LXXVIII. A MICRO-CHEMICAL METHOD FOR DETERMINING THE HEXURONIC ACID (VITAMIN C) CONTENT OF FOODSTUFFS, *ETC.*[1]

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IN an earlier paper from this laboratory [Harris and Ray, 1933] reference was made to a method of estimating hexuronic acid in foodstuffs, depending on modifications in the use of Tillmans's reduction test. It was shown that the hexuronic acid content of orange juice so determined accounted quantitatively for its antiscorbutic activity, and similar results were alluded to for lemon juice, tomato juice, grape-fruit juice and pineapple juice. As was mentioned, the method has since been adapted to a micro-chemical scale, and the purpose of the present paper is to give the detailed description of the method and results for a large number of common foodstuffs.

EXPERIMENTAL.

Method in outline. A small amount of the foodstuff is weighed out and ground up with sand and sufficient 20 % trichloroacetic acid solution to give a final concentration of the latter of about 5 %. The extract is then made up to a suitable volume: *e.g.* when working with materials of the potency of orange, lemon, *etc.* a dilution to about 1 in 10 is suitable; with less potent materials the dilution should be correspondingly less. The filtered extract is transferred to a micro-burette graduated in 0·01 cc. A measured volume, say 0·05 cc. of a recently prepared standardised solution of 2 : 6-dichlorophenolindophenol (about 0·01 *M*), is placed at the bottom of a small pointed tube. (It may be conveniently run out of Krogh and Keys's [1931] micro-syringe, using the screw gauge, the error with a volume of this order being less than 2 %.) The trichloroacetic acid extract is then run in from the burette until the red colour, which the indicator immediately assumes in acid solution, is just discharged. (The titration should be concluded within about 2–3 minutes, otherwise an error may be caused by the fact that the indicator may sometimes tend to fade slowly in the absence of hexuronic acid.) The amount of extract so needed is noted and, since the indicator is standardised in terms of hexuronic acid itself (see below), the hexuronic acid content of the unknown can be immediately calculated. From the known antiscorbutic activity of hexuronic acid, the antiscorbutic activity of the unknown can then be expressed in terms of guinea-pig dosage

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or compared with the potency of a standard material, as previously described [Harris and Ray, 1933].

Standardisation of indicator. For standardisation, a measured volume of the same indicator solution as was used for the estimation is titrated, likewise in acid solution and under the same general conditions, against a solution of hexuronic acid. The amount of pure hexuronic acid in the solution should in turn be checked (in case the specimen is at all impure) by iodine titration. From this relation, 1 cc. of indicator solution is evaluated as equivalent to so many mg. of hexuronic acid. Practical examples illustrating the use of this standardisation method have already been published [Harris and Ray, 1933]. Hexuronic acid is now available commercially, so that other workers should experience no difficulty in carrying out this necessary standardisation. It would however be legitimate to use fresh lemon juice or orange juice instead, if a more rough comparison only is desired.

Preparation of indicator. The indicator solution is made up by dissolving approximately 0·1 g. of the dye in a small amount of boiling distilled water. The residue left undissolved is filtered off and again extracted with boiling water; the combined extracts are then made up to 50 cc. At times trouble was experienced in getting a satisfactory end-point to the titration, the colour change being from red to brown instead of to colourless or pale yellow. This usually occurred when the indicator solution was several weeks old and was overcome when a fresh solution was made up according to the above procedure.

Extraction process. It may be noted that it was found impossible in most cases to remove anything like the full amount of vitamin by a simple expression process. Much of it seems to be retained within the cell structures. Furthermore, an important advantage of adding the trichloroacetic acid is that it appears to stabilise the vitamin and protect it against often fairly rapid destruction by agencies present in the tissue juices, presumably of an oxidative nature and due largely to enzyme systems. As has already been reported from this laboratory, disintegration of cell structure by freezing similarly promotes a considerable destruction of antiscorbutic activity [Mills, 1932].

Choice of titration reagent and conditions of test. Owing to the fact that hexuronic acid possesses outstanding reducing activity, a reduction test seemed the most hopeful method to try. As has already been reported [Harris and Ray, 1933], iodine proved far too unspecific, even at a strongly acid reaction (a condition which tended to inhibit the activity of certain other reducing agents and leave that of hexuronic acid unimpaired). An oxidation-reduction indicator with constants of such an order as to be unaffected by other naturally occurring reductants "weaker" than hexuronic acid was clearly indicated. Study of the literature showed that several oxidation-reduction indicators had in fact been described which from theoretical considerations could be expected to prove superior even to 2 : 6-dichlorophenolindophenol in this respect. However enquiries from many chemical manufacturers here and abroad showed that these were not to be obtained commercially. We decided accordingly that there was some benefit, from the immediate practical point of view, in using 2 : 6-dichlorophenolindophenol, and in actual experience it has been found to give reliable results.

This indicator has previously been employed by Tillmans and co-workers in their important work on the reducing capacity of foodstuffs and food-extracts. A number of points of difference in our use of it should therefore be noted here. Firstly, our object is to determine quantitatively the actual weight of hexuronic acid present in a given amount of material and not merely to obtain arbitrary

numerical values for relative reduction potentials. Secondly, the simple extraction with trichloroacetic acid seems undoubtedly preferable to the elaborate process of heating with sulphuric acid, evaporation, *etc.*, as used by Tillmans. Even more important is the difference that our titration is to be carried out in acid solution. We have shown that certain naturally occurring reducing agents, including notably glutathione and certain phenolic compounds, tend to reduce the indicator in neutral or alkaline solution [Harris and Ray, 1933]. Hence when working with the many natural sources known to contain, *e.g.* glutathione, it seems essential to work in acid solution if the possibility of deceptively high results is to be ruled out. A number of irregularities are in fact apparent in the proportionality between antiscorbutic activity and Tillmans's reduction values, which do not occur in the case of our measurements of hexuronic acid.

We have examined a large number of reducing agents, and found only one occurring naturally, cysteine, which will appreciably reduce the indicator under the conditions described. If working therefore with stale or autolysed tissues, which may contain measurable amounts of free cysteine, it is necessary to make a control test for the latter, *e.g.* by the Sullivan reaction. Adrenaline also may interfere but it is not likely in practice to be often present in sufficiently large concentration to be of any consequence. Yeast also was found to contain a substance which reduced the indicator. Whether or not it is identical with hexuronic acid we have still to determine.

The procedure for titrating a given amount of reagent with the unknown, instead of the more familiar reverse practice, may perhaps be criticised. However we found that if indicator were run into the unknown in the usual manner there was some difficulty in determining the end-point with precision. This is overcome by the method described, which also gives better results than the use of external indicator.

We hope to publish at a later date an account of measurements by the electrometric method. The relative advantages of alternative indicators are also being investigated, as to the sharpness of the end-point, clearness of the colour change, *etc.*

Degree of accuracy attainable. It was found that the end-point colour change was sufficiently sharp to enable duplicate determinations to agree to 97 % when working with solutions of hexuronic acid itself or natural sources of an activity such as lemon juice, orange juice, grape-fruit or the like. As we have shown, the results, taking materials of the widest range of potency, run parallel with the values determined biologically. The actual sensitivity of the method of course far exceeds that possible by the biological method of assay. With sources containing little vitamin C the percentage error naturally tends to be higher than with more active material, but again it is certainly far less than when determined biologically.

Results.

Table I gives the amount of hexuronic acid, as determined by this method, in various natural sources. From this we have calculated the "theoretical" minimum dose, and compared it with the "known" minimum dose, as determined biologically. The calculated, or theoretical minimum dose, is derived from the hexuronic acid content from the known antiscorbutic activity of hexuronic acid itself. For this purpose 0·9 mg. of hexuronic acid is taken as equivalent to 1·5 cc. of orange juice, or 0·9 mg. of hexuronic acid is taken as the minimum

daily dose to protect guinea-pigs from scurvy, both of which relations are in accordance with the experimental biological results of Harris and Ray [1933; also preceding paper]. In other words we have taken as the "calculated" minimum dose the amount of the material in grams which contains 0·9 mg. of hexuronic acid. Table I shows well the agreement between the known biological values and the values calculated from their hexuronic acid content.

Table I.

	Hexuronic acid content mg. per g.	Minimum daily dose for guinea-pigs, g.	
		As calculated from hexuronic acid chemically determined	As measured biologically (newly determined or reputed value)
Cabbage	1·0	0·9	1
Watercress	0·67	1·3	1
Lemon juice	0·62	1·5	1·5
	0·60	1·5	
Orange juice, several types	0·75	1·2	1·5
	0·59	1·5	
	0·48	1·9	
Grape-fruit juice	0·65	1·4	1·5–2
	0·59	1·5	
Pineapple juice	0·30	3	2–3
Imported tomato, juice of	0·21	4·3	3–5
Banana	0·15	6	5–10
Potato	0·15	6	6–10
Rhubarb	0·059	15	12
Carrots	0·028	32	10–35
Grapes	$\leqq$0·030	$\geqq$30	>20, 40
Imported peach, juice of	0·015	60	—
Horse-radish	1·6	0·6	—
Apples:			
Bramley's Seedling, cortex	0·16	5·5	3–5
,, peel	0·77	1·2	1
Newton Wonder, cortex	0·053	17	10
,, peel	0·24	3·7	3
Blenheim Orange, cortex	0·031	29	—
,, peel	0·33	2·7	—
Edward VII, cortex	0·017	53	>20
,, peel	0·12	7·5	2?
Cox's Orange Pippin, cortex	0·016	56	>20
,, ,, peel	0·09	10	—
Suprarenal cortex, ox	1·85	0·5	0·5
Liver, ox	0·68	1·3	—
Milk, cow's (variable)	0·025–0·019	36–47	20–60
"Ostomalt"	0·27	3·3	4
Egg-yolk	0·00	∞	∞
Sussex ground oats	0·00	∞	∞

Summary.

Table I gives the hexuronic acid contents of about 30 food materials and the corresponding "calculated" minimum antiscorbutic doses, which agree well with the minimum doses as actually determined biologically.

The method described entails preliminary grinding with sand and trichloroacetic acid followed by titration of the acid extract against a measured volume of 2 : 6-dichlorophenolindophenol, which has been standardised against a solution of hexuronic acid of known strength, in turn standardised against iodine.

The determination takes only a few minutes to complete; in the case of a material of the activity, *e.g.*, of orange juice, the amount used in titration need be no more than about 0·03 cc., and the sensitivity is such that duplicates agree to within 3 %.

Certain naturally occurring reducing agents, including glutathione, which react with the indicator when the reducing capacity of foods is determined in more nearly neutral solution according to the Tillmans technique do not interfere. Although no claim is made to absolute specificity, it is clear that in practice the method gives accurate results on a large variety of animal and vegetable materials. Many reducing agents were tested with negative results. In the presence of free cysteine, however, as in certain autolysed tissues, a special control is necessary.

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