CCXXXIV. OBSERVATIONS ON THE ESTIMATION OF ASCORBIC ACID BY TITRATION.

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THE work of Tillmans *et al.* [1932], of Harris and Ray [1933] and of others on the estimation of vitamin C by titration against phenolindophenol has offered an opportunity to investigate quickly the antiscorbutic activity of foodstuffs. Since no information in this respect was available regarding Canadian foods, it seemed advisable to make a series of measurements. It was soon found that several modifications in the available procedures could be made to render the estimation more convenient and more accurate. We have employed the procedure of Harris and Ray [1933] with the following modifications.

(*a*) The indicator, 2:6-dichlorophenolindophenol, was made up in a phosphate buffer solution, p_H 7·2. In this solution the indicator was found to be fairly stable, but daily standardisation is essential.

(*b*) The indicator was standardised against ferrous ammonium sulphate according to the method of Tillmans, Hirsch and Hirsch [1932]. Since both ferrous ammonium sulphate and ascorbic acid reduce the indophenol indicator, it was possible, through the medium of the indicator, to standardise the sulphate solution against pure ascorbic acid crystals. The slightly acidified solution of ferrous ammonium sulphate when kept under an atmosphere of nitrogen in an automatic burette showed no deterioration in 6 months.

(*c*) As was first noted by Zilva [1927], acid alone slowly decolorises the indophenol indicator. When titrating extracts rich in ascorbic acid the percentage error caused by the presence of trichloroacetic acid is negligible but in solutions with a low ascorbic acid content the percentage error is considerable. The time required for a given amount of trichloroacetic acid to decolorise a fixed amount of indophenol solution is considerably increased when the indophenol solution is diluted with 15–20 vols. of water. On the other hand, the same dilution only slightly prolongs the time required for the reduction of the indophenol by ascorbic acid. Consequently in this work 1·0 ml. of the indophenol indicator solution was diluted with 15–20 ml. of distilled water just prior to titration with the acidified ascorbic acid extract.

(*d*) When working with solid foods Birch *et al.* [1933] made only one trichloroacetic acid extraction. One extraction yields only about 60% of the free ascorbic acid contained in the tissue and it is essential to make three extractions in order to obtain an estimate of the total amount of free ascorbic acid.

(*e*) A final concentration of 5% trichloroacetic acid was found to have a destructive effect on various ascorbic acid extracts. A 3% solution was found to serve equally well for extraction and considerably lessened the destructive action.

(*f*) The addition of a few drops of potassium cyanide solution during extraction was found to have a stabilising effect on the ascorbic acid present.

(*g*) The use of larger volumes than those recommended by Birch *et al.* increases the accuracy of the procedure. In this work tissues were extracted as follows. To 72 g. of the solid material were added 48 ml. distilled water, 12 ml. 20% trichloroacetic acid solution and 1 ml. 0·2*M* potassium cyanide solution. The mixture was then ground with sand and centrifuged. The residue was twice extracted in the centrifuge cups with 40 ml. of 3% trichloroacetic acid solution to which a little potassium cyanide solution had been added. The combined supernatant fluids were then well mixed, the volume measured and the required amount filtered. The filtrate was titrated from a 5 ml. burette into a measured volume of the indicator solution.

Titration of pigmented extracts.

Several methods of removing the interfering pigment from cherry, raspberry, beet and other extracts were tried, but it was found that in each case in which the colour was removed a part of the ascorbic acid was also removed or destroyed. The mercuric acetate method described by Emmerie and Van Eekelen [1934] proved unsatisfactory in this laboratory, since only about 70 % of added ascorbic acid could be recovered.

In one experiment using the mercuric acetate procedure the following results were obtained. 50 ml. of untreated turnip juice containing 6·8 mg. ascorbic acid were mixed with 5 ml. of an ascorbic acid solution (B.D.H. tablets) containing 13·8 mg. ascorbic acid. 55 ml. of this mixture and 50 ml. of the same turnip juice but without added ascorbic acid were then treated with mercuric acetate as described by the above authors. On titrating the final solutions it was found that the turnip juice alone contained 3·8 mg. and the turnip juice *plus* the ascorbic acid solution contained 13·7 mg. Assuming, as is reasonable, that the 50 ml. of turnip juice in the mixture accounted for 3·8 mg., then only 9·9 mg. or 72% of the added ascorbic acid could be determined.

It was thought that ascorbic acid might have been carried down with the precipitate which is formed. To test this, a solution of pure ascorbic acid was treated with mercuric acetate as before. Again only 70% of the ascorbic acid originally present could be determined. A control solution treated as above except for the addition of mercuric acetate showed practically no change. The fact that ascorbic acid when in the reversibly oxidised state is known to be quite unstable tends to explain the above results and it is likely that ascorbic acid, partially oxidised by the mercuric acetate, is very quickly irreversibly oxidised.

A modification of the method described by Tillmans, Hirsch and Jackisch [1932, 1] for titrating pigmented extracts was finally adopted. The basis of this procedure lies in the fact that most plant pigments are insoluble in chloroform whereas the indophenol indicator is more soluble in chloroform than in water and may be completely removed from aqueous solution with this solvent. About 10 ml. chloroform are placed in a 50 ml. centrifuge-tube. Then 5, 10 or 20 ml. tissue extract, depending on its probable ascorbic acid potency, are added. A pipette connected to a cylinder of carbon dioxide is then lowered into the aqueous layer so that, on bubbling the gas through, the aqueous layer is thoroughly mixed but the chloroform layer is not disturbed. While the aqueous layer is being thus stirred, the indicator solution is added drop by drop from a 5 ml. micro-burette. An approximate preliminary titration is carried out to estimate the amount of indicator required. After the addition of the estimated amount of indicator it is advisable to wait a short time to allow for the complete reaction of the indicator and any ascorbic acid present. Then the tip of the pipette is lowered into the chloroform and carbon dioxide bubbled in for about a minute at a rate sufficient thoroughly to mix the two layers. The two layers are then separated by centrifuging. If the chloroform is practically colourless, insufficient indicator has been added and the titration must be continued. The end-point is reached when just sufficient indicator has been added to give the chloroform a definite red tinge. With practice the titration can be carried out fairly quickly. The red colour in the chloroform is quite stable and does not fade as is the case in acidified aqueous solutions. Using this method it has been found possible to account for 97–99 % of added ascorbic acid.

The pigments of strawberries and red peppers were found to be somewhat soluble in chloroform and for these the above method could not be employed. However, these pigments, in contrast to those of raspberries, cherries *etc.*, may be sufficiently removed to permit ordinary titration by shaking up the tri-

chloroacetic acid extract with butyl or amyl alcohol. If the alcohol is subsequently washed with a small volume of water and the water added to the original extract, the loss of ascorbic acid is not significant.

Rapid destruction of ascorbic acid in minced vegetables.

When shredded turnip is allowed to stand in the open air for a short time, the juice obtained by pressing the pulp is inactive or practically so as far as reducing the indophenol indicator is concerned. This result has also been noted by Ahmad [1935]. If the juice is pressed out immediately after shredding, the activity is retained in the juice fairly well. However, juice expressed from pulp which had been standing about 10 minutes did not retain what little activity it possessed but became inactivated quite rapidly. The inactivation of the pulp is practically complete in 30 minutes and even in 15 minutes the pulp loses about 75 % of its activity.

A quantity of pulp, immediately after shredding, was suspended in acidulated water to which a little potassium cyanide had been added. The mixture was allowed to stand an hour. The liquid obtained on pressing out the mixture was found to be fairly active. When potassium cyanide alone was added to the water the inactivation of the pulp was complete in less than 45 minutes. However, when acid alone was added so that the p_H of the mixture was about 2·5 the juice obtained possessed considerable reducing activity. When the aqueous mixture is saturated with hydrogen sulphide practically no inactivation occurs within 24 hours. Parsnip and cauliflower pulp behaved similarly.

It appears therefore that vegetable pulp has no mechanism for stabilising ascorbic acid. On the other hand, animal tissues were found by Mawson [1935] to possess a marked ability to inhibit the aerobic oxidation of ascorbic acid.

Ascorbic acid content of inner and outer parts of plant tissues.

If there were a relation between ascorbic acid synthesis and photosynthesis in plants, one would expect to find the vitamin more concentrated in those parts of the plant in which photosynthesis is most active. This has been shown to be the case by Bacharach *et al.* [1934], who found that 15 % of the total ascorbic acid content of lemons was in the juice and 85 % in the peel.

This relation has also been demonstrated in the present work. Two samples were taken from the top of a firm head of lettuce and two from the bottom near the root of the same head. The four samples were then extracted and the solutions titrated. The top samples contained 5·6 and 6·2 mg./100 ml. ascorbic acid while the lower ones contained only 3·7 and 4·1 mg./100 ml. One would naturally suppose that the lower part would receive less sunlight than the upper and that consequently photosynthesis would be less active in the lower section.

In the case of cucumbers it was found that whereas the inner edible portion contained 1·5 mg./100 g. ascorbic acid, the outer skin contained 4·3 mg./100 g. or almost three times as much weight for weight.

At first it appeared that the above relationship was reversed in the case of turnips. Juice squeezed from the outer parts of two turnips was found to contain 37 and 47 mg./100 ml. ascorbic acid. Juice from the inner parts of the same two turnips contained 50 and 52 mg./100 ml. respectively. However, since practically all the photosynthesis occurs in the leaves of the turnip and since substances formed in the leaves are transferred down the stalks to the central part of the turnip, the observations in this case are in agreement with the former examples as far as the relationship between ascorbic acid production and photosynthesis is concerned.

Polyploidy and ascorbic acid.

Zilva [1933] reported that the juice obtained by thoroughly macerating tetraploid tomatoes was almost twice as potent with respect to vitamin C as was the juice so obtained from diploid tomatoes. He observed that the former fruits were smaller than the latter but stated that he did not think the variation in potency was a result of this difference in size.

Through the kindness of Dr MacArthur of the University of Toronto it was possible to investigate the ascorbic acid content of these two types of fruit both obtained from the same variety of tomato. The fruits were so selected that all were approximately the same size, about $\frac{3}{4}$ in. in diameter, and appeared to be of the same degree of ripeness. It was found that, considering the weight of the whole tomato, the tetraploids contained 43 mg./100 g. ascorbic acid and the diploids contained 36 mg./100 g. The difference in the two types observed here is not nearly so marked as that observed by Zilva.

It seems possible that at least part of the difference noted by Zilva may have resulted from the difference in size of the tetraploid and diploid tomatoes which he used. Because of this difference in size it is obvious that weight for weight the tetraploids (being smaller) would have a greater surface area than the diploids and as has been pointed out above ascorbic acid is much more concentrated in the surface of such fruits than in the inner section.

The relationship between the ascorbic acid content and the size of tomatoes was illustrated in the fruit obtained from one of the parents of the diploid and tetraploid plants. These tomatoes were extremely small, being only $\frac{3}{8}$ in. in diameter. They contained 68 mg./100 g. ascorbic acid, much more than either of the former and larger samples.

Reduced and reversibly oxidised ascorbic acid.

Tillmans, Hirsch and Jackisch [1932, 2] observed that the titration value of cucumber extracts was considerably increased when the extract was kept under an atmosphere of hydrogen sulphide for several hours. Before titration the hydrogen sulphide was removed by a stream of inert gas such as nitrogen. It appears that in the original extract a part of the ascorbic acid was present in a reversibly oxidised state. In the present work it has been shown that the above result is not confined to cucumber extract but is true for various plant extracts. Table I gives the amounts in mg./100 ml. of reduced and reduced *plus*

Table I. *Reduced and reversibly oxidised ascorbic acid in various tissues.*

mg. ascorbic acid per 100 g. tissue.

Tissue	Reduced	Reduced *plus* reversibly oxidised	Tissue	Reduced	Reduced *plus* reversibly oxidised
String beans	1·4	4·0	Cabbage	15	22
Lettuce	0·30	3·8	Spinach	18	22
Cucumbers	0·80	2·0	Tomatoes	15	15
Onions	5·9	13	Lemon juice	52	52
Broccoli	32	68	Peppers (hot)	167	160
Carrots	1·2	2·1	Bovine adrenal glands	125	121
Parsnips	3·7	5·0			

reversibly oxidised ascorbic acid present in extracts of various tissues. The procedure employed in obtaining these results was to dilute the extract with an equal volume of distilled water so that the concentration of trichloroacetic

acid was about 1·5 %. Hydrogen sulphide was then bubbled in for 5–10 minutes. The extract was corked tightly and allowed to stand overnight. Hydrogen sulphide was then removed by a stream of carbon dioxide; this required about 2 hours. In order to be certain that all the vitamin C present in an extract is determined by the indophenol titration method it is obvious that a preliminary treatment with hydrogen sulphide is essential.

Effect on titration value when certain vegetables are cooked.

During an investigation of the ascorbic acid content of a number of raw and cooked Canadian foods it was found that in the case of certain vegetables, such as cauliflower, carrots, parsnips, beets and potatoes, the titration value was higher for the cooked food than for the raw [McHenry and Graham, 1934]. A similar result in the case of cabbage has recently been reported by Bezssonoff [1934] and by Ahmad [1935]. However, in this laboratory only a decrease in the titration value of cabbage was observed after cooking. This increase when certain vegetables are cooked was reported by Tillmans, Hirsch and Jackisch [1932, 2] but seems to have escaped general notice. The following table gives the amounts of reduced ascorbic acid in extracts of raw and cooked tissues. The values are calculated in mg./100 g. of ascorbic acid. The plant tissues were heated by procedures similar to those used in preparing them for table use.

Table II. *Effect of heating upon titration value.*

mg. ascorbic acid per 100 g. tissue.

Tissue	Raw	Cooked	Tissue	Raw	Cooked
Cauliflower	19	31	Cabbage	15	12
Hubbard squash	3·1	4·1	Onions	8·9	3·1
Potatoes (old)	1·5	4·1	Broccoli	32	22
Beets	2·7	6·2	Corn	7·6	5·1
String beans	1·4	2·1	Peas	14	8·1
Carrots	1·2	2·6	Asparagus	12	8·2
Parsnips	3·7	6·1	Turnip	35	18
Spinach	18	13	Bovine adrenal glands	125	141

In the case of cauliflower, parsnips and lemon juice, determinations have been made at regular intervals during the course of heating in order to study the rate of increase in titration value. The minced pulp of cauliflower and parsnips was suspended in a lightly acidified water to which a little potassium cyanide solution had been added. In order further to inhibit oxidation the heating was done under an atmosphere of hydrogen sulphide. After extraction the solutions were treated with hydrogen sulphide and with carbon dioxide as previously described. The results are shown graphically in Fig. 1. With cauliflower and parsnips the titration value increased rapidly at first and then gradually decreased. There was no increase on heating lemon juice but only a gradual decrease.

It was found possible to bring about this same increase in titration value, without heating, by allowing the pulp mixture to stand several hours under an atmosphere of hydrogen sulphide in 1 % hydrochloric acid solution.

It is unlikely that this increase is due to cellular disintegration as a result of heating and consequently more thorough extraction of ascorbic acid. At first it seemed possible that the results might be attributed to the liberation of some sulphydryl compound. However, colorimetric tests for cystine, cysteine and ergothioneine are practically negative in extracts of the cooked material. The most likely explanation seems to be that a part of the ascorbic acid in the tissue is bound to some other substance and that this combination is split by hydrolysis.

Combined ascorbic acid is capable of reducing the indophenol indicator but is insoluble in trichloroacetic acid solution and for this reason is not determined unless soluble ascorbic acid is first set free. The combined form is, however, soluble in water as the following experiment shows. Cauliflower pulp was well mixed and divided into six 30 g. lots. Three lots were extracted with distilled water. The other three lots were first heated and then extracted with the usual

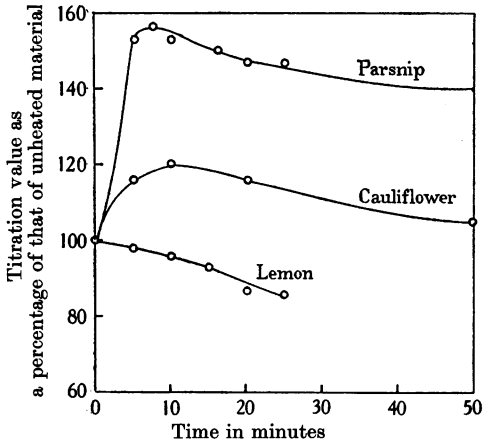

Fig. 1. Change in titration values when cauliflower, parsnip and diluted lemon juice are heated at 58° under hydrogen sulphide.

amount of acid. Part of the extracts were then treated with hydrogen sulphide and carbon dioxide as previously described. The three heated, acid-extracted samples contained 39, 41 and 43 mg. per 100 ml. ascorbic acid. The three unheated, water-extracted samples contained 40, 39 and 41 mg./100 ml. Thus even without a preliminary hydrolysis the ascorbic acid compound is extracted with water and may be determined by titration. However, aqueous extracts are turbid, difficult to clarify and inconvenient for titration.

In another experiment an aqueous extract of cauliflower was divided into two portions. To the first was added sufficient trichloroacetic acid to give a concentration of 3% acid. The precipitate was removed by filtration. To the second portion was added a volume of water equal to that of acid added above. Both solutions were then treated with hydrogen sulphide and carbon dioxide. Calculated on the weight of cauliflower the second solution contained 50 mg./100 g. ascorbic acid and the first only 41 mg./100 g. Evidently the addition of the acid caused the combined form of ascorbic acid to precipitate.

From the above results it is apparent that a simple acid extraction and titration procedure does not give the complete value for ascorbic acid but only measures the free acid. In many plant tissues this amount is supplemented by hydrolysis. There may be present, also, an amount of reversibly oxidised ascorbic acid which is not measured by titration unless it is first reduced by hydrogen sulphide. Ascorbic acid in these three forms may be biologically active but only the free acid can be estimated by simple acid extraction and titration. Bovine adrenal tissue contains no ascorbic acid in the reversibly oxidised form. Acid fruits, such as lemons, oranges and tomatoes, apparently contain only free ascorbic acid.

SUMMARY.

1. A series of modifications in the titration procedure of Harris and Ray which render the estimation of ascorbic acid more accurate have been described.

2. Two methods have been outlined for the removal of interfering plant pigments.

3. Vegetable tissues do not appear to contain a mechanism to prevent the aerobic oxidation of ascorbic acid. Hence they generally contain appreciable quantities of reversibly oxidised ascorbic acid.

4. Several vegetables show an increased titration value after heating for a short period, or after acid hydrolysis. The increase is believed to be due to the liberation of ascorbic acid from a compound which is soluble in water but insoluble in trichloroacetic acid.

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