Determination of Ascorbic Acid in Presence of Interfering Substances by the 'Continuous-Flow' Method*

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Degree of applicability of indophenol test. It is now generally acknowledged that the results of biological and chemical assays for vitamin C on numerous common foodstuffs are in good agreement. Such comparisons of the biological and chemical values are available for virtually all the better known sources of the vitamin, including large numbers of fruits and vegetables, as well as for various less active or inert foods. Likewise there is no evidence that the chemical method is any less satisfactory when used for fruits or vegetables as normally canned, or as cooked by either boiling or steaming. Examples of this kind of correspondence between the chemical and biological data have been cited in previous papers (Harris & Ray, 1933 *b*; Birch, Harris & Ray, 1933; Harris & Olliver, 1942).

In claiming that the chemical method is thus reliable for all such ordinary purposes it is of course understood that the titration with 2:6-dichlorophenolindophenol is carried out by the standard procedure, and that the usual precautions are taken: these include the inactivation of oxidases and the prevention of vitamin loss during the preliminary process of extraction, the final titration itself being rapidly performed, and in a strongly acid reaction, in order to rule out reduction of the indophenol dye by glutathione and certain other substances (e.g. Harris & Ray, 1933 *a*; Birch *et al.* 1933; Harris & Olliver, 1942).

Products containing interfering substances. To this generalization, that for ordinary foodstuffs the simple method of titration gives accurate results, there is probably only one exception of any importance, namely, with certain caramelized and fermented products (Harris, 1933; Mapson, 1943 *a*; Wokes, Organ, Duncan & Jacoby, 1943 *a*). These may contain reducing agents of the nature of reductones, which since they react rapidly with the dye in acid solution will interfere in the titration unless a modified procedure is used. We need not refer here to animal products, including urine, except to mention that the presence in the latter of thiosulphate or other interfering substances does not militate against the practical applicability of the method of determining nutritional status in the human subject by counting the number of days taken to saturate (Harris & Ray, 1935; Harris, 1943; Nutrition Society, 1945). We intend to return to a discussion of the specificity of indophenol titrations for animal materials in a later publication. Also our concern here will be primarily with caramelized foodstuffs rather than fermented preparations.

Methods for use with caramelized products. At this Institute one of the authors had

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been concerned in a study of the nutritional value of dehydrated foods. These, it was found, under exceptional conditions of preparation, namely, if exposed to unduly high temperatures during either processing or storage, were liable to become caramelized with the consequent production of the reductone-like substances. Thus a method for differentiating between the ascorbic acid and the interfering substances in them became necessary. The only quantitative test hitherto available was one depending on the different rates of condensation with formaldehyde (Lugg, 1942; Mapson, 1943*b*). This method, however, had the disadvantage of being time-consuming and relatively laborious. It was felt, moreover, that the results in the formaldehyde test would gain in conviction if they could be shown to agree with those obtained by a second method based on an entirely different principle.

The other author had previously been interested in the possibility of applying to this problem of differentiation between ascorbic acid and interfering substances the principle of the 'continuous-flow' apparatus. This apparatus had been first devised by Hartridge & Roughton (1923) for their physico-chemical investigations on haemoglobin, and provides a means whereby the rate of very rapid chemical changes may be accurately measured. It will be recalled that it was used over 10 years ago by Millikan (1935) for obtaining presumptive evidence of the identity with ascorbic acid of the reducing substances present in the wall of the gut (Hopkins & Slater, 1935); he did not, however, apply it to the actual estimation of ascorbic acid or its differentiation from other substances, which is the problem with which we have been concerned.

We undertook, therefore, a somewhat detailed study of the rates of reaction of ascorbic acid and other reducing substances, as influenced by various changes in the experimental conditions (such as pH, concentration of reactants, solvent, temperature, etc.), using the continuous-flow apparatus for the purpose. As a result we feel able to recommend a reliable and convenient technique which may be used, first, to detect whether or not the interfering substances are present, and secondly, if so, to make a quantitative estimation both of them and of the ascorbic acid. It will be shown that biological tests confirm the accuracy of this new method, and that the results agree also with those obtained by the formaldehyde method.

1. GENERAL ACCOUNT OF PROCEDURE AND FINDINGS

For simplification of presentation, a brief outline will first be given of the analytical procedure which was ultimately adopted, together with a short statement of some of the more important conclusions reached; the more detailed descriptions of the construction of the apparatus, of the technique of its use, and of the various theoretical studies, control tests and checks, can then follow more conveniently in later sections.

The continuous-flow apparatus. The equipment is shown in Fig. 1. Of two exactly similar bottles, one (*A*) contains a solution of indophenol dye of standard strength (1 ml. = 0·04 mg. of ascorbic acid), and the other (*B*) an extract of the 'unknown' made up at such a dilution as to be precisely equivalent in its total reducing capacity to the strength of the dye. The degree of dilution necessary is ascertained by means of a preliminary measurement of the total reducing titre, determined photo-electrically

(the so-called 'static measurement', § II (1), p. 12). Both standard dye and diluted extract are at pH 3·5. By means of the pumping device (*C*), operated by an ordinary filter-pump, the two solutions are forced through the mixing chamber (*D*), and into the reaction tube (*DE*). Instantaneous mixing occurs at the moment when the solutions first impinge on one another head-on, at *D*. By means of the two manometer devices (*F*, *G*) the rate of flow of the solutions out of the two bottles is kept identical, and at any desired figure. Thus the reactants after mixing flow along the reaction tube

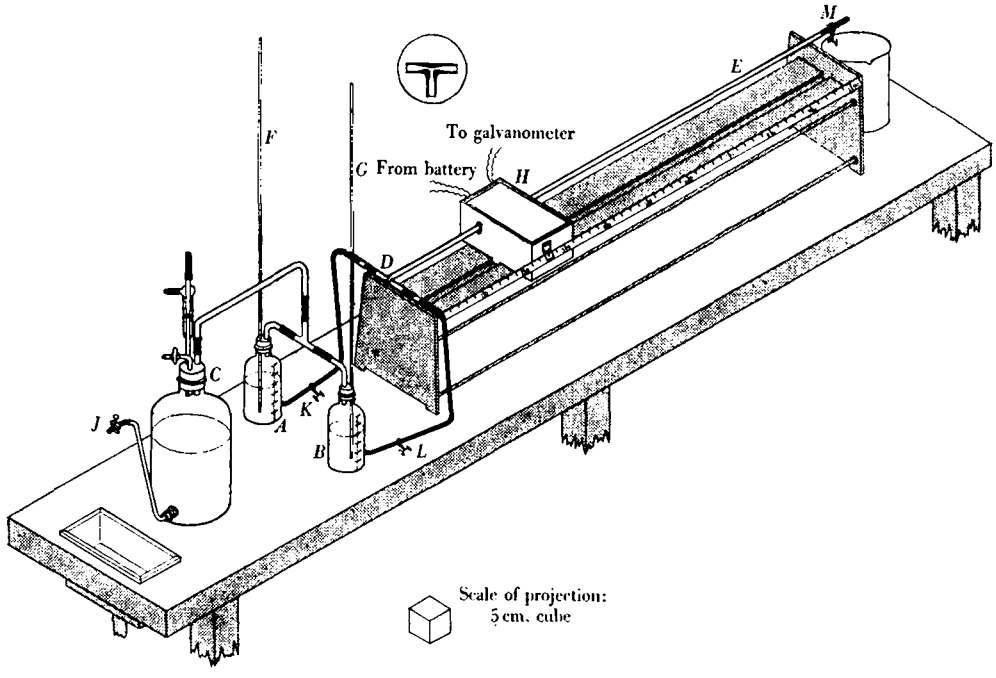

Fig. 1. Apparatus used for determination of ascorbic acid by the continuous-flow method. Isometric projection. Inset shows plan of mixing chamber on double scale.

in equivalent amounts and at a constant velocity. In consequence distances along the tube will also represent the time intervals of the chemical reaction. If, for example, the mixture flows along the tube at the rate of x cm./sec., then at distances x, $2x$ and $3x$ cm. along the tube, the reaction will have proceeded for 1, 2 and 3 sec. respectively. Now the extent of the reaction at any point is indicated by the degree of decolorization of the dye. This is determined by means of a photo-electric colorimeter (*H*) arranged to slide alongside the reaction tube, and parallel with it. Hence by plotting *colour intensities* against *distance along tube*, a continuous record of the time course of the reaction is obtained. When two separate chemical reactions are occurring simultaneously and at slightly different rates, as in the case of the ascorbic acid and the interfering substance both competing for the dye, it is possible to interpret the time curve in such a way as to measure the extent to which each of the two reactions has gone within a given time limit. From this we are able to measure the quantities of both ascorbic acid and interfering substances present.

Method of determining ascorbic acid in the presence of 'gluco-reductone'. Under the standardized conditions described, the reaction between gluco-reductone* and the dye is found to proceed considerably more slowly than that between ascorbic acid and the dye (Fig. 2). After 1·1 sec. it will be seen that only 38% of gluco-reductone has reacted, whereas with ascorbic acid the reaction is almost complete. With mixtures of ascorbic acid and gluco-reductone, the curve is found to lie in an intermediate position, depending on, and in proportion to, the relative amounts of the two substances present (Fig. 2). Hence from the position of the curve it is possible to deduce how much of either constituent is present. For this purpose, and when other interfering substances

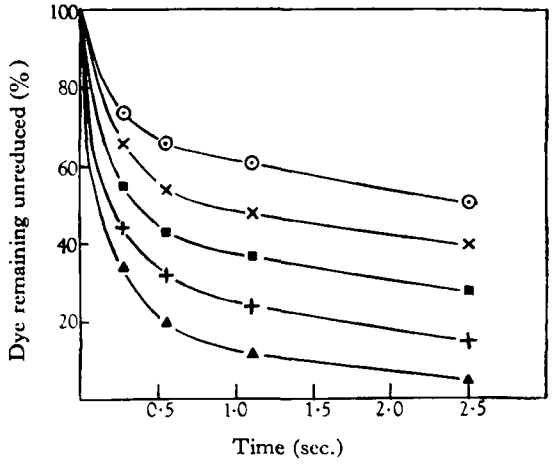

Fig. 2. Rates of reaction of ascorbic acid, gluco-reductone, and mixtures of the two, with the indophenol dye. (pH 3·5, 20°, strength of indophenol dye and total reductants ≡ 0·04 mg. of ascorbic acid/ml.) ⊙——⊙ Gluco-reductone 100%, ascorbic acid 0%; ×——× gluco-reductone 75%, ascorbic acid 25%; ■——■ gluco-reductone 50%, ascorbic acid 50%; +——+ gluco-reductone 25%, ascorbic acid 75%; ▲——▲ gluco-reductone 0%, ascorbic acid 100%.

are absent, all that is necessary is to have a simple reference curve (Fig. 3) relating the proportion of each in the mixture with the percentage of the reaction which is completed after some suitable time limit, e.g. 1·1 sec. Recovery tests indicate that this method gives results of satisfactory accuracy, viz. under the above-mentioned conditions, ascorbic acid and gluco-reductone could be determined with an error of less than 2% of the total reducing titre.

Rates of reaction of various substances with dye. The questions next arise: (1) What other substances are known which resemble ascorbic acid in their ability to reduce the dye? (2) Can they be distinguished from ascorbic acid by differences in their rates of reaction? (3) Do any of these substances actually occur in natural products or processed foods?

To answer these questions, tests will be described in § II (4) (see Fig. 8 and Table 4) to determine the velocity coefficients of the reaction of various known reductants with the dye. The conclusions reached are: (*a*) that it is almost always possible to distin-

* The term gluco-reductone is used throughout this paper to designate the several reductones which are produced when glucose is heated in alkaline solution. Reductone is used to mean the specific substance, enol-tartronaldehyde.

guish between ascorbic acid and other reductants by differences in their rates of reaction, but (*b*) that the matter is of little more than academic interest as there is no evidence of caramelized food containing reducing agents other than those of the gluco-reductone type already referred to.

Tests on ordinary foodstuffs. In the numerous raw fruits and vegetables which we have examined, the figures for vitamin C, as estimated by the flow method, agreed within the experimental error with those determined by the ordinary indophenol test. This again confirms the accuracy of the latter method, which is in agreement, moreover, with the results both of biological assays and of titration by the formaldehyde method. For steamed and boiled fruits and vegetables our conclusions are the same, although our experience here has been less extensive (see § II (5) and Tables 5 and 6).

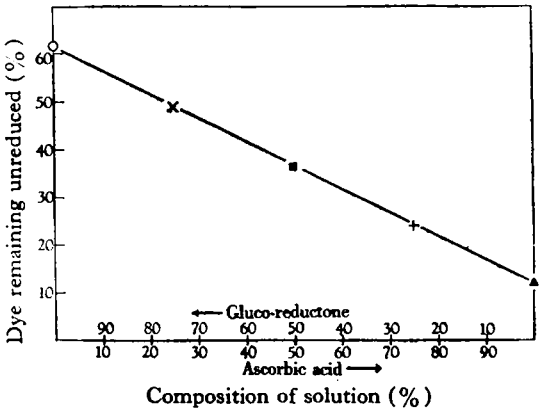

Fig. 3. Reference graph for determining relative amounts of ascorbic acid and gluco-reductone present in mixtures of the two, from the amount of dye found to be reduced in a given time. (Time 1·1 sec., 20°, pH 3·5, solutions of mixed reductants and dye each equivalent to 0·04 mg./ml. of ascorbic acid.)

Caramelized products. Our conclusion that in the case of caramelized dehydrated vegetables the interfering substance resembles gluco-reductone and that both it and the ascorbic acid can be estimated by the flow test, is based on the following observations, among others:

(1) Certain vegetable products devoid of vitamin C, after first being caramelized, were examined by the flow method, and found to respond in the test in a way resembling that of gluco-reductones but not like ascorbic acid.

(2) Conversely, biologically active vegetable products, which had not been subjected to caramelization, reacted in the way typical of ascorbic acid, and not of gluco-reductones.

(3) In recovery tests, ascorbic acid or interfering substance (either artificially prepared gluco-reductone, or the products present in caramelized dehydrated foods), or both, were added to natural foodstuffs, and it was shown that the amounts of both so added could be accurately determined.

(4) The results agreed with those obtained by the formaldehyde method. Also they were checked in a series of biological assays specially designed to be as crucial as possible.

Applicability of the new method. The general conclusion is that the method described can be used, first in a *qualitative* way; that is, to ascertain, when some new type of product is being examined for the first time, whether there is any evidence of interfering substances being present. If the test shows that the velocity of the reaction is the same as that for ascorbic acid, with the corresponding characteristic changes for pH (and if necessary for other variables), then the assumption may fairly be made that ascorbic acid only is present and further search for interfering substances is unnecessary. This is likely to be the case with all ordinary foodstuffs, and then the usual simple method of titration can be regarded as permissible and much labour is saved. If, on the other hand, as may happen with caramelized products, interfering substances are found to be present, then the method may be used in a *quantitative* way to estimate the amounts both of ascorbic acid and of interfering substance.

II. TECHNICAL SECTIONS

(1) *Initial determination of total indophenol-reducing capacity (static measurement)*

In the experiments to be described, velocities of reaction have always been determined by passing exactly equivalent amounts of the given reductant and of the indophenol dye through the flow apparatus. An accurate method for estimating the total indophenol titre of the reductant was therefore the first necessity.

With substances which, like ascorbic acid, react very rapidly, the value could be conveniently determined by simple titration in metaphosphoric acid against indophenol in the ordinary way. With other reductants, such as cysteine, which react relatively slowly, we found it better to titrate against iodine (see Lavine, 1935). With the special foods in which we were interested, however, neither of these procedures was permissible, as the extracts might have contained substances which were oxidized by iodine but not by indophenol, or which reacted only slowly with the latter. We found that the most satisfactory procedure for use in this case (as also in control tests with solutions of reductone and gluco-reductone) was to determine the total indophenol titre by means of a photo-electric colorimeter, used essentially according to the method described by Mindlin & Butler (1937) and by Bessey (1938), sufficient time being allowed, and sufficient excess of the dye added, to ensure the reaction going to completion. By this method the end-point could be determined more accurately in the presence of substances reacting slowly with the dye than by either the visual or the potentiometric methods. A further advantage over the visual method was that it could be operated with deeply pigmented solutions.

(*a*) *Photo-electric colorimeter and method of use.* A photo-electric colorimeter containing a contrast green Wratten filter, 61 N, stable to light, with an absorption range of 490–600 mμ and with a maximum transmission at 520 mμ was used. All solutions were examined in glass cells of 10 ml. capacity.

The principle of the method is to add to the unknown a constant amount of dye, in excess of that required for completing the reaction, and to estimate photo-electrically the amount of it left unchanged after the reaction. The difference between the amount of dye first added and that remaining at the end gives the measure of the total reducing titre.

(*b*) *Reference curve for galvanometer.* In order to be able to convert galvanometer readings into terms of excess of dye, reference charts were constructed as follows.

To 5 ml. of 0·5 M-phosphate-citrate buffer at pH 3·5, 1 ml. of ascorbic acid solution was added, in strengths varying from 0·0 to 0·08 mg./ml. To this mixture 4 ml. of a standard solution of 2:6-dichlorophenolindophenol (equivalent to 0·02 mg./ml. of ascorbic acid) were then added. In each instance the galvanometer reading was recorded after an interval of 45 sec. from the time of the addition of the dye. From the data so obtained a calibration chart was drawn, the ordinates giving the galvanometer readings, and the abscissae the amount of dye left unreduced, and hence by difference the reducing titre (amount of ascorbic acid present).

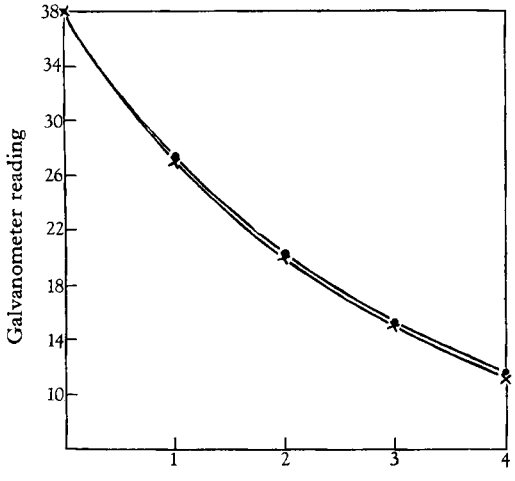

Fig. 4. Static measurement: reference chart for determining excess of dye from galvanometer reading. (Standard dye is equivalent to 0·04 mg. of ascorbic acid/ml.) ×——× After 45 sec. standing; ●——● after 5 min. standing. Spontaneous fading of dye is allowed for in these curves.

(*c*) *'Blank' correction for fading of dye.* The pH of 3·5 was purposely chosen because it is sufficiently acid to ensure a reasonably specific and rapid reduction of the dye by the ascorbic acid, but not too acid to cause any excessive rate of its destruction by bleaching. Indeed, within the time limit of 45 sec. referred to above the amount of fading was in fact ascertained to be negligible. However, in tests on food extracts, to be described below, a wait of longer than 45 sec. was sometimes necessary for the full reduction to occur. Hence an allowance for fading of the dye had to be made, although this correction was still relatively small. This correction was determined for standard periods of (*a*) 3 min. and (*b*) 5 min. For this purpose the same procedure was repeated as that described in the last paragraph, using standard solutions of ascorbic acid and dye, except for the longer periods of standing. In this way three separate reference charts (two of which are shown in Fig. 4) were prepared, one for 45 sec., one for 3 min. and one for 5 min., the two latter automatically correcting for the spontaneous fading of the dye within these respective time intervals.

(*d*) *'Blank' correction for natural colour of unknown.* In applying these reference

charts to determining the total reducing titres of 'unknowns', allowance had sometimes to be made for another factor, namely, any natural pigment present in the extracts. The procedure used was as follows. Five ml. of phosphate-citrate buffer, pH 3·5, were mixed with 1 ml. of the 'unknown', also adjusted to pH 3·5, and 4 ml. of the standard indophenol dye were added. The conditions were thus comparable with those employed for constructing the reference curves. The reading on the galvanometer scale after a chosen time interval (see below) was recorded. The excess of dye was then immediately decolorized by the addition of one drop of a strong solution of ascorbic acid, and the reading of the galvanometer for the corresponding 'blank' again ascertained. Finally, the amount of dye remaining unreduced was determined as the difference between the two galvanometer readings on the reference curve for the appropriate time interval. The difference between this figure and the amount of dye originally added is the amount of dye reduced in the given time interval.

Control tests confirm the accuracy of this procedure (see Table 1).

Table 1. *Estimation of ascorbic acid in coloured solutions*

Foreign dye present	Effect of added dye on percentage transmission ($100I/I_0$)	Ascorbic acid added (mg./ml.)	Ascorbic acid estimated (mg./ml.)	Error of estimation (%)
Edicol red	72	0·0387	0·0390	+0·8
Edicol red	84	0·0196	0·0196	0
Alizarin red	84	0·0388	0·0388	0
Alizarin red	70	0·0388	0·0392	+1·0
Brom thymol blue	85	0·0388	0·0390	+0·5
Brom thymol blue	77	0·0388	0·0392	+1·0
Thymol blue	76	0·0160	0·0157	−2·0
Cresol green	82	0·0160	0·0156	−2·5
Tropaeolin O	44	0·0160	0·0164	+2·5
Edicol brown	86	0·0387	0·0394	+1·8
Edicol brown	86	0·0194	0·0192	−1·0
Fuchsin	90	0·0387	0·0385	−0·5
Fuchsin	90	0·0194	0·0190	−2·0

(*e*) *Conditions necessary for completion of reaction* (*time; concentration of dye*). Since the object of this static measurement is to determine the total reducing capacity of the unknown, it is essential to be assured that the reaction has in fact gone to completion. The important factors are (1) that the time allowed should be sufficient, and (2) that adequate excess of dye should be added at the start.

To demonstrate the effect of concentration of dye on the rate of the reaction with gluco-reductone the following experiments were carried out. The solutions analysed contained 1 ml. of the gluco-reductone solution at pH 3·5. The appropriate amount of buffer solution (pH 3·5) was added, followed by varying amounts of standard indophenol dye (≡0·02 mg./ml. of ascorbic acid), to give a final volume of 10 ml. in each case. The amount of dye reduced was determined in the usual way by the photoelectric colorimeter.

Under these conditions the period of time necessary for complete reduction was found to diminish with every increase in the amount of indophenol dye in excess

(Fig. 5). For the estimation of total reducing power, a period of 45 sec. was too short unless very large excess of dye was added. The latter alternative, however, was not to be recommended since the percentage error of the estimation then became much greater, because of the lack of sensitiveness of the galvanometer to measure the small marginal diminution in the colour of the dye, in proportion to the large total amount present. Our procedure for estimating total indophenol-reducing capacity, therefore, has been to allow a uniform period of 5 min. to elapse after the start of the reaction, and to ensure that, at the end, an amount equivalent to at least 1·5 ml. of the standard indophenol dye is in excess. Unless such precautions are adopted variable values will be obtained, even if a fixed period is allowed for the reaction.

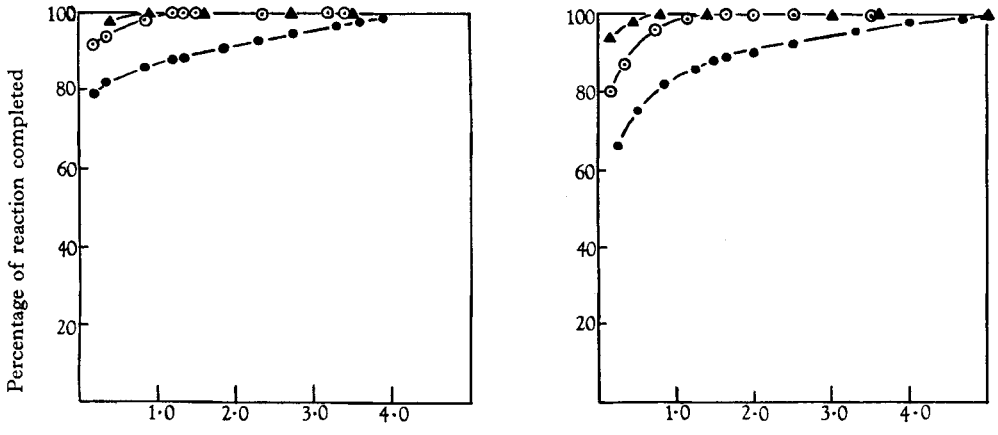

Fig. 5. Reduction of indophenol dye by gluco-reductone, as influenced by: (*a*) the excess of dye added, and (*b*) the time allowed for the reaction. ▲——▲ Reduction time of 5 min.; ⊙——⊙ reduction time of 3 min.; ●——● reduction time of 45 sec. *Left*, 'excess of dye' (abscissa) is expressed as the amount in excess of that which would be needed to complete the reaction; *right*, as the amount in excess of that actually found to have reacted within the given time limit.

(*f*) *Conditions for completion of the static reaction with caramelized vegetables.* Extracts of inert caramelized dried vegetables in metaphosphoric acid were found to reduce indophenol dye relatively slowly, compared with ascorbic acid. In their rate of reaction they resembled gluco-reductone. Moreover, as was also the case with gluco-reductone, the rate of reaction was increased by each increase in the concentration of the dye. Fig. 6 shows the relation between the percentage of the reaction completed and the concentration of dye used, in the case of an extract prepared from caramelized carrots. Other vitamin C-free caramelized vegetables behaved similarly.

As with gluco-reductone, 5 min. was found to be the most convenient period within which to estimate the total reducing titre, shorter periods involving the use of too large an excess of dye.

(2) *The continuous-flow apparatus*

The apparatus we have used is essentially the same in principle as that introduced by Hartridge & Roughton (1923), and we have modified it where necessary to meet our special requirement, namely, a determination of the velocity of reaction of indophenol

dye with ascorbic acid and with various other reductants. The salient points of the apparatus have already been described (p. 8 and Fig. 1). In order to obtain reproducible results, the conditions of use must be accurately standardized, and these will next be described.

(*a*) *Control of rate of flow*

The two reactants, indophenol dye and reductant respectively, are contained in two graduated glass reservoirs (aspirator bottles), *A* and *B* (Fig. 1), of identical shape and size (capacity about 200–400 ml.). Through the neck of each reservoir is attached a manometer tube and an inlet connected to a large aspirator (*C*) attached to a filter-

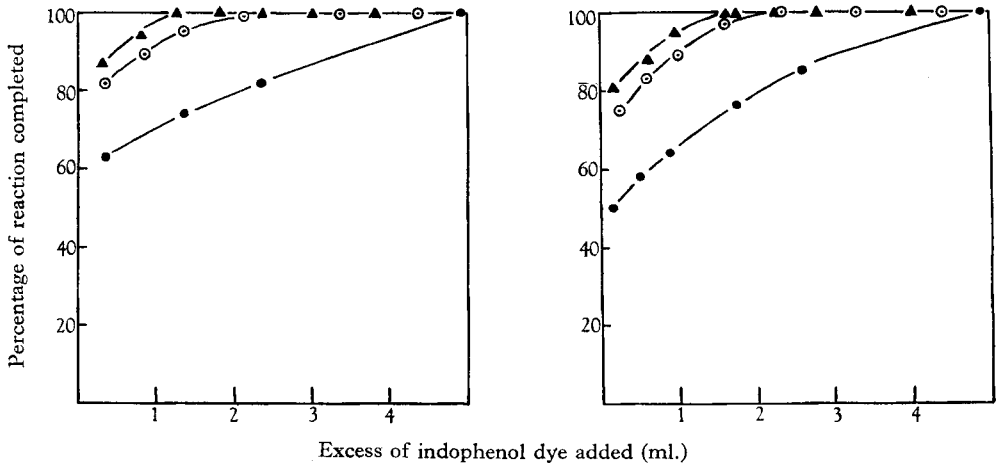

Fig. 6. Reduction of the dye by an extract of caramelized dried carrot, as influenced by: (*a*) the excess of dye added, and (*b*) the time allowed for the reaction (cf. Fig. 5). ▲——▲ Reduction time of 5 min.; ⊙——⊙ reduction time of 3 min.; ●——● reduction time of 45 sec. *Left*, 'excess of dye' is expressed as the amount in excess of that which would be needed to complete the reaction; *right*, as the amount in excess of that actually found to have reacted within the given time limit.

pump. The lower outlets lead to the side arms of the T-shaped reaction tube. The pressure in the aspirator and thus also in the two reservoirs is controlled primarily by means of a screw clip (*J*) attached to the outlet of the aspirator.

Before an estimation is begun, a slightly greater pressure is developed, by means of the pump, than that necessary to force the liquids through the reaction tube at the required rate. Clips (*K* and *L*) on the rubber tubes connecting the reservoirs with the reaction tube are next released, and then the clip (*M*) at the end of the latter. The sudden flow of liquids results in a momentary small drop in pressure; this is immediately adjusted to that required by means of the screw clip (*J*). This pressure must then be kept constant throughout the estimation.

The rate at which the solutions are delivered from the two reservoirs may be varied within wide limits by an alteration of pressure in this manner. In practice we have had occasion to use rates of flow varying from 400 to 1000 ml./min., enabling measurements of chemical change occurring in as short a time as 0·05 sec. to be measured. For most of this work a pressure producing a rate of flow of 430 ml./min. was em-

ployed. With the conditions of our apparatus, and this rate of flow, a distance of 40 cm. along the reaction tube represented a time interval of 1·1 sec. In constructing a new apparatus where conditions may not be exactly the same (as regards volume, interval, pressure, etc.), appropriate rates of flow must first be ascertained.

(*b*) *Equalization of rates of flow of dye and reductant*

In estimating the velocity of reaction between indophenol dye and a reductant, it is clearly of prime importance that the volumes of the two solutions passing through the mixing chamber in any period of time should be equal. Experiments showed that the rate of flow of water from both bottles was nearly proportional to the square root of the pressure applied. From this it can be deduced that the rate of flow was limited more by the kinetic-energy-pressure-drop of the liquid in the mixing chamber than by the viscosity of the solutions in the tube. This implies that the volumes of the two solutions passing from the reservoir were influenced more by their relative densities than by their relative viscosities.

The 2% solution of metaphosphoric acid, used as extractant, had a specific gravity of 1·013, whilst that of the indophenol dye solution was 1·000. This difference in density was sufficient to cause a small but significant difference in the rates of flow from the respective reservoirs. To obtain an equal rate of flow under any pressure, the resistance to the flow of the dye solution was increased slightly by introducing, between the mixing chamber and the reservoir containing the dye, a piece of glass tubing of somewhat smaller bore than that between the mixing chamber and the reservoir containing the metaphosphoric acid.

Procedure with solutions of high density. The densities of solutions, in 2% metaphosphoric acid, of the various pure reducing substances examined, or of extracts of many food materials, did not differ significantly from that of the 2% acid itself, and such solutions passed through the flow apparatus at a rate equal to that of the dye. Extracts of certain food materials may, however, have a higher density (see below), and when this is so additional measures must be taken to ensure equal rates of flow of dye and extract. There are several methods whereby this may be achieved. That which we found most convenient was to add sucrose to the dye solution until its specific gravity differed from that of the extract by no more than the normal margin. This normal difference, between the specific gravity of indophenol dye and of 2% metaphosphoric acid, is 0·013.

Use of sugar. This method was found to be legitimate in use, for the added sucrose had little effect on the rates of reaction of the substances studied. For example, the rate of reaction of ascorbic acid with indophenol dye was slowed by only 1–2% when sucrose was used in concentrations as high as 10%, and we have seldom found it necessary in practice to add as much sucrose as this. If solutions of higher density were to be encountered, it would seem preferable to equalize the flow by the introduction of a further resistance to the flow of dye, in the shape of the tube of smaller bore, already referred to.

An example of an experiment, showing the effect of adding sucrose to equalize the flow, follows.

Dried carrot was extracted with a small volume of metaphosphoric acid. The specific gravity of the resulting extract was 1·042. This solution was forced through the apparatus alongside indophenol dye dissolved in water, under a given pressure, the volume of extract and dye delivered from each reservoir being recorded. A similar experiment was then carried out after sufficient sucrose had first been added to the dye solution to bring its specific gravity to 1·030. The following are the respective volumes of dye and extract delivered during a flow period of 1 min.:

		Volume passing through flow apparatus (ml.)
(*a*)	Indophenol dye (sp.gr. 1·000)	215
	Extract (sp.gr. 1·042)	197
(*b*)	Indophenol dye + cane sugar (sp.gr. 1·030)	200
	Extract (sp.gr. 1·042)	199

Alternative procedures. Another method whereby equalization of flow may be obtained is to alter the relative pressures in each reservoir. We have not found this procedure as simple to operate as the first.

It should be emphasized, however, that the need to take these special measures to equalize the rates of flow only arises exceptionally, when the extract to be examined happens to be of abnormally high specific gravity. In practice this will occur only (*a*) with foodstuffs unusually rich in sugar, such, for example, as jams, or (*b*) with materials of such a low reducing activity that particularly strong extracts have to be prepared from them.

(*c*) *Measurement of rate of flow*

In all instances, no matter whether the special measures just mentioned are needed or not, it is always essential to have a system for standardizing and checking the rates of flow. This consists of: (1) the use of calibrated manometers, and (2) an actual measurement of the volume of each liquid passed into the reaction tube in the course of the experiment.

(1) *Calibration of manometers.* The rates of flow corresponding with various heights of the liquid in the manometer tube are first determined, and plotted for later reference.

When a solution of more than usual density is being used, the complication arises that the rate of flow is lowered for the application of a given pressure. Hence a slightly higher pressure than normal has to be applied to the reservoir to obtain the standard rate of flow. It is therefore convenient to have separate calibration curves for the manometers corresponding with solutions of varying densities.

(2) *Check of volume delivered.* As already indicated, the two reservoirs (*A* and *B*, Fig. 1) are graduated. Before each experiment, a trial run is carried out to ensure that equal volumes of dye and extract are being passed from the reservoir at a constant rate into the mixing chamber. Again, after each experiment the amounts delivered from each reservoir are checked, to ensure that they are equal.

(*d*) *Photo-electric colorimeter*

The photocolorimeter used with the flow apparatus is built so that it can slide alongside the reaction tube. The source of illumination is an electric bulb, operated from a 6 V. accumulator. This is used in conjunction with an appropriate lens which passes the light through an oblong shutter and focuses it on the reaction tube. The shutter is made so that its aperture can be controlled by a vernier screw. By this means, a small rectangular section of the tube (4 mm. in height × 9 mm. in length) is uniformly illuminated. The amount of light reaching the photocell can be controlled by alteration of the size of the aperture. A Wratten filter, 61 N, is incorporated in the instrument.

(*e*) *Galvanometer*

We have found a moving-coil suspension galvanometer provided with an optical lever to be suitable. The galvanometer used has a resistance of 20 Ω, and a sensitivity of 125 mm./μA. at 1 m. distance, and a period of swing of 3 sec.

(*f*) *Determination of strength of dye from galvanometer readings*

To calibrate the photo-electric colorimeter for work both at pH 1·4 and 3·5, the following procedure is used. Indophenol dye of varying strengths (see below) is placed in one reservoir, and a solution of 2% metaphosphoric acid at pH 1·4 or of 2% metaphosphoric acid, adjusted with sodium citrate to pH 3·5, in the other. With no light passing through the apparatus, the galvanometer needle is adjusted to zero on the scale. The reaction tube is then filled with water and the intensity of light falling on the photocell adjusted by means of the movable shutter, so as to bring the galvanometer pointer to the top of the scale. With the instrument so adjusted, dye and metaphosphoric acid solution is run through and corresponding readings recorded. These readings are taken at several parts of the tube; they should not differ significantly for the same dye solution.

The relationship between concentration of dye and galvanometer reading is then determined in the following manner. A solution of dye, of standard strength (exactly equivalent to 0·04 mg. of ascorbic acid/ml.), is passed through the reaction tube from one reservoir, with an equal rate of flow of the acid from the other, and the galvanometer reading is recorded. This is repeated with varying dilutions of the dye down to 10% of the standard strength. From the corresponding galvanometer readings a chart is plotted, which shows that there is an almost linear, inverse relation between the logarithm of the galvanometer reading and the strength of the dye. This is plotted on semi-logarithmic paper and used as a reference curve in subsequent work, for calculating the strength of dye at a given point in the reaction tube from the observed galvanometer reading (Fig. 7). The slope of this curve will differ for each apparatus or for each change in experimental conditions. Alternatively, the relationship can be expressed by the formula:

$$\text{strength of dye (as \% of standard)} = K\,[\log G_w - \log G_d],$$

where G_w = galvanometer reading for tube filled with water, and G_d = galvanometer

reading for dye solution of given strength. In this way the value of K may be ascertained, and used subsequently for the calculation of dye strength from the galvanometer deflexions.

(*g*) *Method of use of flow apparatus for determining reaction velocities*

In carrying out an estimation of the rate of the reaction of a reductant with indophenol dye, the following general procedure was adopted.

The zero adjustment, with no light passing through the apparatus, and the adjustment to the illumination, so as to bring the galvanometer reading to the top of the scale, are made as described in the last section. With the instrument so set and the

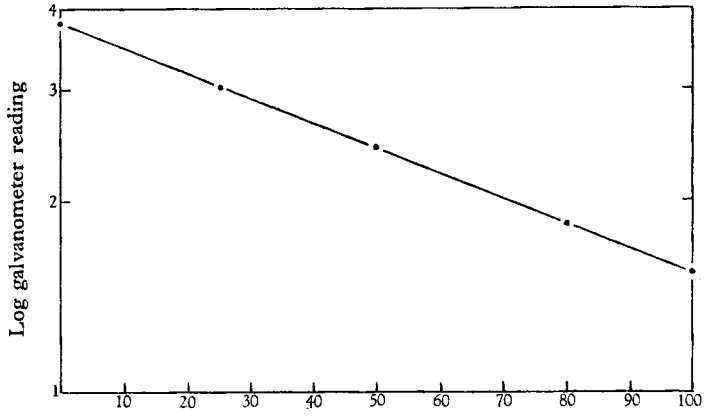

Fig. 7. Example of a reference curve, for determining strength of dye at any point in reaction tube from the galvanometer reading. (Standard strength is equivalent to 0·04 mg. of ascorbic acid/ml.)

photocell at that position on the tube at which a reading is to be recorded, the solution of the reductant and indophenol dye in equivalent strengths are then forced through the reaction tube, at equal rates of flow. First a sufficient quantity of the mixed solutions is allowed to flow through the tube to wash out all remaining water from it. The flow is next arrested and sufficient time permitted to elapse for the reductant to reduce the dye completely. This time will vary from one reductant to another and can be determined by taking periodic readings of the stationary mixed solutions until no further change in potential is registered by the galvanometer. This galvanometer reading for the 'blank' (G_b), as thus obtained, is recorded. (The difference between this reading and that given by water i.e. $(G_w - G_b)$ is a measure of the natural colour of the solution under examination.) Immediately the blank value (G_b) has been determined, the reading of the galvanometer is observed during an actual flow (G_f). The amount of unreduced dye left at this point in the tube is obtained as the difference between these two readings on the calibration chart. Alternatively, it can be calculated by the formula:

$$\text{dye remaining unreduced } (\%) = K\,[\log G_b - \log G_f],$$

K being the constant previously determined during the calibration (§ II (2*f*)). By subtracting it from the total dye originally present, the amount reduced is obtained.

From this figure for amount of dye reduced and from a knowledge of the total reducing capacity (static measurement, § II (1), the extent to which the reaction has proceeded at the given point is determined. From the known rate of flow, and the distance along the tube, the time interval may be calculated. By repeating this same procedure at other points along the tube, the proportion of the total reaction which has been completed during different intervals of time can be determined, and hence the velocity of the reaction calculated.

(3) *Estimation of ascorbic acid in presence of gluco-reductone by the continuous-flow apparatus*

(*a*) *Comparison of reaction velocities of ascorbic acid and gluco-reductone.* In the control tests to determine the rates of reaction of ascorbic acid and of gluco-reductone with the dye, the standardized conditions already specified were followed (viz. equal volumes of ascorbic acid, gluco-reductone and dye solutions; dilutions of each equivalent to 0·04 mg. of ascorbic acid/ml.; temperature 20°; pH 3·5). As has already been noted (Fig. 2) the reaction proceeds much more quickly with ascorbic acid than with gluco-reductone. Thus with ascorbic acid, after intervals of 0·28, 0·55, 0·78 and 1·1 sec. respectively, 64, 80, 84 and 87–89% of the reaction is complete, whereas with gluco-reductone the corresponding figures are 26, 34, 37·5 and 38·5%. With mixtures of the two substances (Fig. 2, p. 10), the resultant rate is in proportion to their relative amounts. It is this fact which makes possible a quantitative estimation of both when they are present simultaneously.

(*b*) *Determination of ascorbic acid in presence of gluco-reductone.* The most convenient procedure for estimating the amounts of ascorbic acid and gluco-reductone in mixtures of the two, on the above principle, was to take readings after one single time interval (1·1 sec.) and to read the answer from a specially constructed reference curve (Fig. 3, p. 11). The latter, it will be understood, relates only to the given standard conditions (namely, total reducing strength equivalent to 0·04 mg. of ascorbic acid/ml., dye at the same relative dilution, temperature at about 20°, reaction normally at pH 3·5 and a time interval of 1·1 sec.), and is not applicable to other conditions. For use with more dilute solutions (equivalent to 0·02 mg. of ascorbic acid/ml.) a second, very similar but not quite identical reference chart, was constructed.

The time interval of 1·1 sec. is chosen because after that period the reaction with ascorbic acid is largely completed (viz. to the extent of 89%), while that with gluco-reductone has not yet gone too far, i.e. there is a sufficiently wide gap between the two individual curves.

(*c*) *Recovery tests.* The validity of this procedure was checked by recovery tests. These showed that both ascorbic acid and gluco-reductone could be determined in mixtures of the two, under the conditions described, with an error of less than 2% of the total reducing titre (Table 2). With more dilute solutions (0·02 mg. of ascorbic acid/ml.) the percentage error was, of course, somewhat greater. Reference is made later (§ II (7) and Table 8) to recovery tests in which known amounts of ascorbic acid and gluco-reductone were added either (1) to inert materials, e.g. oatmeal, or (2) to

extracts of fresh vegetables in which all the reducing material behaves like ascorbic acid, and in which satisfactory recoveries were also obtained.

(*d*) *Results with 'amino-reductone'*. With what we have termed 'amino-reductone', a mixture of substances formed by heating together a solution of glucose, glycine and alkali, the curve of the reaction was similar to that for gluco-reductone, but went slightly faster (Table 3), viz. 29, 38, 41 and 44% reduction after 0·28, 0·55, 0·78 and 1·1 sec. (cf. values in § II (3*a*)).

Table 2. *Determinations of ascorbic acid in presence of gluco-reductone by the continuous-flow method*

Total reducing titre, in equivalents of ascorbic acid (mg./ml.)	Percentage composition: Present: Gluco-reductone (%)	Present: Ascorbic acid (%)	Found Ascorbic acid (%)
0·04	90	10	10
	85	15	16
	79	21	20
	61	39	38·5–41*
	60	40	38
	50	50	49·5–50·5*
	43	57	55·5
	40	60	61
	26·5	73·5	75
	15	85	87·5
0·02	70	30	34
	80	50	47–54*
	35	65	68
	30	70	74
	10	90	87
	57	43	44
	72	28	30
	85	15	16

* Range of values in multiple observations; remaining values are averages of two or three observations.

(*e*) *Effect of change of pH on velocity of reactions*. In the cases of ascorbic acid, gluco-reductone and amino-reductone, a change in the reaction from pH 3·5 to 1·4 had no appreciable effect on the curves of the reaction. This is important in view of the fact that other substances (see below) are subject to pH shifts.

(4) *Reaction velocities of various known substances*

In order to discover whether certain other reducing agents might interfere when present, and if so whether they could be allowed for in a similar fashion, measurements of reaction rates were made in the same way, and the velocity constants calculated (Table 4 and Fig. 8).

It was found that *cysteine* and *sulphite* could both be readily distinguished from ascorbic acid by their much slower rates of reaction. *Dihydroxymaleic acid*, *thiosulphates* and *ferrous* salts could be distinguished by having relatively slow rates at pH 3·5 but much faster at pH 1·4. *Reductic acid* had a velocity constant about one-half that of ascorbic, while *hydroxytetronic acid* alone of the substances examined had

approximately the same constant; there is, however, no evidence that either of these substances, or dihydroxymaleic acid, occurs in natural products or in foods as normally processed. Thus the finding of a reduction rate similar to that of ascorbic acid in these

Table 3. *Rates of reaction of gluco-reductone and amino-reductone with indophenol dye*

Reactant	Time after start of reaction (sec.)	Percentage of reaction completed pH 1·4	pH 3·5
Gluco-reductone*	0·28	28	26
	0·55	37	34
	0·78	38	37·5
	1·1	42	38–39†
	2·5	52	49
	6	63	61
	11	72	71
	16	76	76
	31	82	83
	45	85	86
	60	89	90
	120	94	95
	180	97	97
Amino-reductone*	0·28	38	29
	0·55	46	38
	0·78	50	41·5
	1·1	51·5	44
	6	72	63
	11	79	72
	16	83	76·5
	31	89	83
	60	93	89
	120	99	95
	180	100	98

* Concentrations of reactant and of 2:6-dichlorophenolindophenol equivalent to 0·04 mg. of ascorbic acid/ml.; temperature 20°.

† Range of values in multiple observations; remaining values are averages of two or three observations.

Table 4. *Velocity constants of reactions of various reactants with indophenol dye*, 20°, pH 1·4 *and* 3·5

	K, calculated, in all cases, for 10^{-3}N × sec., and as for a bimolecular reaction pH 1·4	pH 3·5
Ascorbic acid	30 ± 2	30 ± 2
*iso*Ascorbic acid	30 ± 2	30 ± 2
Hydroxytetronic acid	35 ± 2	35 ± 2
Reductic acid	17 ± 1	17 ± 1
Dihydroxymaleic acid	167 ± 2	25 ± 1
Reductone (Euler)	0·54 ± 0·08	0·54 ± 0·08
Cysteine	0·048 ± 0·06	0·048 ± 0·06
Sulphite	0·20 ± 0·02	0·20 ± 0·02
Thiosulphate	25 ± 1	1·5 ± 0·04
Ferrous ions	86 ± 2	7·4 ± 0·8

instances is of little or no consequence in relation to the practical analytical problem of the quantitative estimation of ascorbic acid in foodstuffs and food preparations.

The experimental conditions, and the more detailed conclusions, for each of these substances, were as follows.

(*a*) L-*Ascorbic acid* (Fig. 8). The reaction is bimolecular, and the velocity constant calculated on that basis (0·001 N, 20°, pH 1·4 or 3·5) was found to be 30 ± 4. This agrees well with Millikan's (1935) value, 30 to 34 ± 5.

(*b*) iso*Ascorbic acid* (D-*arabo-ascorbic acid*). This is an artificial product, a stereoisomer of L-ascorbic acid, not known to be present in any natural foodstuff, but having some biological activity. Its velocity constant at pH 1·4 or 3·5 was identical with that of ascorbic acid.

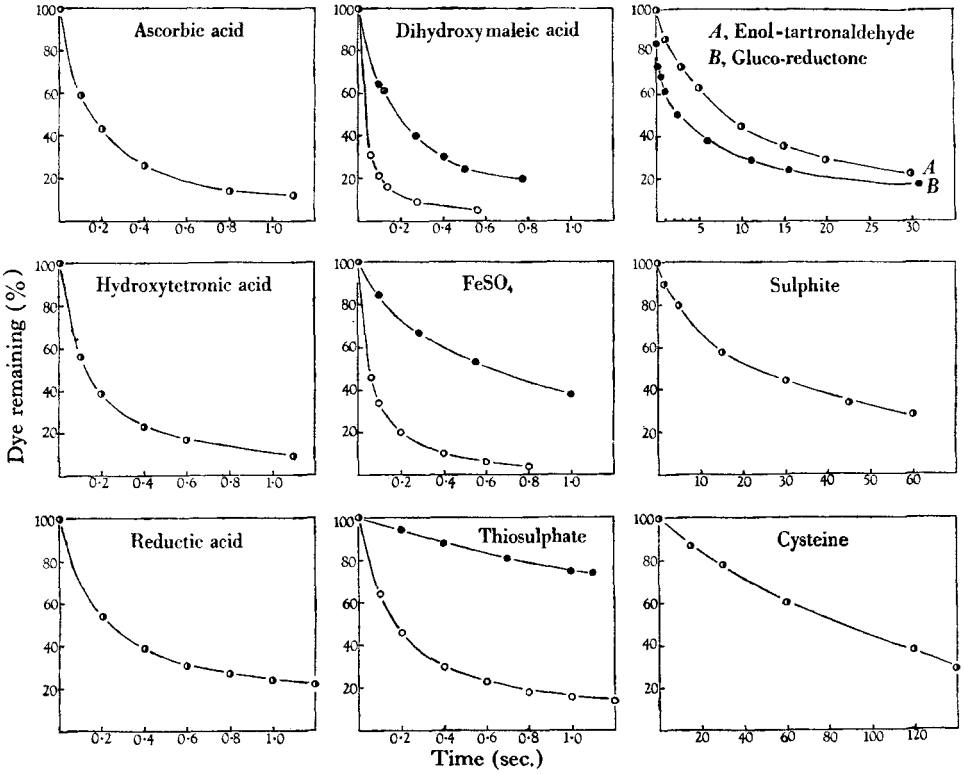

Fig. 8. Rate of reduction of 2:6-dichlorophenolindophenol by ascorbic acid and other substances, under standardized conditions. ●——● at pH 3·5; ○——○ at pH 1·4; ◑——◑ at pH 3·5 and 1·4. Indophenol dye and each reductant present in equivalent strengths (viz. ≡ 0·04 mg. of ascorbic acid/ml.)

(*c*) *Cysteine.* It has previously been pointed out (Harris, Mapson & Wang, 1942) that free cysteine, which is not normally present in fresh foodstuffs, does not in any case introduce any serious error in the estimation of the vitamin, provided that the titration is done rapidly according to the standard procedure (Harris & Ray, 1933 *a*, *b*; Birch *et al.* 1933; Harris & Olliver, 1942). It was, nevertheless, of interest to determine the precise value of the velocity constant. As will be seen (Table 4) it reduces the dye at a rate some 600 times slower than that of ascorbic acid, under the conditions studied.

(*d*) *Sulphite and thiosulphate.* Sulphite is of interest because of its presence in many preserved foods. Methods are already available for estimating it and ascorbic acid in

presence of each other (Harris *et al.* 1942; Mapson, 1942). Thiosulphate is said to be present in urine, and methods have likewise been described for its differential estimation (e.g. van Eekelen & Emmerie, 1935). It will be noted that at pH 1·4 the reaction of thiosulphate is nearly as rapid as that of ascorbic acid, but at pH 3·5 much less rapid; sulphite reduces much less slowly at both pH values.

(*e*) *Ferrous salts.* Since the ferrous ion is slowly autoxidized even in acid solution, it was necessary to take special measures for its protection. These consisted of dissolving $FeSO_4.7H_2O$ in 2% HPO_3, previously rendered free of oxygen, and after checking the strength by the static titration against indophenol, determining the velocity coefficient immediately in the flow apparatus. The distinguishing feature again is the much slower rate of reaction compared with that of ascorbic acid at pH 3·5, although at pH 1·4 the rate of reaction is faster than that of ascorbic acid. When ferrous salts are present together with ascorbic acid, several alternatives are of course available for estimating both, e.g. the formaldehyde method (Mapson, 1946).

(*f*) *Reductone.* A specimen was isolated from its lead salt from a caramelized solution of glucose (Euler & Martius, 1933) and recrystallized five times from ethanol. The rate of reaction (Table 4) is some 50 times slower than that of ascorbic acid, and is unaffected by change of reaction from pH 1·4 to 3·5.

(*g*) *Gluco-reductone.* A solution was prepared: (*a*) by treating a 2% solution of glucose with a few mg. of KCN, and heating at 90° for 2½ min. in presence of 0·17N-NaOH (Euler & Martius, 1933), or (*b*) by heating a 0·5% solution of glucose with 0·05N-NaOH for 10 min. at 80° (Kertesz, 1934). Variations in the time of heating or in the strength of the soda, within these limits, were found to produce no significant changes in reducing properties. The solutions were acidified with 20% HPO_3, cooled, brought to pH 3·5 by addition of sodium citrate solution, and diluted to approximately the correct reducing titre when tested by the static method, and finally more accurately adjusted.

To determine the rate of reaction after periods greater than 2·5 sec. the flow was arrested in the reaction tube, and readings taken at intervals at a fixed point.

It will be noted (Figs. 8, 9) that with gluco-reductone the reaction is relatively rapid during the first sec., and that a slower reaction then follows. This indicates again that a solution of this kind behaves differently from a single, pure substance. Apparently at least two reductants are present. For this reason it is not possible to calculate a velocity constant.

In comparison with reductone itself (enol-tartronaldehyde), the rate of reduction of the gluco-reductone mixture is greater initially. There was a scarcely perceptible increase in the rate of reaction on changing the pH from 3·5 to 1·4.

(*h*) *Other reductones.* Similar reducing solutions were prepared from xylose, fructose, galactose, mannose and arabinose by heating with alkali. These solutions reduced the indophenol dye at a rate not significantly different from that of gluco-reductone.

(*i*) *Amino-reductone.* The production of reductones is known to be influenced by the presence of amino-acids in solution (Enders, 1943). A solution of amino-reductone was prepared by treating a solution of glucose (1%) with a few mg. of KCN and NaOH

(0·17N) at 90° for 2½ min. The product was acidified and the concentration adjusted to standard strength as found necessary after the usual static measurement (§ II (1)). This solution differed from gluco-reductone by (1) the development of an appreciable orange or brown colour on standing, and (2) a slight fall in total indophenol titre during the first 1–2 hr. after preparation. The rate of reaction was a little greater than that of gluco-reductone at pH 3·5, and was increased slightly by a change of pH from 3·5 to 1·4.

(*j*) *Reductic acid.* This substance is formed when certain sugars are heated in presence of strong mineral acid (Reichstein & Oppenauer, 1933; cf. Snow & Zilva, 1944). From its structure it might be expected to react with indophenol in a manner similar to ascorbic acid. The constant was found to be about half that of ascorbic acid, and it was not affected by change of pH from 3·5 to 1·4.

(*k*) *Dihydroxymaleic acid.* Determination of the velocity constant was rendered difficult by the fact that this substance decomposes into glyco-aldehyde and CO_2 in acid solution (Franke & Brathuhn, 1931). In order to minimize the decomposition, a strong solution was prepared in ethanol, a small portion of it diluted with sufficient HPO_3, and the strength quickly determined, and then diluted to the necessary degree. The noteworthy feature of the reaction was the great increase in the velocity constant with raised acidity (Table 4).

(*l*) *Hydroxytetronic acid.* Again, as was to be expected from the closely similar chemical structure, the rate of reaction of hydroxytetronic acid was almost the same as that of ascorbic acid, and was unaffected by a change of reaction from pH 3·5 to 1·4 (Table 4).

(5) *Tests on fresh and cooked fruits and vegetables*

Fresh fruits and vegetables

Extracts prepared from various fresh fruits and vegetables were examined by the procedure which we have described above. It was found that the reducing substances present reacted with indophenol dye, both at pH 3·5 and 1·4, at a rate indistinguishable from that of ascorbic acid (Table 5). This gives further evidence for the view that in such foods there are no significant amounts of indophenol-reducing substances other than ascorbic acid (Birch *et al.* 1933; Harris & Olliver, 1942; Mapson, 1943*b*).

Experimental details. The technique used was as follows. Extracts of the fresh material were made by grinding up a weighed quantity of the fresh fruit or vegetable with a small amount of 20% metaphosphoric acid. Sufficient water was then added to reduce the concentration of metaphosphoric acid to 2%, and the reaction adjusted to pH 3·5 by sodium citrate solution. The total reducing activity of the extract was determined by the static measurement (§ II (1)), and the solution diluted to the extent necessary to make it equivalent to 0·04 mg. of ascorbic acid/ml. The rate of reaction was then determined in the flow apparatus. The same procedure was repeated with the reaction at pH 1·4.

Reducing substance in black-currant skins. The only exception which we have so far

encountered to the generalization that the reducing substance in fresh fruits and vegetables has the characteristics of ascorbic acid, is that a small amount of some other unidentified reductant was found in the skin of black currants but not in their juice. Since the skin of the fruit represents so small a proportion of the total berry, this finding, although of interest, seems of little practical significance.

Table 5. *Rates of reaction of ascorbic acid, and of extracts made from fresh fruits and vegetables, with indophenol dye* (20° *and* pH 3·5)*

Material tested	Percentage of reaction completed after 0·28 sec.	0·55 sec.	0·78 sec.	1·1 sec.
Ascorbic acid	64	80	84	87–88
Parsley	65	78	84	86
Brussels sprouts	65	80	85	89
Marrow-stem kale	63	79	84	86
Potatoes	63	78	83·5	86
Curly kale	64	79	84·5	86·5
Broccoli leaf	64	76	83	87
Spring cabbage	66	78	83	86
Broccoli	63·5	77	82	86·5
Spinach	66·5	79·5	84	88
Lettuce	65	79	83·5	88
Peas	65	80	84	88
Nasturtium leaves	64	80	83·5	88
Carrot tops	64	78	83·5	86
Orange juice	65	79	84	88·5
Tomatoes	63	78	83·5	86
Cress	66	79	84	87
Lemon juice	64	77	82	86·5
Grass	64·5	79	84	87
Gooseberries	66	79	84	88
Strawberries	63	80	84	88
Raspberries	64·5	78	—	86
Red currants	65	77	83	86
Black currants: whole (sample 1)	62	73	80	83
whole (sample 2)	58	72	—	81
skinned	64·5	78	83	87
Black-currant skins: sample 1	44	59	—	73
sample 2	41	48	—	62
Walnuts, unripe whole: sample 1	64·5	78·5	84	87
sample 2	64	78	—	87

* Concentrations of reactant and dye as in Table 3.

Cooked vegetables

A few experiments were conducted with vegetables cooked by the ordinary methods of boiling or steaming, and with various canned fruits and vegetables. The results (Table 6) give no evidence of the presence of any interfering substance.

It is recognized, of course, that when vegetables are cooked in the presence of excess of alkali with the pH above 8·5 (Allen & Mapson, 1944), or raised to a sufficiently high temperature to cause caramelization, as, for example, in the preparation of chip potatoes, interfering substances resembling gluco-reductones are formed. These may be differentially estimated by the technique described in this paper.

(6) *Tests on caramelized dehydrated vegetables*

The object of this section will be to describe control experiments in which caramelized dried vegetables were used as the test material.

Dried vegetables were taken which contained no vitamin C, or in which, if present, it could be destroyed by exposure to light. This dried product was then caramelized by heat, with concomitant production of the interfering substances. These, it was shown (e.g. Fig. 9 and Table 7) closely resembled gluco-reductone or amino-reductone in their rate of reaction with indophenol dye and could be estimated in the same way.

Table 6. *Rates of reaction of extracts made from cooked fruits and vegetables with indophenol dye* (20° *and* pH 3·5)*

	Percentage of reaction completed after		
Cooked fruit or vegetable	0·28 sec.	0·55 sec.	1·1 sec.
Spring cabbage (boiled)	65·5	78·5	87·5
Spring cabbage cooking liquor	65	79	87·5
Savoy cabbage (boiled)	64	78·5	86·5
Brussels sprouts (boiled)	65	78	87
Brussels sprouts cooking liquor	65	78	87·5
Brussels sprouts (steamed)	64·5	78	87
Bottled gooseberries	64	78·5	86
Spinach (boiled)	62·5	76·5	86

* Concentrations of reactant and dye as in Table 3.

Materials used. The dried vegetables studied included carrots, swedes, potatoes and cabbage. In the case of the three first-mentioned products, specimens were specially selected in which the amount of indophenol-reducing substance was nil or negligible. In the case of the cabbage, and where necessary with the other products also, the ascorbic acid present was destroyed by the following procedure. The powdered dried vegetable, after being well moistened with water, was exposed to a 500 W. lamp. The ascorbic acid rapidly fell to an insignificant figure (e.g. after 12–15 hr. illumination to less than 6 mg./100 g. of dried cabbage) without the production of other indophenol-reducing substances.

Production of caramelization. The reducing substances with which we are concerned are produced when dried vegetables are (*a*) dried at abnormally high temperatures or (*b*) stored for moderate periods at temperatures above 25° (Mapson, 1943*b*). The amount formed varies for each vegetable and is affected by other factors (Brightwell & Mapson, 1946); at 100° the formation is rapid.

In our experiments, vitamin C-free dried products, as described above, were finely ground, sieved, and heated on shallow trays in an oven at 100° for periods varying from 1 to 48 hr. Under these conditions browning quickly occurred (see also Tomkins, Mapson, Allen, Wager & Barker, 1944). With carrots and swedes the product was a dark brown powder, and with cabbage and potatoes light brown. These powders were extracted with 2% metaphosphoric acid. The colour of the resulting filtrates ranged from light yellow to deep brown. The amounts of reducing substance produced are shown in Table 7, second line of figures. It is unlikely that in practice any more highly

caramelized vegetables would be encountered than those here described. The reducing titres of the metaphosphoric-acid extracts decreased slowly on standing, unless the solution was rendered free of oxygen (as by means of a stream of nitrogen).

Reaction curves of the interfering substances present. The total indophenol-reducing capacities of the metaphosphoric-acid extracts of these caramelized dried vegetables

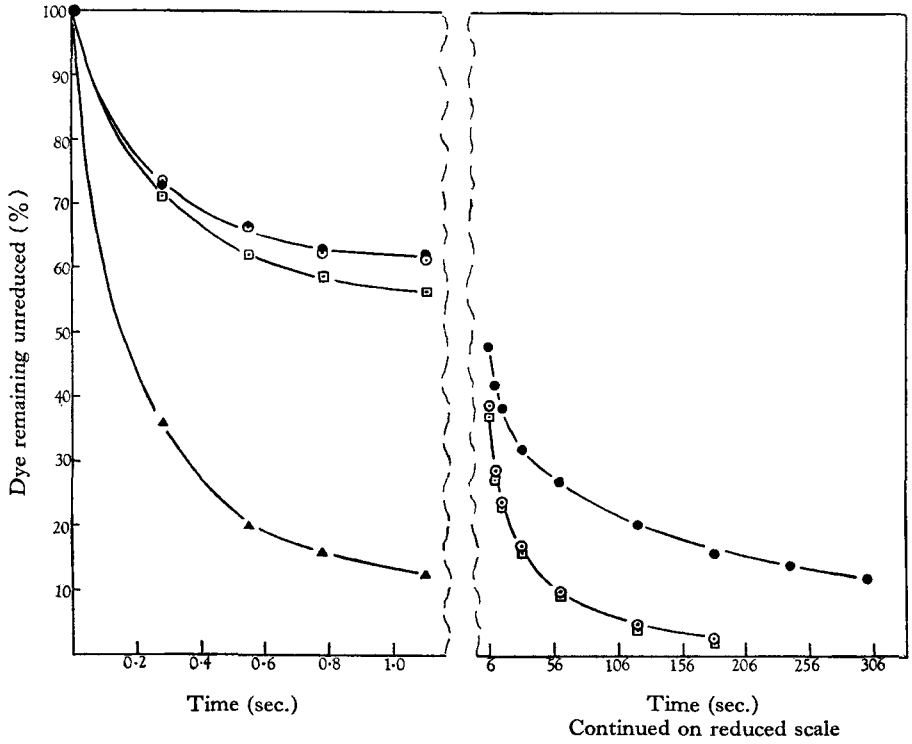

Fig. 9. Rates of reaction of gluco-reductone, amino-reductone, extract of caramelized carrot, and ascorbic acid for comparison. (pH 3·5, 20°, strength of indophenol dye and total reductant ≡ 0·04 mg. of ascorbic acid/ml.) ⊙——⊙ Gluco-reductone; ⊡——⊡ amino-reductone; ●——● extract of caramelized carrot; ▲——▲ ascorbic acid.

were first determined in the static photo-electric colorimeter (§ II (1)), and the extracts diluted as necessary to bring them to the standard strength (equivalent to 0·04 mg. of ascorbic acid/ml., § II (2)). The reaction curves were then followed in the flow apparatus at pH 1·4 and 3·5.

It was found, as with gluco-reductone, and in contrast with ascorbic acid, that the rate of reaction was slightly faster at pH 1·4 than at 3·5. Moreover, within the first 1–2 sec. of the reaction at pH 3·5, the reaction curve was almost indistinguishable from that of gluco-reductone, and after the standard time period of 1·1 sec. the percentage reduction was identical with it within the experimental error.

Although, as just mentioned, the reaction curve of the caramelized material at pH 3·5 so closely resembled that of gluco-reductone during the first second or two of the reaction, an appreciable difference could be detected after more prolonged periods, the reaction at this stage proceeding somewhat more slowly (Fig. 9). Also, the longer

Table 7. *Rate of reaction of extracts made from inert caramelized dried vegetables with indophenol dye, at* pH 3·5 *and* 1·4 (20°)
(For comparison with values for gluco-reductone and amino-reductone, see Table 3.)

		Percentage of reaction completed. Product (with no. of hours heated at 100°, and indophenol titre)*													
		Swede					Carrot				Potato			Cabbage		
Time after start of reaction (sec.)	pH	1½ hr. 1·36 mg.	2 hr. 1·56 mg.	3½ hr. 2·30 mg.	4½ hr. 2·45 mg.	10–16 hr. 3·10 mg.	3 hr. 0·92 mg.	6 hr. 1·57 mg.	12 hr. 1·77 mg.	24 hr. 2·24 mg.	16 hr. 0·95 mg.	29 hr. 0·99 mg.	48 hr. 1·00 mg.	1 hr. 0·75 mg.	3 hr. 1·27 mg.	27 hr. 2·20 mg.
0·28	3·5	28	28	28	28	33	27	29	30·5	33·5	29	29	30	—	—	30
0·55		33·5	34·6	33	36	38	33·5	34·7	36	39·5	36	36	37	—	—	36
0·78		37	37	37	37	39	37·5	—	—	—	37	37	—	—	—	—
1·1†		**38**	**38**	**38·5**	**38**	**40**	**38·5**	**37·5**	**39**	**42**	**39**	**38·5**	**40**	**37**	**36·5**	**39**
6·0		48	49	51	48	53	52	48	49	53	53	53	53	51	51	53·5
11		55	55	59	55	59	58	54·5	55	59·5	61	60	59·5	58	58	60
16		59	60	61	59	62	61·5	57·5	59	62·6	65	65	63	62	62	63
31		66	65·5	68	65	68	68	63	65	68·6	71	70	69	68	68	68·5
60		74	74	75	74	74	73	70	69·5	75	76	76	74·5	75	79	75
120		80	81	81	81	81	79·5	79	79	81	81	80	81	82·5	83	81
180		84	84	84	84	86	83·5	83	83	86	84·5	83	85	88	87	86
240		88	89	88	88	88	86	86	88	89	87	88	87	—	90	88
300		89	90	90	90	90	88	88	90	92	90	90	90	94	92	90·5
0·28	1·4	—	34	—	35	40	—	35·5	—	40	36	40	39	—	—	—
0·55		—	40	—	42	46	—	44	—	46	46	47	46	—	—	—
0·78		—	41·5	—	—	—	—	46	—	49	—	—	—	—	—	—
1·1		—	43	—	45	50	—	47	—	52	52	53	53	—	—	—
6·0		—	56	—	59	65	—	59	—	68	69	67	70	—	—	—
11		—	62	—	65	73	—	66	—	75	75	74	75	—	—	—
16		—	66·5	—	69	78	—	70	—	78	80	78	79	—	—	—
31		—	71	—	76	85·5	—	77	—	84	86	84	86	—	—	—
60		—	81	—	82·5	90	—	83	—	88	89	87	88	—	—	—
120		—	90	—	91	96	—	91	—	96	95	95	95	—	—	—
180		—	96	—	96·5	99	—	96	—	99	97	99	99	—	—	—
240		—	—	—	—	—	—	97	—	—	—	—	—	—	—	—
300		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* As equivalent of mg. of ascorbic acid/g. of heated product (estimated in static, photo-electric colorimeter 5 min. after addition of dye).
† Standard time interval, for comparison with the corresponding values in Table 3.

the dried vegetable had been heated, and the greater the consequent degree of caramelization, the more closely did the reductant produced resemble, in the initial stages of the reaction at pH 1·4, an amino-reductone solution, and the less a simple gluco-reductone solution (Table 7). However, even with highly caramelized products, the rate of reaction at pH 3·5 within the first 1–2 sec. is not appreciably different from that for those which are less highly caramelized, or from that of a gluco-reductone solution itself; and, in any case, such excessively caramelized products are not likely to be encountered in practice, because the maximum time of heating dried vegetables, such as swede, carrot or cabbage, is not likely to exceed 6 hr. at 100°.

We are thus able to conclude that, as with gluco-reductone solutions, a reaction of pH 3·5 and a time interval of 1·1 sec., as in Fig. 3, serve for the quantitative differentiation of the interfering substance in caramelized vegetables from the ascorbic acid.

(7) *Validity of results. Recovery tests. Biological comparisons*

The evidence so far presented indicates:

(*a*) That gluco-reductone could be quantitatively distinguished from ascorbic acid by comparing their reaction-time curves with indophenol dye, most simply by measuring the extent of the reaction after 1·1 sec. at pH 3·5 (§ II (3*a*)).

(*b*) That other reducing agents, particularly those known to be present in natural foods, could generally be distinguished from both ascorbic acid and gluco-reductone by the reaction curves, also at pH 3·5 and 1·4 (§ II (4)).

(*c*) That in fresh, or boiled, steamed or canned fruits and vegetables, the time curve is characteristic of ascorbic acid, and there is no evidence of the presence of interfering substances (§ II (5)).

(*d*) That in caramelized products the interfering substance formed resembles gluco-reductone (or amino-reductone), and no evidence could be obtained of the presence of other types of reductants (§ II (6)).

This leads us to the conclusion that both the ascorbic acid and the interfering substances mentioned in the last paragraph should be capable of estimation by the procedure we have described. Further evidence for this conclusion will be presented in this section, as follows:

(*e*) Whereas caramelized dehydrated products known to be devoid of vitamin C on being submitted to test by the flow method were shown to contain the interfering substance but no ascorbic acid, other dehydrated vegetables of known antiscorbutic activity, but not caramelized, were shown by the result of the flow test to contain ascorbic acid but no interfering substance.

(*f*) In recovery tests, a quantitative estimate was found possible of ascorbic acid and gluco-reductone or both added to various dehydrated vegetable products or other foodstuffs already containing:

(1) ascorbic acid without the interfering substances,

(2) the interfering substances without the ascorbic acid, or

(3) both ascorbic acid and interfering substances together.

(*g*) The results of the determination of ascorbic acid, both in caramelized dehydrated foods in presence of the interfering substance, and also in certain other foods, agreed

with the results obtained by the independent methods of (1) formaldehyde test and (2) biological assay.

Limits of error. Some account must also be given of the percentage accuracy of the method. It was found that, under the conditions we employed, the recovery of added ascorbic acid or interfering substance was of the order of 97–103% of the theoretical, expressed as a percentage of the total reducing titre.

Table 8. *Recovery of added ascorbic acid and gluco-reductone*

	Reducing activity, percentage of total*		
Extract	Gluco-reductone added	Ascorbic acid added	Ascorbic acid found
Oatmeal	0	100	100
	100	0	0
	50	50	49
	20	80	79
	80	20	19
	Gluco-reductone added	Natural ascorbic acid present	Ascorbic acid found
Kale	0	100	99
	48·5	51·5	55
	64	36	35
	84	16	15
	20	80	80
Brussels sprouts	0	100	99
	53	47	50
	41	59	59
Potatoes	0	100	98
	29	71	69

* Total concentration of reductants and dye as in Table 3.

Recovery tests: experimental details. Dehydrated vegetables containing no ascorbic acid were heated at 100° for varying periods of time. Extracts of the caramelized products were made in 2% metaphosphoric acid, and brought to pH 3·5 with sodium citrate buffer. Their total indophenol-reducing activity was estimated in each case in the photo-electric colorimeter at pH 3·5, and the solution then diluted to strengths equivalent to 0·04 mg. of ascorbic acid/ml. To these solutions known amounts of solutions of ascorbic acid (0·04 mg./ml.) were added, and the mixtures examined in the flow apparatus, the standard strength of indophenol dye being used. The amount of dye left after 1·1 sec. was determined in the usual way and the reference curve (Fig. 3) used to estimate the amount of ascorbic acid present.

For the other recovery tests (p. 31) the technique used was similar. The findings are given in Tables 8 and 9.

The results of individual analyses generally showed an agreement within about ±3% (Tables 8, 9). In practice, however, duplicate or even triplicate analyses on each solution were made, and an average taken. In most instances these average values, so determined with the use of the standard reference curve, agreed with the amount of ascorbic acid added to within ±2%.

Agreement with formaldehyde method. Comparisons of the results by the flow or formaldehyde method were made, with a large number of fresh, processed and dried caramelized and uncaramelized dehydrated fruits and vegetables and with certain other foods. Some of these preparations had been processed or stored in such a way as to produce caramelization of varying degrees; others were subjected to heat treatment in the laboratory. The agreement between the results of the two methods (see

Table 9. *Estimation of ascorbic acid added to caramelized dehydrated vegetables*

Caramelized material	Serial no.	Ascorbic acid as percentage of reducing titre: Added	Found	Av. found
Dried carrot, heated 24 hr. at 100°	1	11·5	11, 9, 10	10
	2	28	26, 27·5, 29	27·5
	3	46	47·5, 48·5	48
	4	63	61·5, 62·5	62
	5	82	80, 80	80
Dried carrot, heated 3 hr. at 100°	1	10	13, 12·5, 13	13
	2	19·5	19, 18·5	19
	3	40	40, 42	41
	4	71·5	71, 72·5	72
Dried potato, heated 36 hr. at 100°	1	12	10, 10	10
	2	20	17·5	17·5
	3	37·5	32·5, 34·5	33·5
	4	63	65	65
	5	84	87, 84	85·5
Dried swede, heated 12 hr. at 100°	1	12	16, 11·5	14
	2	26·5	28·5, 26·5	27·5
	3	42	43, 41·5	42
	4	75·5	74·5, 73·5	74
	5	90	93, 91	92
Dried swede, heated 1½ hr. at 100°	1	7·5	7, 6·5, 7	7
	2	10·5	8·5, 8, 8	8
	3	15	12, 14	13
	4	19	14·5, 14·5, 15·5	15
	5	32·5	29·5, 32	31
	6	72·5	74, 70, 75	73
	7	80	78, 81·5, 82·5	81

Table 10) seems very satisfactory, and illustrates also that a large amount of interfering substance may sometimes be produced in the caramelized foods, but not in the other products examined.

Comparison with biological assays. Probably the best criterion of the accuracy of any new chemical method of analysis is its agreement with results obtained in biological assays. The fuller details of the method of technique used in the biological assays (mostly by the semi-curative growth test (see Harris & Olliver, 1942, p. 168)) are given by Cruickshank, Harris & Mapson (1947), but it may be stated here that those materials were chosen for test which were considered to afford as stringent a check as possible of the reliability of the new method. Among these (either assayed afresh, or tested on previous occasions) were: (1) raw, fresh fruit or vegetable foods containing only ascorbic acid and no interfering substance, (2) cooked or processed foods, likewise containing ascorbic acid only, (3) caramelized vegetable foods containing varying amounts both of the interfering substance and of the vitamin, (4) caramelized products

containing only the interfering substance and no vitamin, and (5) finally, food preparations devoid of both.

The results (Table 11) seem to be in very satisfactory agreement.

Table 10. *Estimation of ascorbic acid in caramelized and non-caramelized dehydrated foods. Comparison of results by* HCHO *and the continuous-flow method*

Dehydrated material (with code no.)	Treatment	Titration result expressed as equivalents of ascorbic acid (mg./100 g.) Total	By HCHO method	By continuous-flow method
Cabbage (A. 584*b*)	Stored in N_2 at 37° for 5 months	265	180	184
Cabbage (A. 584*c*)	Stored in N_2 at 25° for 5 months	435	374	357
Cabbage (A. 584*d*)	Stored in N_2 at 25° for 5 months	450	342	334
Cabbage (A. 584*d*)	Stored in N_2 at 37° for 5 months	149	81	88
Cabbage (A. 640–8*d*)	Stored in N_2 at 28° for 6 months	360	335	324
Cabbage (A. 640–10*d*)	Stored in N_2 at 28° for 6 months	310	282	274
Cabbage (A. 667*a*)	Stored in N_2 at 37°	101	5	11
Cabbage (A. 667*b*)	Stored in N_2 at 37°	117	29	27
Cabbage (A. 667*c*)	Stored in N_2 at 37°	95	34	32
Cabbage (A. 667*d*)	Stored in N_2 at 37°	116	13	7
Cabbage (A. 730)	Stored in air at 37°	70	17	16
Cabbage (A. 730)	Stored in air at 37°	59	8	3
Cabbage (A. 662*c*)	Stored in N_2 at 25°	296	250	251
Cabbage 1	Stored at 15° for 2 months	243	209	211
Cabbage 1	Heated 1 hr. at 100°	193	37	38
Cabbage 2	Freshly dried	400	371	367
Cabbage 2	Heated 30 min. at 100°	346	220	224
Cabbage 2	Heated 60 min. at 100°	300	174	172
Cauliflower (A. 559*b*)	Stored in N_2 at 25°	89	55	41
Meat and vegetable soup	Stored at 28°	38	2	2
Tomato 1	Sun-dried	120	0	5
Tomato 2	Roller-dried flakes	145	6	4
Tomato 3	Spray-dried powder	79	34	38
Guava 1	—	920	740	760
Guava 2	—	1800	1420	1440
Carrot	Stored at 37° for several months	61	1	3
Swede, raw (A. 720*a*)	—	170	148	144
Swede (A. 663*c*)	Stored in N_2 at 37°	43	7	7
Swede (A. 663*d*)	Stored in N_2 at 37°	46	15	11
Swede 1	Freshly dried	136	122	122
Swede 1	Heated 30 min. at 100°	97	64	66
Swede 1	Heated 60 min. at 100°	68	4	5
Whole orange		235	194	192
Whole orange	Sulphited	357	310	325
Spinach	Dried 65° without scalding	202	111	122
Spinach	Dried 65° after scalding	186	147	149
Whole grapefruit 1	—	284	249	250
Whole grapefruit 1	Heated at 80° for 2½ hr.	169	128	120
Whole grapefruit 2	Sulphited	320	263	272
Cape gooseberries		38	31	28

III. WORKING DIRECTIONS FOR ROUTINE USE OF THE FLOW METHOD

The following procedure is suitable for use with caramelized dehydrated vegetables and similar products.

(*a*) A weighed sample is ground in presence of sand with a small quantity of 20% metaphosphoric acid, and the mixture diluted with water to reduce the concentration

of the acid to 2%. After filtration, with spinning if necessary, the reaction of the clear fluid is adjusted to pH 3·5 by sodium citrate and the total reducing titre estimated as described in § II (1).

(*b*) On the basis of this analysis the extract is diluted to be equivalent in total reducing strength to 0·04 mg. of ascorbic acid/ml., and passed through the flow apparatus with an equal rate of flow of indophenol dye of exactly equivalent concen-

Table 11. *Examples of agreement with biological assays*

Test material	Category (see p. 33)	Presence of: Ascorbic acid	Presence of: Interfering substance	Result of chemical analysis (mg./100 g. (av.)): Ascorbic acid	Result of chemical analysis (mg./100 g. (av.)): Interfering substance (expressed as equiv. of ascorbic acid)	Result of biological assay, ascorbic acid (mg./100 g.)	Biological value as % of chemical value	Reference
New potatoes	(1) Raw	+	o	35	o	37	106	(*a*)
Cabbage water	(2) Cooked	+	o	25	—	25	100	(*a*)
Black-currant purée	(2) Processed	+	o	120	—	138	115	(*a*)
Cabbage, dehydrated	(2) Processed (not caramelized)	+	o	460	o	463	101	(*a*)
Cabbage, dehydrated	(3) Excessively caramelized	+	+	115	160	114	99	(*b*)
Carrot, dehydrated	(4) Excessively caramelized and inactivated	o	+	o	220	Nil	—	(*b*)
Dried egg yolk	(5) —	o	o	o	o	Nil	—	(*c*)

Gluco-reductone, reductic acid and hydroxytetronic acid were tested biologically and found inactive (*b*).

References: (*a*) Harris & Olliver (1942).
(*b*) Cruickshank *et al.* (1947).
(*c*) Unpublished work.

tration. The percentage of dye remaining unreduced after a reaction period of 1·1 sec. is determined as described in §§ II (2) and II (3). From this value and the reference graph (Fig. 3) relating percentage of unreduced dye to the proportion of ascorbic acid and gluco-reductone in the mixture, as previously determined for the apparatus used, the percentages of ascorbic acid and of interfering substances (expressed in terms of gluco-reductone) present in the mixture are obtained.

In the case of weak solutions where the reducing strength may have to be made up to 0·02 mg. instead of 0·04 mg./ml. the dye has also to be made up to the same equivalent concentration, and the reference curve (Fig. 3) to be determined afresh. Strengths of less than 0·02 mg. are not recommended, however, and the standard concentration of 0·04 mg. should always be used where possible. The curve must also be redetermined for each change in experimental conditions, and should be rechecked from time to time.

IV. APPLICABILITY OF METHOD

The technique here described was devised in the first place for estimating ascorbic acid in caramelized vegetable foods. It has, however, some wider uses, which can be classified as (1) qualitative and (2) quantitative.

(1) *Qualitative use.* In the first place, the method enables a rapid qualitative test to be done on any food to ascertain whether the indophenol-reducing material has the properties of ascorbic acid, or whether significant amounts of interfering substances can be shown to be present. It deserves to be emphasized that, of the known organic reductants examined, hydroxytetronic acid alone cannot be differentiated from ascorbic acid or its isomers, but that there is no evidence that this substance is produced in any normal method of food processing. If, therefore, the test on any food extract shows that the velocity of the reaction with the dye at pH 1·4 and 3·5 is identical with that of ascorbic acid, it seems a fair assumption to make that ascorbic acid alone is present. In this case the ordinary simple method of titration with indophenol can be used straight away.

(2) *Quantitative application.* Secondly, the method may be used to determine ascorbic acid quantitatively in caramelized dried vegetable foods. In addition, with many dried fruits (e.g. guava, orange, grapefruit, tomato, see Table 10) the results have been in close agreement with those obtained independently by the formaldehyde method, and also with those of a number of biological tests. It will, of course, be necessary to test a more extended range of dried fruits before it can be claimed to be of universal applicability to them all. Further work (now in progress) will be needed also to check the applicability of the method to other types of processed foods, particularly to fermented products (Harris, 1933). In such cases the available evidence suggests the possibility that materials distinct from reductones may be present (cf. Wokes *et al.* 1943*b*). Finally, the only other limitation of the method that can be foreseen at present is that the procedure cannot be used with foods so poor in ascorbic acid, or other reducing substances, that their extracts contain less than about 0·02 mg. equivalent/ml.

SUMMARY

1. Dehydrated vegetables, caramelized by exposure to unduly high temperatures during either processing or storage, may contain reducing agents of the nature of reductones, which, since they react rapidly with indophenol dye, interfere in the chemical estimation of ascorbic acid if the ordinary method of direct titration is used. A procedure has been devised for the differential determination of both ascorbic acid and reductones present in such materials, and has been used satisfactorily for routine determinations. It depends on the use of an apparatus employing the principle of the continuous flow (Hartridge & Roughton, 1923), by which the complete time course of a reaction can be followed.

2. Control tests with mixtures of equal volumes of ascorbic acid (0·04 mg./ml.) and 2:6-dichlorophenolindophenol (in equivalent strength) at pH 3·5 and temperature 20° indicated that after 0·28, 0·55, 0·78 and 1·1 sec. respectively, 64, 80, 84 and 87–89 %

of the reaction was complete, whereas with gluco-reductone (the mixture of substances formed by heating a solution of glucose with alkali) in place of ascorbic acid the corresponding values were 26, 34, 37·5 and 38·5%. With amino-reductone (the mixture of substances formed by heating a solution of glucose with glycine and alkali), they were 30, 38, 41 and 44%. The values when plotted lay on well-separated smooth curves, and thus ascorbic acid could readily be distinguished from these interfering substances. Moreover, mixtures of gluco-reductone and ascorbic acid gave curves in intermediate positions, in proportion to the relative amounts of each present; hence both substances could be accurately estimated when present simultaneously. In recovery tests, ascorbic acid and gluco-reductones could be determined under the above conditions with an error of less than 2% of the total reducing value.

3. Other substances were examined in the same way and their velocity constants determined. Cysteine and sulphite could be readily distinguished from ascorbic acid by their much slower rates of reaction, while dihydroxymaleic acid, thiosulphate, and the ferrous ion were distinctive in having relatively slow rates at pH 3·5 but much faster at pH 1·4. Reductic acid had approximately half the velocity constant of ascorbic acid, while hydroxytetronic acid, alone of the substances examined, had approximately the same constant; there is, however, no evidence that either of these substances, or dihydroxymaleic acid, occurs in natural products.

4. The reductants (interfering substances) formed when dehydrated vegetables are caramelized resembled gluco-reductones closely in their rate of reaction with 2:6-dichlorophenolindophenol at pH 3·5, and no indication was obtained of the presence of the other type of reductants mentioned in paragraph 3 above. Both the ascorbic acid and the interfering substance could therefore be estimated by the use of the continuous-flow technique, the experimental evidence for this conclusion being as follows:

(*a*) Various dehydrated vegetables known to be devoid of antiscorbutic activity were heated for differing periods at 100° so as to cause caramelization to varying extents; application of the flow test then accurately showed the presence of interfering substance and no ascorbic acid.

(*b*) On the other hand, dehydrated vegetables which had known antiscorbutic activities, but had not been subjected to caramelization, were shown by the test to contain ascorbic acid and no interfering substance.

(*c*) Furthermore, in recovery tests, it was shown that a quantitative estimate was possible of the ascorbic acid or gluco-reductones, or both, added to various dehydrated vegetable products or other food preparations, already containing (i) ascorbic acid without the interfering substances produced on caramelization, or (ii) the interfering substances without the ascorbic acid, or (iii) both present simultaneously.

(*d*) Results of determination of ascorbic acid in caramelized dehydrated vegetables and other products by the continuous-flow method agreed well with those obtained by the formaldehyde method (Mapson, 1943*b*) and by biological assays.

5. The limit of error was low, the ascorbic acid or interfering substance, determined in recovery tests, ranging from about 97 to 103% of the theoretical value, expressed as a percentage of the total reducing content.

6. No significant amount of interfering substances was found in numerous fresh vegetables, in certain common fruit juices, or in vegetables cooked by the usual methods of boiling or steaming, or processed or dehydrated in absence of caramelization. Hence, for the usual run of fruit and vegetable materials it is confirmed that the ordinary indophenol titration is accurate, since it gives the same result as that obtained by the continuous-flow method. Only with plant products heated in the presence of alkali, or heated to exceptionally high temperatures as, for example, in the preparation of chip potatoes, are interfering substances encountered. In doubtful cases, or when new types of products are being examined, the flow method provides a rapid and convenient means of ascertaining whether or not certain interfering substances are present.

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