19. Vitamin Methods

5. A Note on the Determination of Ascorbic Acid in Fruits and Vegetables in the Presence of SO_2

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Sulphur dioxide is extensively used for preserving fruits, fruit juices and fruit pulps. Since it reduces 2:6-dichlorophenolindophenol almost instantaneously, it is impossible to determine ascorbic acid in the treated products unless special precautions are adopted. Two simple procedures for overcoming this difficulty are described in the present communication.

The two methods are, in principle, as follows: (1) An extract of the material in 5% metaphosphoric acid is subjected to exhaustion *in vacuo* to remove the SO_2 . (2) Acetone is added to form the acetone-bisulphite complex, which neither reduces the dye itself nor interferes with the reduction of the dye by ascorbic acid. Both methods are relatively simple to carry out and yield reliable results if certain precautions are observed.

EXPERIMENTAL

In all the experiments reported below SO_2 was added in the form of NaHSO_3 ; frequently in commercial practice metabisulphite is used, but control experiments have shown that it is immaterial whether the SO_2 is added as sulphite, bisulphite or metabisulphite. The ascorbic acid was estimated either by the direct visual method of Birch *et al.* [1933], or electrometrically by the method of Harris *et al.* [1942].

A 5% solution of metaphosphoric acid was used to extract the material according to the method described in greater detail by Harris & Olliver [1942] and Harris *et al.* [1942].

Method I

The 5% metaphosphoric acid extract of the sulphited tissue is placed in the vacuum apparatus shown by Harris & Olliver [1942, Fig. 1] and the SO_2 is eliminated by evacuating it for 30–40 min. to remove O_2 ; the solution is agitated before evacuation by a stream of O_2 -free N_2 , and during evacuation to facilitate boiling. The SO_2 which interferes with the titration of ascorbic acid by the indophenol dye is thereby removed. It is not claimed that complete removal of the SO_2 is effected, but any reduction of the dye due to residual SO_2 is so small as to be well within the limits of experimental error of the indophenol method of estimation of ascorbic acid in natural products.

Method II

This method depends upon the formation of the bisulphite compound of SO_2 with acetone, in acid solution. To the 5% metaphosphoric acid extract of the tissue, acetone is added to a concentration of 20%,* and the resultant solution is titrated against the dye in the usual manner, save that the titration should not be completed in less than 40 sec.

* When acetone in a concentration of 20% is added to a 5% metaphosphoric acid solution there is a slight decrease in volume. In practice, to 20 ml. of the unknown solution 5 ml. of acetone are added and volume after mixing is made up to 25 ml. in a graduated flask.

The ideal time for completion is between 45 and 60 sec. If completed too rapidly low results are obtained, if completed too slowly high results are obtained. The explanation of these phenomena is given below.

Influence of acetone on the rate of reaction between ascorbic acid and indophenol. In aqueous solution pure ascorbic acid, if added in excess, reduces 2:6-dichlorophenol-indophenol at pH values below 4.0 virtually instantaneously. The lower half of Fig. 1 shows the time in seconds required for a solution of ascorbic acid to reduce an equivalent amount of the dye at different pH values in aqueous and in 20% acetone solutions. Acetone in a concentration of 20% causes a significant reduction in the rate of the reaction. In view of this finding it was of interest to determine how rapidly it was necessary to complete the titration in order to determine ascorbic acid in solutions containing acetone.

Table 1 shows some typical results obtained with solutions of ascorbic acid in water and 20% acetone.

The lower value obtained in the presence of acetone, when the titration is done too rapidly, viz. 20 sec. or less, is due to the slower rate of reduction of the dye, and for this reason it is inadvisable, as already indicated, to complete an acetone titration in less than 40 sec. The higher value resulting when the titration is completed at too slow a rate is due to the slow fading of the dye, which always occurs in acid solutions. Acetone retards this fading, and no error results from this cause if the titration is completed within 1 min.

An alternative procedure is that in which the dye is run from a burette into an aliquot of the unknown as in Tillmans' method of titration. With this procedure the rate of titration of the ascorbic acid in acetone-water solution is without much influence on the result, provided that the end-point is

taken as that at which a faint pink colour persists for 15 sec.; this is evident from Table 2. However, in the author's experience, whilst the first appearance of a faint pink colour is easily detected when the dye is titrated against a fixed amount of a pure solution of ascorbic acid, the end-point is much more difficult to detect when extracts from biological tissues are to be tested. This is especially true when such extracts are slightly coloured. For this reason, despite the precautions necessary for completing the acetone titration within a time limit, this method of titrating unknown against a fixed amount of dye is preferred. These objections to the former procedure do not, however, apply to the electrometric method of titration [Harris *et al.* 1942] in which the end-point is determined by a change in e.m.f., not in colour. In actual practice the electrometric method yields excellent results with the acetone procedure described above.

Accuracy of methods. In Table 3 are given four examples illustrating the accuracy of the method. In all cases the acetone titrations were completed within 45–60 sec., the

Fig. 1. The influence of acetone on the rate of reduction of 2:6-dichlorophenolindophenol by ascorbic acid, cysteine and reduced glutathione.

Table 1. *Effect of acetone on rate of reduction of dye by ascorbic acid (ascorbic acid added to dye)*

Time for completion of titration	Ascorbic acid in solution used mg./ml.	Description of solution	Ascorbic acid mg./ml. as found by titration (av. of duplicate titrations)
20-25 sec.	0.05	Ascorbic acid in 5% metaphosphoric acid	0.050
40-60 sec.	0.05	" " " "	0.0505
1 min. 40 sec.-2 min.	0.05	" " " "	0.0535
20-25 sec.	0.05	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.0483
40-60 sec.	0.05	" " " "	0.050
1 min. 40 sec.-2 min.	0.05	" " " "	0.052
20-25 sec.	0.04	Ascorbic acid in 5% metaphosphoric acid	0.040
40-60 sec.	0.04	" " " "	0.0404
1 min. 40 sec.-2 min.	0.04	" " " "	0.0415
20-25 sec.	0.04	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.0375
40-60 sec.	0.04	" " " "	0.0402
1 min. 40 sec.-2 min.	0.04	" " " "	0.0405
20-25 sec.	0.03	Ascorbic acid in 5% metaphosphoric acid	0.030
40-60 sec.	0.03	" " " "	0.0302
1 min. 40 sec.-2 min.	0.03	" " " "	0.0317
20-25 sec.	0.03	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.0285
40-60 sec.	0.03	" " " "	0.0304
1 min. 40 sec.-2 min.	0.03	" " " "	0.0313

Table 2. *Effect of acetone on rate of reduction of dye by ascorbic acid (dye added to ascorbic acid)*

Method of titration	Time for completion of titration	Ascorbic acid in solution used mg./ml.	Description of solution	Ascorbic acid mg./ml. as found by titration (av. of duplicate titrations)
Visual titration (modified Tillmans' procedure)	30 sec.	0.50	Ascorbic acid in 5% metaphosphoric acid	0.50
	1 min.	0.50	" " " "	0.50
	30 sec.	0.50	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.50
	1 min.	0.50	" " " "	0.50
	35 sec.	0.20	Ascorbic acid in 5% metaphosphoric acid	0.201
	2 min. 10 sec.	0.20	" " " "	0.202
	35 sec.	0.20	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.203
	2 min. 10 sec.	0.20	" " " "	0.201
	—	0.20	Ascorbic acid in 5% metaphosphoric acid	0.2009
	—	0.20	Ascorbic acid in 5% metaphosphoric acid + acetone 20%	0.200

concentration of acetone in the solution being 20%. The pH of the solution was approximately 1.5-2.0, a value which is favourable for the formation of the bisulphite compound. The ascorbic acid solutions to which bisulphite had been added were mixed with the required amount of acetone and allowed to stand 5-15 min. before titration. This delay is not however necessary, for if the solutions are titrated within 3-4 min. after the addition of acetone, the same result is obtained, within the limit of experimental error.

The results given in Table 3 indicate that even when the concentration of SO_2 is nearly 40 times greater than that of ascorbic acid, the presence of 20% acetone allows the ascorbic

acid to be estimated with an accuracy within the usual limits of experimental error of the indophenol method. Equally good results are obtainable with the alternative 'evacuation' method.

Table 3. *Estimation of ascorbic acid in presence of SO₂*

Exp.	Ascorbic acid added to solution mg./ml.	SO ₂ added to solution mg./ml.	Treatment	Ascorbic acid as found by titration mg./ml.
SO ₂ added as NaHSO ₃	0.080	—	—	0.0803
	0.080	0.32	20 % acetone added	0.0804
	0.080	0.32	Evacuation on pump in N ₂ for 30 min.	0.0798
	0.080	0.32	—	0.162*
	0.040	—	—	0.0405
	0.040	0.64	20 % acetone added	0.0403
	0.040	0.64	Evacuation on pump in N ₂ for 30 min.	0.0409
	0.040	0.64	—	0.179*
	0.040	—	—	0.0401
	0.040	1.52	20 % acetone added	0.0404
	0.040	1.52	Evacuation on pump in N ₂ for 30 min.	0.0404
	0.040	1.52	—	0.605*
	0.040	—	—	0.0402
	0.040	1.28	20 % acetone added	0.0401
	0.040	1.28	—	0.32*
SO ₂ added as Na ₂ S ₂ O ₅	0.040	—	—	0.0402

All acetone titrations completed in 45–60 sec., SO₂ added as bisulphite or metabisulphite.

* Apparent ascorbic acid values of solution.

An indication of the limits of concentration of SO₂ in the final extract for titration within which it is possible to estimate ascorbic acid with accuracy, using the acetone method, is shown in Table 4. When the concentration of SO₂ is as high as 3.7 mg./ml.

Table 4. *Limits of 'acetone' method for removing influence of SO₂*

Ascorbic acid added to solution mg./ml.	SO ₂ mg./ml. added as NaHSO ₃ to solution before titration	Treatment	Ascorbic acid as found by titration mg./ml.
0.03	—	—	0.0301
			0.0300
			0.0304
	0.9	20% acetone titration	0.0300
		" " "	0.0302
	1.44	" " "	0.0299
		" " "	0.0301
	2.3	" " "	0.0317
		" " "	0.0320
	3.7	" " "	0.0357
		" " "	0.0350

the error becomes serious. This concentration in the final extract is certainly never likely to be met with in determinations of ascorbic acid in foodstuffs in analytical practice, for even if SO₂ is present in this concentration in the original foodstuff, which is equivalent to 3700 parts of SO₂ per million, during the grinding and extraction of the food with 5 % metaphosphoric acid before titration, the concentration of the SO₂ will be considerably reduced by the action of the acid and by the dilution.

If, however, there is need to estimate ascorbic acid in the presence of a very high concentration of SO₂, most of the SO₂ can be taken off under the vacuum very quickly, as in the first method outlined in this paper, and the resulting liquid can then be diluted with acetone to immobilize any trace of SO₂ remaining. By combining both procedures

it is possible to estimate ascorbic acid by indophenol titration in any solution, no matter how much SO_2 it contained originally.

Of the two methods, that employing acetone is quicker and more convenient to use, and for these reasons is preferred by the writer.

Estimation of ascorbic acid in sulphited pulps

Table 5 gives some examples of biological materials examined. In all cases the SO_2 was added to a weighed amount of the fruit juice or pulp in the form of bisulphite and the acid extract prepared in the usual way. The amount of ascorbic acid was then estimated

Table 5. *Results with sulphited foods*

Exp.	Material	SO_2 added as NaHSO_3 mg./g.	Treatment	Ascorbic acid as found by titration mg./g.
A	Black currant purée	— 1.5	— 20% acetone added	0.77 0.77
		mg./ml.		mg./ml.
B	Orange juice (dil.)	— 0.64 0.64	— 20% acetone added Evacuated on pump in N_2 for 30 min.	0.106 0.107 0.1065
C	Orange juice	— 0.64 0.64	— 20% acetone added Evacuated on pump in N_2 for 30 min.	0.407 0.402 0.406
D	Cabbage, 4% metaphosphoric extract	— 0.64 0.64	— 20% acetone added Evacuated on pump in N_2 for 30 min.	0.0895 0.0900 0.0894
		mg./g.		mg./g.
E	Raw black currants	— 0.5	— Evacuated on pump in N_2 for 30 min.	1.74 1.76 1.86 1.81
F	Cooked black currants	— 0.5	— Evacuated on pump in N_2 for 30 min.	1.53 1.56 1.56
G	Raw raspberries	— 0.5	— Evacuated on pump in N_2 for 30 min.	0.405 0.44 0.43
H	Cooked raspberries	— 0.5 0.5	— 20% acetone added Evacuated on pump in N_2 for 30 min.	0.355 0.345 0.347
I	Plum pulp, Victorias (canned)	— 1.52 1.52	— 20% acetone added Evacuated on pump in N_2 for 30 min.	0.0413 0.0416 0.0411
		mg./ml.		mg./ml.
J	Plum juice, Victorias (canned)	— 1.5	— 20% acetone added	0.0500 0.0502

after (a) addition of acetone to, or (b) after evacuation of, the metaphosphoric acid extract of the pulp. Here again the results obtained indicate that the accuracy with which the ascorbic acid can be estimated is well within the general limits of accuracy of the method.

The influence of non-specific reducing substances. As already mentioned, acetone in a concentration of 20% causes a reduction in the rate at which the indophenol dye is re-

duced by ascorbic acid. Ascorbic acid is not, however, the only substance in biological materials which reduces the dye. Cysteine, glutathione and ergothioneine are the chief organic constituents of tissues which will reduce the dye. The two latter, however, to a less extent than the first, reduce the dye so much more slowly in acid solutions than does ascorbic acid itself that interference by them, unless in very high concentrations,

Table 6. *Estimations in presence of cysteine and glutathione*

Ascorbic acid solution mg./ml.	Cysteine added mg./ml.	Description of solution	Ascorbic acid mg./ml. as found by titration (av. of duplicate titrations)
0-0325	—	Ascorbic acid in 5 % metaphosphoric acid	0-0326
	0-0320	" " " "	0-0330
	0-0320	Ascorbic acid in 5 % metaphosphoric acid + acetone 20 %	0-0328
0-0310	—	Ascorbic acid in 5 % metaphosphoric acid	0-0310
	0-03	" " " "	0-0310
	0-03	Ascorbic acid in 5 % metaphosphoric acid + acetone 20 %	0-0314
			0-0306
			0-0314
			0-0306
	Reduced glutathione added mg./ml.		
0-0310	—	Ascorbic acid in 5 % metaphosphoric acid	0-0310
	0-03	" " " "	0-0314
	0-03	Ascorbic acid in 5 % metaphosphoric acid + acetone 20 %	0-0310
0-0350	—	Ascorbic acid in 5 % metaphosphoric acid	0-0352
	0-0350	" " " "	0-0354
	0-0350	Ascorbic acid in 5 % metaphosphoric acid + acetone 20 %	0-0349

is of little or no practical significance. Fig. 1 shows the influence of acetone on the rate at which the dye is decolorized by solutions of cysteine and glutathione in water and in 20 % acetone. From this the following conclusions are clear: (1) The presence of acetone in the solution at all pH values either decreases the rate at which the dye is decolorized by cysteine or glutathione or does not appreciably affect it. (2) Even with acetone the time for completion of the reaction in acid solutions (pH 2.0) is some 50 times more rapid with ascorbic acid than with equal concentrations of cysteine or glutathione. As already shown [Harris *et al.* 1942] this great difference in rate leads to an error of only 1 % when cysteine is present in a concentration equal to that of ascorbic acid.

An actual determination of the error involved in estimating ascorbic acid in the presence of acetone in which cysteine or glutathione are present is shown in Table 6. From this it will be seen that the error (<1 %) is no greater than when the titration is carried out in an aqueous solution.

SUMMARY

1. SO₂ in acid solution reduces 2:6-dichlorophenolindophenol instantaneously and may cause serious error in the estimation of ascorbic acid.

2. Two methods for estimating ascorbic acid in vegetable extracts containing SO₂ are described, depending in principle on:

(a) Prior removal of the SO₂ from a 5 % metaphosphoric acid extract of the food, by means of a current of N₂ and exhaustion *in vacuo*.

(b) Addition of acetone to a concentration of 20 % to the metaphosphoric acid before titration, to form the acetone-bisulphite complex which does not reduce indophenol dye. Precautions must be taken in the rate of titration; it must be completed in not less than 40 sec. and not more than 60 sec.

3. Using both methods, the limits of error in the estimation of ascorbic acid in fruit and vegetable tissues containing SO_2 are on the average less than 1 %.

4. The acetone method of titration gives results which are as reliable as those obtained by the normal method, even when cysteine or glutathione is present in equal concentration to that of ascorbic acid.

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