The Application of the Spekker Photoelectric Absorptiometer to the Determination of Vitamin C

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In a footnote to their recent paper on the reliability of the chemical estimation of vitamin C by titration with 2:6-dichlorophenolindophenol, Harris & Olliver [1942] mention the application of a procedure involving the addition of an excess of dye and instantaneous measurement of the excess of colour, in order to differentiate the rapid reduction effected by ascorbic acid from the slower reduction due to non-specific reductants. Several methods employing this principle have already been described [Meunier, 1937; Guthe & Nygaard, 1938; Evelyn, Malloy & Rosen, 1938; Bessey, 1938; Bukatsch, 1939]; in every case, however, the authors have relied on measurements made on a single mixture of test solution and dye. The measurement is complicated by the fact that, in the presence of non-specific reductants, the colour is fugitive owing to the progressive decolorization of the excess dye by the interfering materials. Meunier [1937] investigated kinetically the decolorization of 2:6-dichlorophenolindophenol by ascorbic acid, glutathione and cysteine and concluded that in the presence of the latter substances the loss of colour due to ascorbic acid could be satisfactorily assessed by making a plot of residual colour against time and extrapolating back to zero time. Subsequently other workers have also employed this device to allow for the fugitive character of the excess dye coloration.

When a Spekker photoelectric absorptiometer was employed for the determination of ascorbic acid in fruits and jams by this method it was found that fading of the colour of excess indophenol was sometimes very rapid, especially with solutions which had been treated with hydrogen sulphide for the estimation of the partially oxidized dehydroascorbic acid [McHenry & Graham, 1935]. Accuracy of extrapolation in such cases was not high and for this reason it was considered inadvisable to rely on readings of the colour due to a single addition of indophenol. An improved method, involving readings on a number of solutions with different amounts of excess indophenol, has therefore been developed.

METHOD

To a series of increasing amounts of a solution of 2:6-dichlorophenolindophenol, each diluted with distilled water to a fixed volume, are added uniform

aliquot portions of the test solution. The colour of each mixture is measured by means of a Spekker photoelectric absorptiometer, a further similar aliquot of the test solution diluted to the same volume with distilled water only being used as a standard. The reading on each mixture is corrected for fading by making three observations over about a minute and extrapolating back to zero time. The colour values of the mixtures after correction are then plotted against the amount of indophenol reagent added; the appearance of excess reagent is marked by a sudden increase in the slope of the resulting curve, the point of flexure corresponding with the end-point of the titration.

Solutions employed. (a) *Indophenol reagent.* A solution of 2:6-dichlorophenolindophenol containing 0.25 mg./ml. was found to be most suitable for the Spekker titration. To stabilize the dye 10% of Clark's phosphate buffer of pH 7.0 was incorporated. This reagent was stored in a dark bottle in the refrigerator; it was renewed after about a month and standardized every 2 or 3 days in the usual manner [Harris & Olliver, 1942].

(b) *Sample solution.* The sample solution, which may be prepared in any suitable manner, should contain preferably from 5 to 8 mg. and in any case not more than 10 mg. ascorbic acid per 100 ml.

Titration procedure. The Spekker instrument measures the residual light transmission of the test solution and in examining a series of solutions of progressively increasing colour the instrument reading diminishes continually. The instrument must therefore be adjusted to allow of a suitable range of readings being obtained before complete extinction is reached. To this end 4 ml. of test solution are added from a pipette with a rapid delivery to 4 ml. of distilled water in a 1 cm. Spekker cell and mixed in with a quick shake. The instrument is adjusted to give a reading of 1.000 on this mixture with the Spekker no. 6 light blue filter in position. If the sample solution is itself of a red colour, it may be found that the sensitivity of the instrument with the light blue filter is very low. The sensitivity can be improved by substituting a green or even a red filter, but the titration curve obtained is of much less satisfactory shape. It is preferable to retain the blue filter and balance the instrument as accurately as the reduced sensitivity will permit.

The mixture in the cell thus prepared is retained as a 'zero' check.

Readings are then made on a series of solutions to which progressively increasing amounts of indophenol reagent have been added, the amount of water being correspondingly reduced to keep the total volume at 8 ml. in each case. The end-point of the titration must be known approximately, either from a preliminary titration or from previous experience with the particular material under examination, and the additions of indophenol reagent should be chosen to allow at least two additions before the end-point is reached. After the end-point, at least five further additions should be made, the amount of indophenol reagent being increased by 0.2 ml. each time. The observations on these solutions are made in the following manner.

To the appropriate indophenol reagent-water mixture in a second 1 cm. Spekker cell are added 4 ml. of test solution from the pipette previously used and at the same time a stop-watch is started. The solutions are mixed with a quick shake and the instrument reading is observed as rapidly as possible; the reading and time are noted. The instrument is thrown out of adjustment and readjusted, and a second reading and time, and finally a third, are obtained in the same way. These readings should be made as rapidly as possible, especially if appreciable fading, indicated by a steady increase in the instrument reading, is observed; all three readings should be completed within a minute at the most. In difficult cases a convenient device to enable the first reading to be obtained rapidly is to set the instrument to the approximate reading expected, as judged from the previous readings, before adding the test solution to the indophenol reagent-water mixture. The 'zero' reading should be checked after the observations on each mixture.

Interpretation of results. A plot is first made of Spekker reading against time for each mixture examined and the curves so obtained are extrapolated back to zero time to indicate the probable initial Spekker reading of each mixture. A second plot is then made of the extrapolated initial Spekker readings against the amount of indophenol solution added for the whole series of mixtures examined. This second plot represents the variation in colour of the sample solution, diluted to a fixed volume, as progressively increasing amounts of 2:6-dichlorophenolindophenol are incorporated. It comprises two relatively straight portions: one, horizontal, corresponds to the more or less uniform colour of the mixtures in which all the colour of the added indophenol has been discharged by the ascorbic acid present; the other, inclined fairly steeply, corresponds to the progressively increasing colour of the excess indophenol. These straight portions intersect at a relatively sharp angle and the point of inter-

section corresponds with the end-point of the titration.

The accuracy of the determination of the point of intersection depends primarily upon the correct drawing of the steeper portion of the plot. In practice the individual results deviate somewhat from linearity owing to random fluctuations; it was thought possible, therefore, that visual assessment of the optimum straight line to represent these results might not be sufficiently exact to avoid the introduction of appreciable errors. This point was examined by comparing, for a number of typical plots, the results obtained by such visual assessment and by application of the method of least squares to the calculation of the slope of the steeper portion of the plot. Of seventeen results examined, only two showed a deviation exceeding 2%, while the mean deviation was $\pm 1\%$. Thus direct visual assessment, if carefully carried out, is sufficiently accurate for all practical purposes.

According to Meunier [1937] extrapolation to zero time is itself sufficient to eliminate errors due to interference by more slowly reacting non-specific reductants. If this were so, it would be possible, after a suitable calibration of the instrument, to calculate the ascorbic acid content of any mixture from its initial Spekker reading, obtained by extrapolation of experimental readings to zero time; methods of this type have, in fact, already been described [Evelyn *et al.* 1938; Bessey, 1938]. In practice, however, fading of the mixtures is sometimes extremely rapid, and this necessarily means that the accuracy of the extrapolation is correspondingly limited. In fact, as remarked above, the individual results on the steeper portion of the plot frequently deviate somewhat from the theoretical straight line, indicating that these individual results are not free from appreciable errors. The determination of the first appearance of excess indophenol, as indicated by the point of intersection on the plot, is subject to much less uncertainty. In this connexion it may be remarked that during the examination of a large number of materials by the method it was observed that, even when fading of the mixtures was very rapid and extrapolation correspondingly difficult, the shape of the final plot was always quite satisfactory.

EXAMINATION OF THE METHOD

Titration of cysteine and glutathione. Of the various non-specific reductants which may give rise to interference in the titration of ascorbic acid with 2:6-dichlorophenolindophenol, the most common are probably cysteine and glutathione. An examination of the effects of these materials on the estimation of ascorbic acid by the Spekker titration method was therefore made. In the first place titrations were carried out on solutions containing cysteine (in the

Table 1. Titration of solutions of cysteine (hydrochloride) and glutathione with 2:6-dichlorophenolindophenol

Amount present* mg./100 ml.	Indophenol required for 4 ml. test solution (ml.)				
	Cysteine (hydrochloride)		Glutathione		
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration	
6.5	0.045 (1 drop)	Nil	0.045	-0.01	
26.0	Pink colour after 1 drop, but fugitive in about 20 sec. Similar effect with further additions. Gradual increase in time required to discharge colour, but no true end-point	-0.04	0.045	Nil	
65.0	Dye added in 0.2 ml. portions. Pink colour after first addition, but fugitive within 15 sec. Similar behaviour with subsequent additions, complicated by appearance of greenish yellow end-colour. Permanent pink colour at about 12 ml.	-0.04	0.045	Nil	

* These figures represent the amount of cysteine present; the amount of hydrochloride taken was appropriately greater.

Fig. 1. Fading of solutions containing 2:6-dichlorophenolindophenol in admixture with cysteine (hydrochloride) and glutathione. (Figures attached to curves indicate amount of indophenol solution added to 4 ml. portion of cysteine or glutathione solution.) See note attached to Table 1.

form of its hydrochloride) and glutathione alone. The solutions were prepared in 6% acetic acid and contained approximately one, five and ten times the amount of ascorbic acid normally aimed at in the test solution; comparative visual titrations with in-

dophenol were made. The results are given in Table 1. Fig. 1 shows the fading curves of these mixtures; it is to be noted that, since the Spekker absorptiometer measures the amount of residual light trans-

Table 2. Recovery of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione (Paired results for the visual titration were obtained one before and one after the photoelectric titration)

Amount of interfering material present mg./100 ml.*	Ascorbic acid found, mg./100 ml.				
	In presence of cysteine (hydrochloride)		In presence of glutathione		
	Visual titration	Spekker photoelectric titration	Visual titration	Spekker photoelectric titration	
Nil	6.28 } 6.32 6.35 }	6.10	6.28 } 6.32 6.35 }	6.10	
6.5	6.40 } 6.40 6.40 }	6.16	—	—	
26.0	6.52 } 6.49 6.45 }	6.10	6.53 } 6.47 6.40 }	6.13	
65.0	7.8 } 7.8† 7.8 }	6.10	6.40 } 6.42 6.43 }	6.10	

* See note attached to Table 1.
† This figure is very approximate: no definite end-point could be obtained.

It will be observed that even in the absence of any interfering material the visual titration yields higher results than the Spekker photoelectric titration; this is to be expected, since in a visual titration a small excess of indophenol is required to produce a visible colour, whereas the photoelectric procedure should yield the exact end-point of the titration. As the amount of interfering material increases, with its attendant difficulty in the observation of the visual end-point, the apparent recovery of ascorbic acid by the visual titration increases progressively. Recovery by the photoelectric method, apart from slight random variations, remains constant.

The photoelectric titration curves of the mixtures are given in Fig. 2: all the curves are of quite satisfactory shape, although the mixtures containing large amounts of cysteine showed very rapid fading (cf. Fig. 1).

Fig. 2. Photoelectric titration curves of ascorbic acid in admixture with cysteine (hydrochloride) and glutathione. See note attached to Table 1.

mitted, a diminution of colour is indicated by an increase in the instrument reading. In both cases the rapidity of fading depends upon the concentration of each of the reactants; as indicated by the visual observations, however, fading of mixtures containing cysteine is very much more marked than that of mixtures containing corresponding amounts of glutathione; even in greatly excessive concentrations, fading due to the latter material is relatively

of reducing substance other than ascorbic acid (as shown by the shape of the fading curves in the Spekker titration) and in these cases the Spekker titration gave considerably lower results than a visual titration with indophenol. Table 3 shows some typical results.

The lemon juice showed little variation between the results obtained by the two methods, the small difference observed being attributable to the excess

Table 3. Comparison of visual and Spekker titrations of plant materials with 2:6-dichlorophenolindophenol

Material	Ascorbic acid found, mg./100 g.	
	Visual titration	Spekker titration
Lemon juice	58.2	55.7
Unripe walnuts, solution in 5% metaphosphoric acid	1628.0	1415.0
Banana honey (concentrated banana juice), solution in 6% acetic acid	Fading prevented definite end-point. First colour corresponded to about 58 mg.; fairly permanent colour corresponded to 70-75 mg.	16.0

slow. It is satisfactory that, even with the very rapid fading observed in the presence of large amounts of cysteine, the final plot approached closely to linearity and indicated only a slight negative departure from the theoretical zero titration value.

Recovery of ascorbic acid from mixtures containing cysteine and glutathione. Solutions were prepared in 6% acetic acid containing 6.5 mg./100 ml. of commercial synthetic ascorbic acid in admixture with varying amounts of cysteine (hydrochloride) and glutathione; a control solution was prepared containing ascorbic acid alone. These solutions were titrated with 2:6-dichlorophenolindophenol, both visually and by the Spekker photoelectric method. The results are given in Table 2.

APPLICATION OF THE METHOD TO NATURAL MATERIALS

The method has been applied to the examination of a range of plant materials, including both natural and manufactured products. Some of the materials examined apparently contained appreciable amounts

of indophenol required to produce a visible colour. The other materials, however, showed considerable discrepancies and, since there seems no reason to suppose that the Spekker titration could yield low results, it appears that with these materials the visual titration may be considerably in error.

SUMMARY

The Spekker photoelectric absorptiometer has been applied to the determination of ascorbic acid in the presence of non-specific reductants by a step-titration method; satisfactory recovery of ascorbic acid was obtained in the presence of cysteine and glutathione in amounts up to ten times that of the ascorbic acid itself.

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