CLXXVI. VITAMIN C IN *CITRUS* JUICES.

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The recent work of Svirbely and Szent-Györgyi [1933] has brought almost conclusive proof of the identity of vitamin C and the ascorbic acid which can be prepared from orange juice, paprika, or suprarenal glands, and it has been shown by several workers that there is a close connection between the amount of ascorbic acid as determined by titration with Tillmans's reagent, dichlorophenolindophenol, and the antiscorbutic value of the material examined.

In the experiments described in the following pages this method of titration has been applied to the examination of lemon and orange juices with the object of ascertaining the degree of natural variation in the content of ascorbic acid and the conditions which determine its preservation or disappearance in storage.

Harris and Ray [1933] give reasons for preferring to carry out this titration in acid solution (p_H 2·5) rather than in the nearly neutral solution proposed by Tillmans. They find that with fresh juice the results are practically identical but that if the juice is boiled or aerated in order to destroy the vitamin little diminution is found in the reducing power as determined by titration in neutral solution, whereas acid titration shows a much more marked falling-off.

The titrations here to be described were made in very slightly acid solution, p_H about 6. At this acidity the indicator gives a violet-blue coloration whereas in more acid solution the colour is red, and in neutral solution indigo blue. At this reaction—p_H 6—the end-point is much more satisfactory than in the acid state, the red coloration being difficult to see, while in neutral solution the reduction of the indicator takes place slowly so that the titration becomes rather tedious. At p_H 6 the indicator can be run in rapidly and the colour disappears instantaneously until near the end when it slows down, and the titration is considered to be finished when the violet-blue colour is permanent for half a minute.

The titrated liquid left exposed to the air gradually becomes dark blue from reoxidation of the indicator.

It is found that the indicator solution if made up in accordance with the suggestion of Tillmans, Hirsch and Hirsch [1932], in Sørensen's phosphate buffer solution of p_H 7 keeps better than in water and this solution was generally used for the titrations. As described by these authors the solution was standardised by titration with titanous chloride, this, in turn, being standardised against ferric ammonium sulphate.

The results are given as cc. of $N/1000$ solution of the indicator per 1 cc. of juice, and are not calculated to ascorbic acid since it is possible that other substances capable of reacting with the indicator may be present in the juice and that further work may lead to some correction for these.

Procedure. 2 cc. of juice are measured into a small beaker and diluted with about 10 cc. of water. Add 3–4 cc. of 10 % sodium acetate solution and titrate with approximately $N/1000$ solution of dichlorophenolindophenol until the violet-blue colour is persistent for half a minute. In the case of orange juice,

whose acidity is so much lower than that of the lemon, the sodium acetate should be followed by a drop of acetic acid.

Some of the results obtained with lemon juice are shown in Table I. (The fruit was obtained from various districts and squeezed in the laboratory using an ordinary glass lemon squeezer. The juice was passed through a strainer and titrated immediately.)

Table I.

Sample	cc. *N*/1000 indicator per 1 cc. juice	Acidity g. citric acid per 100 cc.	Sample	cc. *N*/1000 indicator per 1 cc. juice	Acidity g. citric acid per 100 cc.
1	9·1	6·85	12	8·45	7·0
2	10·7	7·35	13	8·1	6·65
3	10·5	7·40	14	8·25	7·07
4	8·5	6·85	15	9·05	6·95
5	9·7	7·65	16	6·25	7·35
6	8·25	6·80	17	8·00	6·65
7	10·45	7·85	18	7·5	6·80
8	10·65	7·70	19	8·6	7·25
9	9·55	7·30	20	7·2	7·05
10	8·1	7·35	21	8·0	7·65
11	7·7	7·60			

It is seen that the variations are very considerable, the lowest value being only about 60 % of the highest and the doses of juice used in biological trials may have quite different values in different cases.

It has not been possible so far to see any relation between the reducing value of the juice and its acidity or other properties. Nor is there any clear connection with the degree of ripeness of the fruit, results of similar value being found in December and in March.

Even more striking variations were found between individual lemons. Twenty lemons gathered from the same tree on March 2nd and squeezed separately gave results varying from 4·9 to 10·0.

Orange juice is more nearly uniform and the results are distinctly higher. All the samples examined have been found to lie between 10·3 and 11·8.

Effect of preservatives. It was found that lemon and orange juices without any addition of preservatives could be kept for long periods without any appreciable diminution in their reducing power. Although they ferment, and even when the surface is covered with a growth of mould, the reducing power is almost unaltered.

In the presence of preservatives this is not the case. In Table II are set out the results of an experiment in which freshly prepared lemon juice was divided into a number of portions. One was left without any addition and to the others were added the indicated quantities of various preservatives. The bottles were left at the ordinary laboratory temperature (between 16° and 22° during the period of the experiment) and titrated at intervals. In the unpreserved juice, fermentation sets in within 48 hours and is tolerably complete within a week.

The sugars were added in the form of 80 % syrups so that the juice was diluted to twice its volume. Fermentation began in the first few days and the titration value remained practically constant thereafter.

It is seen that formaldehyde has an immediately destructive effect on the reducing factor. The other preservatives do not seem to act on it directly, but in all the unfermented juices the reducing factor had practically disappeared in 30 days.

The experiments in presence of sugar were repeated with addition of sulphur dioxide or benzoic acid as preservatives (Exps. 10 and 11).

Table II.

Preservative employed	Titrations: Original	After: 2 days	7 days	30 days	95 days
1. None	7·80	7·50	7·50	7·35	6·90
2. Sulphur dioxide 0·035 %	7·80	7·40	6·90	0·60	—
3. Benzoic acid 0·063 %	7·80	6·30	5·60	0·50	—
4. Sodium fluoride 0·10 %	7·80	5·80	2·20	0·30	—
5. Formic acid 0·30 %	7·80	5·60	2·00	—	—
6. Formaldehyde 0·30 %	7·80	0·50	—	—	—
7. Salicylic acid 0·05 %	7·80	6·20	3·20	0·50	—
8. Glucose 40 %	3·90	3·60	3·60	3·50	—
9. Sucrose 40 %	3·90	3·75	3·75	3·60	—
	Original	3 days	8 days	16 days	37 days
10. 40 % sucrose + SO_2 0·035 %	4·55	4·0	4·0	3·2	1·2
11. 40 % sucrose + benzoic acid 0·063 %	4·55	3·8	2·9	1·0	—

In this and in other experiments the juices preserved with sulphur dioxide retained their reducing power longer than the others, but even in this case it disappeared almost entirely within 30 or 40 days. The sulphurous acid itself reduces the indicator though more slowly than the reducing factor of the juice and no good end-point can be obtained in its presence. The method followed is to distil off the sulphur dioxide in a current of carbon dioxide, cool rapidly, dilute to the original volume and titrate. It was found that this procedure had no effect on the titration value of pure juice or of juice to which sulphurous acid had been quite recently added.

The experiment was tried of adding much larger quantities of sulphurous acid, but this had little effect on the rate of loss of the reducing factor.

Amount of SO_2 added 2·85 g. per litre (10 times usual addition):

Titration.	Original	7·5
,,	After 12 hours	7·5
,,	,, 5 days	7·4
,,	,, 12 ,, ...	5·8
,,	,, 19 ,, ...	4·1
,,	,, 28 ,, ...	2·9
,,	,, 42 ,, ...	1·1

In 4 weeks the reducing factor had diminished to one-third of its original value but the amount of SO_2 still present was 1·6 g. per litre—five times as much as is usually employed as preservative.

Effect of alcohol. The addition of alcohol to juice caused a rapid loss of reducing power.

	Original titration	3 days	8 days	16 days	37 days
Juice containing 10 % of alcohol	8·55	6·9	5·0	1·1	—

With 50 % of alcohol all reducing power was lost in 5 days.

Effect of acidification.

	Original titration	18 hours	8 days	15 days
90 cc. of juice + 1 cc. hydrochloric acid, p_H 1·8	8·75	7·8	1·4	—
90 cc. of juice + 5 cc. hydrochloric acid, p_H 1·2	8·3	7·2	0·9	—

The reducing power falls off rapidly and is practically gone within a week. The acidity is too high for fermentation to take place.

Effect of traces of metals.

	Original	24 hours	96 hours	5 days	12 days	28 days
Juice + 0·001 % copper	8·0	6·5	5·6	5·6	5·6	5·6
„ + 0·003 % „	8·0	6·2	4·3	—	—	4·3
„ + 0·001 % iron	8·0	7·8	7·8	7·8	7·8	7·7
„ + 0·001 % aluminium	8·0	7·8	7·8	7·8	7·8	7·7

Iron and aluminium have no effect. Copper causes a rather rapid decrease until fermentation begins and then has no further effect.

Effect of pasteurisation. Juice contained in a series of test-tubes was heated at 65° for an hour. The tubes were stoppered with cotton-wool and tested at intervals.

Original titration	8·0
Immediately after pasteurisation	7·5
After 16 hours	7·2
„ 2 days	6·8
„ 4 „	5·1
„ 6 „	4·7
„ 8 „	3·5
„ 11 „	3·1
„ 13 „	2·2
„ 18 „	—

A steady diminution of the reducing factor occurs, leading to disappearance in 18 days. Boiling juice under a reflux condenser for several hours causes only a small decrease in reducing power. The boiled juice, if protected from fermentation, follows the same course as the pasteurised juice shown above. In this respect we do not find any important difference when the titrations are carried out in acid solution as suggested by Harris and Ray.

Titration of original juice	9·7
„ after boiling for 2 hours under reflux	9·5
„ after boiling for 2 hours under reflux (titrated at natural acidity of juice)	9·3

Effect of lemon oil. The results in presence of lemon oil are somewhat irregular and this is to be expected. The oil is only soluble to a very slight extent in the juice so that anything beyond a very small quantity forms a layer on the surface of greater or less thickness and this layer may to some extent be protective. Oil is not very efficient as an anti-fermentative, but affords considerable protection against the growth of moulds.

Experiments were made with 0·2, 0·5 and 1·0 % of oil. In all cases there was a fairly rapid diminution in reducing power in the first few days. With the smaller quantities this fall was arrested after the fifth day and the value then remained constant for several weeks at about two-thirds of the original figure. With 1 % the reducing power continued to diminish slowly and in 2 months had almost disappeared.

In experiments with juice pasteurised by heating for an hour at 65° similar results were obtained. The juice thus treated loses its reducing power at about the same rate as when treated with benzoic acid or similar preservatives. If, however, at any time ferments are allowed access to the liquid the loss is arrested and the reducing power remains nearly constant at the point it had reached before fermentation began.

The loss of reducing power while in the sterilised state is greatly retarded if precautions are taken to extract any dissolved air from the juice and to keep it thereafter out of contact with the atmosphere, and experiments are now in course to determine the duration of the reducing factor in these circumstances.

All these observations are in close agreement with the work of Williams and Corran [1930] who used the biological method and concluded that those substances which exert the strongest preservative action against gross fermentation possess the greatest destructive action on the antiscorbutic vitamin system.

It seems probable, however, that the preservatives, except formaldehyde, do not directly attack the reducing factor, but that they inhibit the action of another factor which in the untreated juice protects the vitamin from atmospheric oxidation.

This protective agency being destroyed by all the usual anti-fermentatives and by heat, and being restored if fermentation is set up in the once sterilised liquid before the reducing factor has disappeared, must be of the nature of an enzyme [*cf.* Zilva, 1928].

Orange juice. The behaviour of orange juice is, in general, similar to that of lemon juice although one or two inconsistencies have been observed which are still the subject of study.

	Titrations			
Preservative employed	Original	After 8 days	20 days	40 days
1. None	11·25	10·95	10·95	10·65
2. Sulphur dioxide 0·035 %	11·25	9·40	8·10	6·65
3. Benzoic acid 0·063 %	11·25	7·80	0·75	—
4. Sodium fluoride 0·10 %	11·25	7·70	0·60	—

In this series it is seen that the reducing power is maintained in the presence of sulphurous acid to a greater extent than was found to be the case with lemon juice, quite half of it remaining after 6 weeks.

With fluorides or benzoates on the other hand the loss of reducing power was almost complete in 3 weeks. In other experiments, however, when, after addition of benzoate, the juice was placed in a vacuum for some time in order to extract as far as possible any air, dissolved or adhering in minute bubbles to the floating pulp, the diminution of reducing power took place much more slowly. After 3 weeks the titration was 6·7, and after 6 weeks 6·25. The loss continued slowly and final disappearance of the reducing power required 4 months.

Further experiments were made with juice from blood oranges squeezed in April and therefore much riper than the fruit previously used. After addition of the usual proportion of benzoic acid the juice was stirred gently *in vacuo*, and was then preserved in ordinary corked bottles. To one of these was added sufficient citric acid (5 %) to bring its acidity up to that of average lemon juice.

	Titrations			
Treatment	Original	After 10 days	15 days	24 days
1. None	11·25	10·0	10·0	9·8
2. Benzoic acid 0·063 %	11·25	8·1	6·5	6·1
3. Do.	11·25	8·0	6·5	5·4
4. Do. + 5 % citric acid	11·25	5·0	3·1	0·4

The acidified juice behaves like lemon juice. The unacidified juice loses its reducing power at a slower rate.

Acidification with hydrochloric acid has, as with lemon juice, the effect of

accelerating the loss of reducing power provided the amount added is sufficient to arrest fermentation.

	Original titration	5 days	8 days	12 days	18 days
1. 90 cc. of juice + 1 cc. hydrochloric acid, p_H 2·1	10·3	9·5	9·5	9·3	9·3
2. 90 cc. of juice + 4 cc. hydrochloric acid, p_H 1·2	10·1	8·5	7·0	4·0	2·6

In No. 1 the acidity was not sufficient to prevent fermentation and the reducing power, in consequence, remained nearly constant.

The p_H of the orange juice before addition of acid was 3·6. That of lemon juice is about 2·1 to 2·3.

Fractionation. The fractionation of the reducing or antiscorbutic factor in lemon juice when this is treated with lead acetate at various p_H values has been the subject of considerable discussion. Zilva [1932] is of opinion that there is no definite relation between the reducing powers of such fractions and their antiscorbutic values, while at the same time there is no clear-cut separation of vitamin in the different precipitates. These in fact, voluminous and difficult to wash, are of the type most likely to retain small quantities of other substances, and any extended manipulation of them is to be avoided since the reducing factor is here in a highly unstable state. In the course of a few hours the solution of one of these precipitates loses a large part of its reducing power.

It is not clear from Zilva's paper what proportion of the total antiscorbutic factor he found to be contained in the precipitate at p_H 5·4. From the observations of Tillmans, Hirsch and collaborators [1932] it seems that about one-fifth of the original reducing power was precipitated at this point.

Working with small quantities of juice which permit rapid manipulation we have found about 10 % of the reducing power to be removed in this precipitate.

A second precipitate was obtained by adding ammonia to the first filtrate until the p_H reached 8·0 and the filtrate from this had no reducing power. The second precipitate (p_H 5·4–8·0), redissolved in acetic acid, was found to contain over 60 % of the reducing factor originally present.

Taken 250 cc. of lemon juice of which 1 cc. requires 10·5 cc. $N/1000$ indicator solution.

Total reducing power ... ... 2625 cc.

1. Added 13·5 g. calcium carbonate, stirring well. Heated to 85°, filtered and washed. Filtrate 500 cc. of which 1 cc. requires 5·15 cc. indicator.

   Total reducing power of filtrate ... ... 2575 cc.

   p_H of filtrate 5·4.

2. Added 10 g. lead acetate, filtered and washed. Filtrate 800 cc. of which 1 cc. requires 2·93 cc. indicator.

   Total reducing power of filtrate ... ... 2340 cc.

   Loss between 1 and 2 ... ... 235 cc.

   i.e. 9 % of total present.

3. Added ammonia to p_H 8·0; filtered and washed. Filtrate had no reducing power.

4. Precipitate redissolved in dilute acetic acid. Volume of solution 170 cc. 1 cc. required 9·5 cc. of the indicator solution.

   Total reducing power ... ... 1615 cc.

   i.e. 61·5 % of the reducing power originally present.

The precipitate was decomposed with hydrogen sulphide, filtered, washed, the hydrogen sulphide expelled in a current of carbon dioxide and the liquid concentrated in vacuum to 60 cc. without further loss of reducing power.

In another experiment with 500 cc. of juice nearly 70 % of the original reducing power was obtained in the concentrate but with larger volumes of juice it has not, so far, been possible to get more than about 40 %, the loss taking place during the formation and manipulation of the second lead precipitate.

Commercial juices. Mr F. K. Donovan has been kind enough to make some titrations of commercial juices and of juices pressed by himself in London for comparison with our results.

Lemon juice freshly pressed in London gave titrations of from 7·7 to 9·45, results well within the range we have found in Sicily. Commercial juices, generally preserved with sulphurous acid, and the syrups made from them, gave, as would be expected from the foregoing observations, much lower results. The highest titration found was 4·7 and in other cases hardly any reducing power remained.

Imported lime juice, preserved by its oil only, gave 2·2 and 1·35. When freshly pressed from limes the figures found were 4·85 and 4·9. Hassan and Basili [1932] have published experiments with Egyptian limes which led them to the conclusion that fresh juice is active but loses its activity more rapidly than lemon juice, and that the loss begins in the fruit itself during ripening or storage. The limes from which juice was pressed in London were of unknown history and probably juice from newly gathered fruit might have considerably higher values.

Imported West Indian grape fruit juice gave titrations between 4 and 5 and freshly pressed juice was not much higher. Sicilian grape fruit pressed shortly after gathering, however, gave a result comparable with that of lemon juice—7·5.

Experiments are now in course in which the reducing power of juices preserved in various ways, including juices sterilised by the Matzka process, will be followed during a period of some months and will be compared with the results of biological trials carried out with the same material.

It is hoped that the results may be the subject of a further communication.

Summary.

1. The method of titration with dichlorophenolindophenol has been applied to the examination of a number of samples of lemon and orange juices, both freshly prepared and preserved in various conditions.

2. It has been found:

(*a*) That the reducing power of fresh lemon juice is subject to considerable variation, the lowest samples examined having only 60 % of the reducing power of the highest.

(*b*) That the reducing power of orange juice is more constant and rather higher than that of lemon juice.

(*c*) That the reducing power of both juices does not diminish much in storage in the absence of preservatives, but that the use of any preservative which is efficient in preventing fermentation is followed by the gradual diminution of the reducing power which totally disappears in, at most, a few weeks.

(*d*) That the same result is brought about by strong acidification, pasteurisation or boiling.

3. It is concluded that in untreated juice the reducing factor is protected from atmospheric oxidation by the action of an enzyme, and that when this action is inhibited by any of the usual means the reducing power is rapidly lost.

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