But leaving the shredded vegetable exposed to the warm atmosphere may destroy over 67 % of the vitamin during the course of 2 hours. Storing the vegetable as such also has a very deleterious effect.

The results shown in Table VII refer to a single extraction. By repeating the extractions more reducing substance is obtained as shown in Table VIII.

Table VII.

10 g. of karela[1] were ground up with sand and trichloroacetic acid and filtered. The extract together with washings was made up to 25 ml. The final concentration of trichloroacetic acid in the diluted extracts was maintained at 5 %.

Concentration of trichloroacetic acid used for extraction	Volume required for 0·25 ml. of indicator ml.	Ascorbic acid value as calculated from the reading mg./100 g.
100	3·35	18·65
75	3·25	19·23
50	2·85	21·93
25*	1·80	34·72
20*	1·80	34·72
10†	3·0	20·83
5	3·5	17·86

* Boiling the vegetable for 5–10 minutes with water or trichloroacetic acid and then making the extracts had no effect upon the titration values.

† Cutting up or shredding the vegetable and leaving it for some time before making the extracts profoundly affected the values, *e.g.*

	Reading	Ascorbic acid value mg./100 g.
Fresh	1·20	52·08
After 10 minutes	1·35	46·30
After 20 minutes	1·45	43·10
After 2 hours	3·50	17·85
Keeping whole vegetable for 3 days at room temperature	6·50	10·00

Table VIII.

Vegetable and treatment	Reading for 0·1 ml. indicator ml.	Ascorbic acid value mg./100 g.
10 g. karela extracted in cold with 20 % trichloroacetic acid, extract made up to 25 ml. Concentration of trichloroacetic acid in the extract 5 %.		
1. First extraction of vegetable	0·50	50·0
2. Extraction of the residue	1·90	13·1
3. Further extraction of the residue	20·0	1·25
4. ,, ,, ,,	50·0	0·50
10 g. cabbage treated in the same manner as karela.		
1. First extraction of vegetable	2·5	11·0
2. Extraction of residue	25·0	1·0
3. Further extraction of the residue	40·0	0·62
4. ,, ,, ,,	—	0

This simple experiment shows that a single extraction with trichloroacetic acid does not extract anything like the full amount of vitamin C present in the vegetable tissues. Supposing the four repeated extractions to represent the full amount of vitamin C, then the first extraction represents only 77·1 % in the case of karela and 87·1 % in the case of cabbage.

(4) *The effect of boiling or cooking on cabbage.*

Cabbage behaved differently from most other vegetables in our experience. Usually boiling the vegetable with water or trichloroacetic acid did not profoundly affect the ascorbic acid value of the extract. But boiling the cabbage

[1] Karela (*Momordica charantia*, Curcurbitaceae) is a common Indian vegetable, selected on account of its richness in vitamin C.

for 10 minutes raised the ascorbic acid value almost threefold (Table IX). Boiling for a longer time resulted in the destruction of the reducing substance in the extract.

Table IX.

10 g. cabbage extracted with 6·25 ml. of 20 % trichloroacetic acid either in the cold or with boiling. Extracts made up to 25 ml.

Treatment	Ascorbic acid value mg./100 g.
Cold extraction	13·3
Boiling 10 minutes	40·5
„ 15 „	29·15
„ 30 „	22·30
„ 1 hour	14·8
„ 1½ „	5·0
„ 2 „	1·56

It may appear from these observations that either the cell walls of cabbage are not broken up by grinding with cold trichloroacetic acid or the vitamin is more firmly bound within the tissues.

Conclusions.

The experiments described in the text of this paper show that this method can be of practical value for the estimation of ascorbic acid in its solutions and in natural materials. Under definite conditions it is capable of giving accurate and quantitative results. Trichloroacetic acid which slowly reduces the indicator by itself does not interfere in the estimation if the titrations are carried out quickly and finished within 1–2 minutes. On the other hand, in the presence of trichloroacetic acid and by titrating the solutions against the indicator instead of the reverse procedure of Tillmans, a better and sharper end-point is obtained. Solutions of pure ascorbic acid, natural fruit juices and mixtures of both in varying dilutions show theoretically correct results under the following conditions:

(i) The concentration of ascorbic acid in solutions or extracts in which estimation is desired must be higher than 2 mg./100 ml.

(ii) The concentration of trichloroacetic acid must not be higher than 5 %.

(iii) 1·0–0·1 ml. of 0·01 *M* solutions of the indicator are to be placed in the titration flask according to the potency of the extract, and the solution is to be run in from the burette steadily with constant shaking of the flask.

(iv) The titration is to be finished within 1–2 minutes.

Difficulty arises when the solutions to be titrated are weak in ascorbic acid content, containing less than 2 or even 1 mg./100 ml. Vegetables and fruits poor in vitamin C often yield extracts of this quality. In these cases the reaction is slow, the end-point indefinite, and consequently the values are only roughly approximate.

The next question which needs consideration is whether reversibly oxidised ascorbic acid, which is probably as good an antiscorbutic as the reduced form, can be estimated by the chemical method. Ordinarily the indicator will not react with this form of ascorbic acid and, when the latter constitutes an appreciable proportion of the whole, the results may be quite misleading. Some authors have utilised the action of H_2S over a prolonged period to bring the acid back to the reduced form [Eekelen *et al.*, 1933]. But it appears undesirable to treat a complex natural material with H_2S for such a long period as 6 hours, on account of the

unknown changes that the treatment may induce. Further, it is not known whether this reversibly oxidised ascorbic acid exists in appreciable amounts in natural vegetable tissues.

The experiments summarised in Table IV show that a certain proportion of ascorbic acid which ordinarily does not react with the indicator does so in the presence of trichloroacetic or glacial acetic acid.

With respect to the extraction of vitamin C from fruits and vegetables, 20–25 % trichloroacetic acid appears to give the best results. Higher concentrations probably destroy the vitamin and lower concentrations do not extract it completely. Boiling the vegetable tissues with water or trichloroacetic acid solutions does not materially raise the quantity of ascorbic acid in the extracts excepting in that of cabbage. On repeated extraction of the residue, however, more of the vitamin is extracted. In the two vegetables investigated the first extract represented only 77–87 % of the total ascorbic acid. This throws considerable doubt on the value of this method of extraction, as only a proportion of the total vitamin is extracted, and this proportion may vary from material to material. It is clear that this method does not give quantitatively the vitamin present in the vegetable material. At the same time we cannot be sure that in the biological estimation of vitamin C the whole of ascorbic acid present in the food is absorbed and taken account of by the organism. Probably the efficiencies of the two processes run more or less parallel, and hence the striking agreement between the values obtained by the two methods.

Attention may also be drawn here to the ready destruction of vitamin C in vegetable tissues when left exposed to the atmosphere after being cut or shredded. In karela over 67 % of the vitamin was destroyed during 2 hours. A similar observation has been recorded by Kohman *et al.* [1931] for carrots. Storing karelas for three days at room temperature reduced their vitamin C by over 80 %. These points are of practical importance from the point of view of nutrition, for they show how considerably vegetables may vary in their vitamin C content according to their freshness.

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