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Chromatographic Determination of Ascorbic Acid

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A description is given of a quantitative, chromatographic method for the determination of ascorbic acid. According to this method ascorbic acid is separated from interfering substances by chromatography on a cellulose column and determined in the eluate by means of diazotised nitroanisidine. Determinations of ascorbic acid contents down to 1 mg per 100 g in various materials have been tabulated to show the applicability of the method.

A survey of the literature on chemical assay of ascorbic acid in biological materials shows that the specificity of most of the methods used is not very satisfactory.

Thus the method most widely used, *i.e.* titration with an oxidizing reagent, as for instance dichlorophenol-indophenol [1,2], depends solely on the power of reduction of ascorbic acid, and this has certainly proved to give reliable results in the assay of, for instance, most fresh vegetables and fruits, but too high results in the assay of many other materials, for instance dried foods, which contain foreign, reducing substances. Descriptions of methods endeavouring to overcome this difficulty, thereby improving the specificity of the method, abound in the literature (*cf.* review by Sebrell and Harris [3]), but since the chemical nature of the reducing substances concerned are often unknown, it will generally be impossible with absolute certainty to eliminate the process of reduction resulting from their presence.

Another method is to oxidize ascorbic acid to form dehydroascorbic acid and determine the resulting carbonyl function by means of 2,4-dinitrophenylhydrazine [4]. There is a risk, however, that other aldehydes and ketones, partly resulting from the process of oxidation, may react with the reagent, leading to falsely high ascorbic acid values.

A third method for chemical assay of ascorbic acid, devised by Schmall *et al.*[5], depends on the condensation of ascorbic acid with a diazotised *o*-nitroaniline derivative in acid solution and spectrophotometric measurement in alkaline solution of the resulting hydrazide of oxalic acid. Moor [6] has modified the method, adapting it for the determination of ascorbic acid in foods. In this laboratory it has been found, however, that Moor's method may fail when the extract is low in ascorbic acid while at the same time containing substances

which produce high blank values. In a few cases the extinction determined for the blank was found to be higher than the extinction determined for the analytical sample.

A generally applicable way of improving the specificity of the assay involves chromatographic purification of the ascorbic acid prior to the chemical assay. The literature contains many examples of paper-chromatographic separation of ascorbic acid from interfering substances [7-9], but it appears to be difficult to avoid loss of ascorbic acid during the fairly protracted chromatographic separations. Moreover, it is possible to use a limited volume of extract only for this chromatographic separation, since the paper cannot "carry" any large amount of accompanying substances or of the metaphosphoric acid required for the extraction. As a consequence the sensitivity obtained is not satisfactory.

According to the method described in the following these difficulties have been overcome. The method depends on the purification of the ascorbic acid by chromatography on a column, and this has made possible the determination of ascorbic acid contents as low as 0.4 mg ascorbic acid per 100 ml extract. The content of the ascorbic acid in the purified solution is determined according to a modified version of the method devised by Schmall *et al.*[5] mentioned above.

EXPERIMENTAL

Principle of method. The procedure consists in:

(1) extraction of ascorbic acid with metaphosphoric acid,

(2) preliminary treatment of the extract, enabling it to be transferred to a cellulose column,

(3) chromatography of the extract, elution with a mixture of butanol-water-acetic acid,

(4) determination of ascorbic acid in the eluate by means of diazotised 2-nitro-4-methoxyaniline, which interacts with ascorbic acid to form — after shaking with a sodium hydroxide solution — the disodium salt of oxalic acid 4-methoxy-2-nitro-phenylhydrazide, the extinction of which at 570 mμ is proportional to the content of ascorbic acid.

Reagents

(1) *Metaphosphoric acid, 5 %.* Dissolve 5 g "metaphosphoric acid", analytical grade (HPO_3 containing 50—60 % $NaPO_3$), in 100 ml water. Prepare fresh solution on the day of using.

(2) *Acetone,* pure grade.

(3) *Acetone, aqueous solution, 50 % v/v.*

(4) *Acetic acid,* analytical grade.

(5) *n-Butanol,* analytical grade.

(6) *Ethylenediamine-NNN'N'-tetraacetic acid, disodium salt, 10 %.* Dissolve 10 g ethylenediamine-NNN'N'-tetraacetic acid, disodium salt ($C_{10}H_{14}O_8N_2Na_2$, $2H_2O$) in 100 ml water.

(7) *Ethylenediamine-NNN'N'-tetraacetic acid, 0.2 %.* Mix 2 ml 10 % ethylenediamine-NNN'N'-tetraacetic acid (reagent 4) with 2 ml acetic acid and dilute to 100 ml with water. This solution is supersaturated with regard to the free ethylenediamine-NNN'N'-tetraacetic acid, but is good for one week.

(8) *Eluant.* Mix 5 ml ethylenediamine-NNN'N'-tetraacetic acid, 0.2 %, (reagent 7) with 14 ml water, 1 ml acetic acid and 100 ml *n*-butanol by shaking vigorously until the mixture is (apparently) homogeneous. Prepare on the day of using.

(9) *Ascorbic acid,* reference standard, 100 μg/ml. Dissolve 50.0 mg ascorbic acid in 0.2 % ethylenediamine-NNN'N'-tetraacetic acid (reagent 7) and dilute to 50 ml with

this reagent. To a 2 ml portion of this solution add 1 ml acetic acid and dilute with *n*-butanol to 20 ml. This may cause some ethylenediamine-NNN'N'-tetraacetic acid to precipitate.

(10) *Diazotised nitroanisidine.* Mix 1 ml of a solution of 4-amino-3-nitroanisole ($C_7H_8N_2O_3$; dissolve 190 mg in 6 ml acetic acid and dilute to 100 ml with 1 N hydrochloric acid) and 0.1 ml sodium nitrite solution (dissolve 800 mg $NaNO_2$ in 100 ml water). Dilute the colourless solution with 1 ml 96 % ethanol and 2 ml *n*-butanol with stirring. The reagent is now ready for use and will keep for about one hour.

(11) *Cellulose powder.* Whatmann Cellulose Powder, standard grade.

(12) *Sodium hydroxide, 2 %.*

PROCEDURE

Cellulose column for chromatography. The chromatographic tube (*cf.* Bro-Rasmussen and Hjarde [10]) is about 40 cm long (internal diameter 12 mm) with a widening at the top holding 20—30 ml eluant and at the bottom terminating in a capillary tube, about 1 cm long (internal diameter 1 mm, external diameter about 7 mm).

Suspend 10 g cellulose powder in about 100 ml acetone, transfer directly to the chromatographic tube and force through the latter by means of carbon dioxide (at about 0.5 atm). Whenever the capillary tube gets choked with cellulose powder, and a little powder has collected at the bottom, clean the capillary tube with a piece of wire, providing free passage for the acetone. When all the powder has been transferred to the column — using as much acetone as required for this purpose — and the acetone has been forced out without allowing air to enter the cellulose, force the following liquids through the column in the order stated: 2 ml 50 % acetone (reagent 3), 2 ml 10 % ethylenediamine-NNN'N'-tetraacetic acid (reagent 6), 2 ml 50 % acetone (reagent 3), and, finally, 25 ml eluant (reagent 8), each liquid being pressed down to the surface of the column before the next is transferred to the column.

Extraction. Accurately weigh out a sample of not more than 30 g, if necessary under a 5 % solution of metaphosphoric acid, transfer to a Waring blendor with 100 ml 5 % metaphosphoric acid, bubble carbon dioxide through the solution and homogenize. Fill up to 250 ml with water and centrifuge.

From this stage it is necessary to protect the ascorbic acid from daylight, the effect of which is to decompose ascorbic acid. This decomposition causes most trouble in highly diluted solutions.

Preliminary treatment of extract. For the chromatography use, preferably, a volume of extract that contains about 20 μg ascorbic acid. If 0.5 ml extract (or an even smaller volume) is sufficient, add 1 ml acetic acid and a little cellulose powder (up to 0.5 g) to the sample drawn, stir and dilute with 5 ml *n*-butanol.

If more than 0.5 ml extract is used for the analysis, evaporate to a volume which is less than 0.5 ml by means of a stream of electrically heated carbon dioxide, and continue as described above.

In case the volume of the extract sample is more than 0.5 ml, it may be necessary to add hydrochloric acid. As regards this, *cf.* comments on the method.

Chromatography. Stir the suspension carefully and transfer quantitatively to the column by means of a pipette. Begin measuring the volume of the collected eluate from the moment the sample is transferred to the column. Force the sample through the column by means of carbon dioxide (at about 0.5 atm) and wash with three to four 1—2 ml portions of eluant, the liquid being pressed down to the surface of the column following each transference of eluant. Any lumps of cellulose from the sample left in the column should immediately be disintegrated with a spatula. Following the quantitative transference of ascorbic acid material to the column, pour eluant directly into the chromatographic tube and collect the following fractions:

(a) 34 ml, which contains the liquid front and is discarded.
(b) 10 ml, which should not contain any ascorbic acid.
(c) 13 ml, main fraction, which should contain all the ascorbic acid present.
(d) 10 ml, which should not contain any ascorbic acid.

Transfer fractions b, c and d to 50 ml separatory funnels, washing down with 5, 2 and 5 ml eluant, respectively, the volume of liquid in the separatory funnels being thus, in all cases, 15 ml.

Determination of ascorbic acid. To each of the samples b, c and d and to a reference sample (15 ml eluant to which has been added 0.200 ml ascorbic acid standard solution (reagent 9)) in separatory funnels add 0.200 ml diazotized nitroanisidine (reagent 10) and mix by swirling the separatory funnels for 0.5 min following each addition. The period of reaction should not be less than 5 min. Then carefully add 10 ml 2 % sodium hydroxide solution from a pipette, running the solution along the wall of the separatory funnel so as to prevent the two phases from mixing before the subsequent shaking. Now, as quickly as possible, shake vigorously for about one minute. Run out the aqueous solution and centrifuge for 5 minutes. Measure the extinction in the spectrophotometer in a 5 cm cell at 570 mμ.

Since the content of ascorbic acid in the sample is proportional to the extinction measured, the content of ascorbic acid can be calculated from the extinctions of the reference standard and the main fraction, the mean value of the extinctions of the fractions immediately before and after the main fraction being used as blank, *cf.* Discussion.

COMMENTS

re extract. The problems connected with the extraction does not differ from those met with in other methods for ascorbic acid analysis. Metaphosphoric acid solution is now generally acknowledged to be the best extraction agent, oxalic acid, which is otherwise frequently applied, having proved to be unsuitable [11].

To ensure reproducibility of the chromatography, the pH of the extract must be adjusted at about 1, too high pH values resulting in too high retention volumes, while too low pH values (caused for instance by excess of strong acids as trichloroacetic acid) result in too small retention volumes, *i. e.* ascorbic acid will be present in the fractions immediately after or immediately before the main fraction. When the above procedure is followed for the preparation of the extract, the acidity of the latter will in most cases be as required, and the chromatography will proceed satisfactorily without ascorbic acid in the fractions before and after the main fraction. This applies to all the vegetables mentioned in Table 1. It has been found, however, that in the chromatography of a product as cod roe some ascorbic acid will be present in the fractions immediately after the main fraction (5 ml extract was reduced by evaporation before being chromatographed), which is presumably due to a too high pH value of the extract. For this reason 0.1 ml hydrochloric acid (about 3 N) was added per 5 ml extract. This addition is based on qualitative experiments which have shown that chromatography of pure ascorbic acid solutions to which had been added 100 mg "HPO_3" (an analytical preparation containing about 60 % $NaPO_3$) — which corresponds to the quantity of "HPO_3" present in 5 ml extract — results in complete separation, *i. e.* no ascorbic acid in the fractions before and after the main fraction. If about half of the $NaPO_3$ present is neutralized by means of 0.1 ml hydrochloric acid (about 3 N), the ascorbic acid will still be retained quantitatively in the main fraction. This means that whenever the pH of the extract is expected to be too high, it is possible to ensure complete separation by adding hydrochloric acid in the above quantities. Only in the case of extracts which required evaporation did it prove necessary to add hydrochloric acid.

A reasonable accuracy is ensured by transferring a quantity of extract which contains 10—30 μg ascorbic acid to the column. If less than this amount of ascorbic acid is present in 0.5 ml of the extract, a suitable volume must be withdrawn and evaporated to a volume which is less than 0.5 ml. Such evaporation is conveniently performed in a 10 ml sample tube by means of a slight stream of electrically heated carbon dioxide. Temperature and rate of flow must be adjusted so that the tube with the sample is just tepid to the touch. The quantity of extract which may be transferred to the column depends on the nature of the extract, as excessive quantities of extracted substances may in the first place impede the quantitative transference of the sample to the column, because after the butanol addition the cellulose powder may become very tacky, and, in the second place, delay the passage of the ascorbic acid so that traces of ascorbic acid may be present in the fractions following the main fraction. The aim of the abovementioned addition of

cellulose powder to the sample is to enable this cellulose powder to adsorb the components of the extract which are precipitated by the 5 ml *n*-butanol added. Qualitative experiments show that when using 100 mg glucose with 0.3 ml water, 100 mg "HPO_3" and 0.1 ml 3 N hydrochloric acid, the fractions before and after the main fraction will be free from ascorbic acid, while when 250 mg glucose is used instead of 100 mg, the fraction after the main fraction will contain traces of ascorbic acid. The sensitivity of the method depends on these factors. As it appears from Table 1, the volume of the largest sample withdrawn for evaporation and chromatography in the course of the present work was 5 ml.

re chromatographic column. The presence of ascorbic acid in the different fractions collected depends, of course, on the dimensions of the chromatographic tube. Experience has shown that the reproducibility is very good from chromatogram to chromatogram using the same chromatographic column. Whether the dimensions of the column are correct may be checked by measuring the length of the column of cellulose powder, which should be between 31 cm and 33 cm, and by test chromatography as described in the following.

Chromatograph 200 μl of the "strong" standard solution (reagent 9), which contains 200 μg ascorbic acid (adding 1 ml acetic acid, a little cellulose powder, and 5 ml *n*-butanol and pouring this mixture into the tube). Collect the following fractions: 1 (34 ml), 2 and 3 (2 × 5 ml), 4 (2 ml), 5 (5 ml), 6 (4 ml), 7 (2 ml), and 8 (5 ml). Discard fraction 1, and to each of the following add 200 μl diazotised nitroanisidine (reagent 10). Extract the dye by shaking with 1 ml 5 % sodium hydroxide solution. Fractions 5 and 6 should contain the ascorbic acid, while fractions 4 and 7 must show only faint traces of blue colour. If tubes of other dimensions are to be used, the procedure of this qualitative chromatographic separation should be modified to indicate the fractions in which the ascorbic acid occurs in this case.

When acetone is used for charging the tube with cellulose powder, a tightly packed and readily reproducible column will be obtained. Treatment with 2 ml of 10 % aqueous solution of the disodium salt of ethylenediamine-NNN'N'-tetraacetic acid prevents oxidation of the ascorbic acid in the column due to catalytic action of copper and, more-

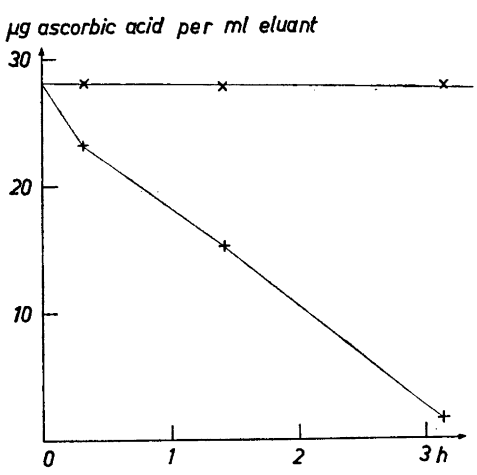

Fig. 1. The stability of ascorbic acid in the eluant (×), compared with the stability in the same medium without ethylenediamine-tetraacetic acid (+). Both solutions were kept at room temperature in artificial light.

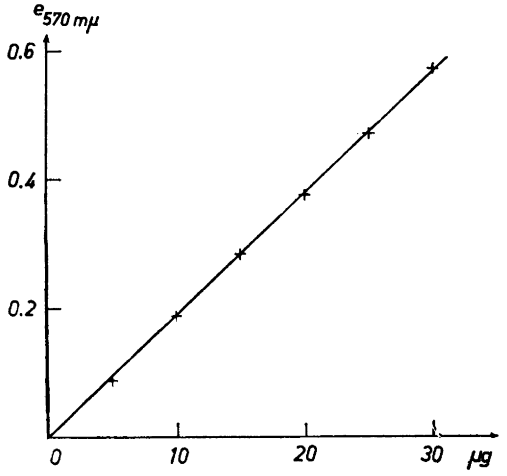

Fig. 2. Standard curve. Abscissa: μg ascorbic acid per 15 ml eluant. Ordinate: extinction at 570 mμ in a 5 cm cell.

over, due to its content of water, ensures that the cellulose powder is saturated with water, which is essential to obtain a reliable chromatographic separation.

re eluant. The eluant should be stabilized by means of ethylenediamine-NNN'N'-tetraacetic acid. The solubility of this substance in the butanol phase is very low, but the stabilizing effect appears clearly from the curve in Fig. 1. Ethylenediamine-NNN'N'-tetraacetic acid does not protect ascorbic acid against the action of light, and artificial illumination should therefore be used throughout. The eluant should be freshly prepared. All distilled water used in these experiments has been condensed in metal equipment and has not been redistilled.

The chromatographic separation is performed rapidly, only 30 min passing from the moment when the sample is transferred to the column and until the final fractions have passed out of the column.

re ascorbic acid determination. The reagent has been chosen with a view to ensure maximum specificity. Experience shows that the extinction of the interfering substances accompanying ascorbic acid in the chromatographic separation generally decreases with increasing wavelength in the range 400 mμ to 600 mμ, consequently a reagent should be chosen which forms a hydrazide of oxalic acid with absorption maximum at the greatest possible wavelength. From this point of view 2-nitro-4-methoxyaniline was the best reagent available.

The interaction of diazotised nitroaniline and ascorbic acid is not instantaneous, maximum yield is obtained by allowing 5—20 min for the reaction.

It is important to follow closely the instructions given with regard to the extraction of the reaction products by means of sodium hydroxide solution. If the mixing of the phases is effected by a slow and cautious shaking, the yield of hydrazide will be reduced.

The colour will not persist for an unlimited period. Following the procedure described, the reduction will be about 1 % per hour at an extinction of 0.400 (corresponding approximately to the 20 μg standard).

In the above description of the procedure the use of only one reference standard sample is prescribed. This has been shown to be justified, as the standard curve (see Fig. 2) has been found to be linear within the range 0—30 μg.

RESULTS AND DISCUSSION

Table 1 summarizes the results of determinations of ascorbic acid obtained by the chromatographic method and by titration with dichlorophenol-indophenol in various biological materials. The following discussion of the specificity and accuracy of the method is based on these measurements taken together with recovery experiments and chromatographic determinations of substances of special nature.

One of the requirements which must be fulfilled to obtain a correct determination of an ascorbic acid content by the chromatographic method is that the extinction determined at 570 mμ in the main fraction derives exclusively from the interaction between the reagent and ascorbic acid. Without a more thorough examination it is not possible to determine whether this is the case. There may be coloured substances in the extract which pass out together with ascorbic acid in the main fraction and is transferred from here to the sodium hydroxide phase, and there may be ascorbic acid analogs which are not separated by chromatography and which react with the reagent in the same way as ascorbic acid.

As it appears from the table illustrating the chromatographic separations performed (Table 1), experience shows, however, that the extinctions measured in the fractions before and after the main fraction are small in the case of nearly all materials. It has been assumed, therefore, that coloured substances

Table 1. Content of ascorbic acid in some natural products and foodstuffs.

Sample	ml extract for chrom-ato-graphy	Extinction at 570 mμ in 5 cm cell			mg ascorbic acid per 100 g determined by	
		Fraction before main fraction	Main fraction	Fraction after main fraction	chrom-ato-graphy	titration
Strawberries	0.2	0.005	0.506	0.001	89.5	88.5
Cauliflower (white head)	0.2	0.002	0.520	0.002	90.5	88.0
Parsley	0.1	0.002	0.547	0.001	192	186
Dill	1	0.039	0.421	0.001	33.5	35.0
Gooseberries (green)	0.2	0.006	0.319	0.000	54.0	51.5
Peas (green)	0.4	0.011	0.445	0.010	47.0	52.0
Potatoes (new)	0.4	0.007	0.375	0.002	27.5	30.0
Oranges	0.2	0.006	0.399	0.005	49.5	47.5
Orange juice (canned)	0.5	0.007	0.318	0.006	31.0	33.5
Broccoli	0.2	0.006	0.309	0.004	145	148
Tomatoes	1	0.010	0.460	0.006	12.5	12.6
Black currant syrup	0.5	0.011	0.354	0.016	55.0	59.0
Beetroot	2	0.005	0.200	0.223	3.6 (max.)	8.9
Milk	4	0.022	0.388	0.017	0.97	1.12
Liver, calf	0.5	0.013	0.357	0.011	22.5	27.5
Kidney, calf	2	0.007	0.565	0.006	9.6	11.2
Rat skin	5	0.012	0.337	0.009	2.8	3.5
Cod roe 1	5	0.001	0.332	0.011	2.9	4.4
» » 2	5	0.025	0.446	0.013	3.9	6.4
Cauliflower (a)	0.5	−0.006	0.231	0.015	19.6	36.2
Tomatoes (a)	1	0.004	0.219	0.020	8.9	22.1
Vegetables, unspecified (a)	1	0.015	0.083	0.009	6.1	35

(a) Dehydrated products, commercial

do not generally cause interference and that the blank value of the main fraction may therefore with reasonable accuracy be determined as the mean value of the extinctions of the fractions immediately before and after the main

Table 2. Recovery of ascorbic acid after chromatography.

Ascorbic acid chromatographed μg	Extract ml	Ascorbic acid recovered μg	Ascorbic acid recovered %
20.0	0.2	20.1	100
20.0	0.2	20.4	102
20.0	0.2	19.9	100
20.0	5 [a]	20.4	102
20.1	5 [b]	20.0	100

a. 5 ml extract of cod roe in 2 % metaphosphoric acid + 0.1 ml appr. 3 N hydrochloric acid + a solution of dichlorophenolindophenol until total oxidation of ascorbic acid. Excess of oxidizing agent is destroyed by allowing the acid solution to stand overnight. 20.0 μg of ascorbic acid is added and the solution is reduced by evaporation to a volume of less than 0.5 ml.

b. 5 ml of a solution of 2 % metaphosphoric acid to which 20.2 μg ascorbic acid is added, is evaporated till the volume is less than 0.5 ml.

fraction. As an exception may be mentioned the highly coloured beet root extract, in the case of which the extinction of the fraction after the main fraction is too high to be used as a blank, and that the ascorbic acid value found therefore must be considered merely to represent a maximum value. Incidentally, this very low value as compared to the titration value tends to confirm the results obtained by Somer *et al.*[12]

*Iso*ascorbic acid and glucoascorbic acid interact with the diazonium reagent and with sodium hydroxide to form the same hydrazide as ascorbic acid. It is impossible by chromatography to separate *iso*ascorbic acid and ascorbic acid, while glucoascorbic acid will follow immediately after ascorbic acid in the chromatogram, *i.e.* in the fraction after the main fraction. Consequently it is not possible according to this method to distinguish between ascorbic acid and *iso*ascorbic acid. Other substances, related to ascorbic acid, which might be expected to interfere in the analysis in this way have not been studied.

In addition to the condition mentioned in the above, which must be fulfilled to ensure correct results, the main fraction must not contain substances the presence of which results in a reduction of the yield obtained from the reaction.

Sulphur dioxide may interfere in this way, since it passes the chromatographic column together with ascorbic acid, resulting in too low ascorbic acid values. If thus the molar ratio of sulphur dioxide to ascorbic acid is as 1:1, the yield of hydrazide will be 5 % too low. However, sulphur dioxide may be removed from the acid extract by bubbling carbon dioxide through the solution.

The reliability of the method in general may be judged by comparing the results obtained when applying the titration method and the chromatographic

method to vegetables and fruits and also by the results of recovery experiments, as those represented in Table 2.

All ascorbic aid determinations hitherto made according to the described method have been included in Table 1. Agreement between the results obtained according to the two methods are seen to be fairly good with regard to most of the vegetables, while considerably lower values are found in the case of products of animal origin and dehydrated vegetables. It should be mentioned that the titrations have been performed by means of dichlorophenol-indophenol, if necessary after extraction with chloroform (a modification of the xylene method [13] generally used with coloured or turbid extracts), without any attempt at improvement of the specificity.

It will be seen that in the case of nearly all the samples the extinction values obtained from fractions before and after the main fraction are small; this applies also to the samples with regard to which chromatography of 5 ml extract was necessary.

It appears from the recovery experiments (Table 2) that the losses resulting both from chromatography and evaporation are very small. An experiment with cod roe extract, in which the ascorbic acid was first oxidized with dichlorophenol-indophenol before the addition of the 20 μg ascorbic acid, also shows that the content of ascorbic acid is maintained quantitatively throughout the procedure.

The sensitivity of the method depends on the nature of the sample and on the demands made as to accuracy. It appears from Table 1 that in protein-containing materials, as for instance cod roe, it is possible to determine ascorbic acid contents down to a concentration of about 3 mg per 100 g without reducing the demands as to a reasonably large deflection of the measuring instrument.

Note added in proof. Apples contain a colourless substance (or several substances) which very nearly follows ascorbic acid on the column and gives a reddish colour with the reagent, thereby interfering with the determination. The chemical nature of the substance has not been investigated.

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REFERENCES

1. Tillmans, J. *Z. Untersuch. Lebensm.* **60** (1930) 34.
2. Harris, L. J. and Olliver, M. *Biochem. J.* **36** (1942) 155.
3. Sebrell, W. H. and Harris, R. S. (Eds.) *The Vitamins*, Academic Press inc., New York 1954, Vol. I, p. 253.
4. Roe, J. H. and Kuether, C. A. *J. Biol. Chem.* **147** (1943) 399.
5. Schmall, M., Pifer, C. W. and Wallish, E. G. *Anal. Chem.* **25** 1486.
6. Moor, H. *Mitt. Gebiete Lebensm. u. Hyg.* **47** (1956) 20.
7. Ulmann, M. *Pharmazie* **9** [6] (1954) 523.
8. Chen, Y. T., Isherwood, F. A. and Mapson, L. W. *Biochem. J.* **55** (1953) 821.
9. Kadin, K. and Osadca, M. *Agr. Food. Chem.* **7** (1959) 358.
10. Bro-Rasmussen, F. and Hjarde, W. *Acta Chem. Scand.* **11** (1957) 34. (Fig. 1.)
11. Baker, L. C., Lampitt, L. H. and Wittenberg, E. *J. Sci. Food Agr.* **6** (1955) 682.
12. Somers, G. F., Kelly, W. C., Thacker, E. J. and Redder, A. M. *Food Research* **16** (1951) 62.
13. Pepkowitz, L. P. *J. Biol. Chem.* **151** (1943) 405.

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