Since the original isolation of myrobalanitannin in 1910 this tannin has also been identified in witch-hazel bark (Edwards & Nierenstein, 1943), and, as shown in this communication, in a large number of other plants. As will be seen (Table 1), the bark of *T. chebula* also contains myrobalanitannin, which thus disproves the contention of Meyer (1909) that

the tannin yielding ellagic acid is not present in the bark.

EXPERIMENTAL

I. *Confirmation of formula (I).* Myrobalanitannin was methylated with excess diazomethane in an apparatus similar to that described by Malkin & Nierenstein (1930) and the methanol solution refluxed for 6–8 hr. on a boiling water-bath with a solution of sulphuric acid in dilute methanol. Recrystallization of the product from methanol gave tetramethylellagic acid in microscopic needles, m.p. 289–290°. (Found: C, 60.6; H, 4.1; OCH₃, 34.2%. Calc. for C₁₈H₁₄O₈: C, 60.4; H, 4.0; OCH₃, 34.1%.)

II. *Distribution of myrobalanitannin.* The finely powdered materials were extracted with a mixture of chloroform and carbontetrachloride, so as to remove fats, waxes, etc., and then percolated with ethanol until no more tannin could be qualitatively detected. The residue left on evaporation of the alcohol crystallized from distilled water in faintly brownish microscopic needles, which did not melt below 360°. For myrobalanitannin Edwards & Nierenstein (1943) record $[\alpha]_D^{21} = +21.98^\circ$ (water); we found $[\alpha]_D^{21} = +21.77^\circ$ (water), $[\alpha]_D^{28} = +37.16^\circ$ (ethanol) and $[\alpha]_D^{17} = +29.03^\circ$ (methanol). The distribution of myrobalanitannin is given in Table 1. Glucose and ellagic acid were estimated by the method of Nierenstein, Spiers & Geake (1921).

SUMMARY

The original formula of myrobalanitannin has been confirmed, and the distribution of this tannin studied.

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Interfering Substances in the Roe and Kuether Method for the Determination of Ascorbic Acid

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Roe & Kuether's method (1943) is based upon the red coloration given by the 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid with 85% H₂SO₄. This reaction is very sensitive

and its specificity, as will be shown, is high. We investigated the method in connexion with another inquiry and record some of our observations.

METHODS

General technique

Our technique was that of Roe & Kuether (1943), but we allowed the final coloured solution to stand for $\frac{1}{2}$ hr. at constant temperature, in order to minimize the possibility of error due to fading when investigating interfering substances. All estimations were carried out in 4% trichloroacetic acid and the colour developed was measured in an Evelyn type photoelectric colorimeter. The standard error of percentage deviation from the mean of individual *l*-ascorbic acid determinations over a period of 6 months for the range 0.001–0.04 mg. *l*-ascorbic acid/4 ml. was 2%. The use of different batches of reagents did not influence the results. A linear relationship between log *R* and concentration was found up to about 0.01 mg. *l*-ascorbic acid/4 ml., in reasonable agreement with the above authors. When the Evelyn type of photoelectric colorimeter was replaced by the 'Spekker' Absorptiometer (standard or sensitive type), or when King & Haslewood's (1937) visual photometric technique was employed, a lower limit of only 0.01 mg./4 ml. could be satisfactorily attained.

Reagents

l-Ascorbic acid, reductic acid and d-glucosascorbic acid (see Snow & Zilva, 1941).

Reductone (see Snow & Zilva, 1938).

d-Araboascorbic acid (see Snow & Zilva, 1943).

2:3-Diketogulonic acid solution (see Penney & Zilva, 1943).

Laboratory reagent quality 2:4-dinitrophenylhydrazine, thiourea, and trichloroacetic acid were used. The sodium carbonate, bicarbonate and hydroxide, and the glucose, were Analar reagents. Analar sulphuric acid was used to prepare an 85% solution (Roe & Kuether, 1943). The norite was a commercial sample of activated vegetable charcoal washed with acid before use.

Table 2. *Recovery of l-ascorbic acid in the presence of reductic acid*

<i>l</i> -Ascorbic acid added (mg.)	Reductic acid added (mg.)	'Ascorbic acid' found		<i>l</i> -Ascorbic acid after correction (mg.)	Error	
		at 25° (mg.)	at 37° (mg.)		Before correction (%)	After correction (%)
0.020	0.008	0.0087	0.0288	0.0201	+44	+ $\frac{1}{2}$
0.020	0.016	0.0174	0.0383	0.0209	+91	+4 $\frac{1}{2}$

Heat treatment of alkaline solutions of glucose

Alkaline solutions of glucose were prepared using: (1) NaHCO₃, (2) Na₂CO₃, (3) 0.3N-NaOH, (4) 0.02N-NaOH.

(1) NaHCO₃: (a) a 1% solution of glucose in 0.5% NaHCO₃ was boiled for 30 min. (see Roe & Oesterling, 1944); (b) equal volumes of 2% glucose and 1% NaHCO₃ solutions were heated in a boiling water-bath until a constant temperature was attained. After mixing, the heat treatment was continued for 30 min.; (c) equal volumes of 2% glucose and 1% NaHCO₃ solutions, after being heated to 90°, were mixed and allowed to react at that temperature for 30 min. After heating, the solutions were made slightly acid with HCl, cooled and diluted to the required volume.

(2) Na₂CO₃: glucose solutions in Na₂CO₃ were treated as above.

(3) 0.3N-NaOH (see Snow & Zilva, 1944): equal volumes of 4% glucose solution and 0.6N-NaOH were heated to 90°

in a water-bath and then mixed. After $\frac{1}{2}$ min. at this temperature, the mixed solutions were removed, acidified, cooled and diluted to the required volume.

(4) 0.02N-NaOH (see Snow & Zilva, 1944): equal volumes of 4% glucose solutions and 0.04N-NaOH were treated as above except that the mixed solutions were allowed to remain at 90° in the water-bath for 10 min.

All the above alkaline solutions were coloured yellow-brown after the heat treatment, the intensity of the coloration varying directly with the severity of the treatment. On acidification some, but not all, of the colour was dispersed.

Direct titrations with indophenol were carried out at pH 1.5.

RESULTS

Interfering substances

Reductic acid. When the Roe & Kuether reaction is applied to reductic acid, a colour develops which is similar to that obtained with *l*-ascorbic acid. There is, however, one essential difference, namely, that if the reaction is carried out at 25° for 15 min. about 92% of the colour given after heating at 37° for 3 hr. is generated. This can be seen from Table 1.

Table 1. *The production of colour by reductic acid after 15 min. at 25° and 3 hr. at 37°*

Exp.	Reductic acid (mg.)	'Ascorbic acid' found		'Ascorbic acid' at 25° at 37° (%)
		at 25° (mg.)	at 37° (mg.)	
1	0.008	0.0084	0.0093	90
2	0.008	0.0089	0.0096	93
3	0.008	0.0091	0.0096	95
Average	0.008	0.0088	0.0095	92

Ascorbic acid under the same conditions generates only about 2.5% of its final colour. It should thus be possible to estimate *l*-ascorbic acid in the presence of reductic acid if no other interfering substances are present. That this can be done within certain limits will be seen from Table 2. The figures show that in pure solutions the error due to reductic

Table 3. *The influence of increasing amounts of reductic acid on the calculated error*

<i>l</i> -Ascorbic acid (mg.)	Reductic acid (mg.)	<i>l</i> -Ascorbic acid after correction (mg.)	Error (%)
0.01	0.01	0.0106	+ 6
0.01	0.02	0.0114	+14
0.01	0.03	0.0122	+22
0.01	0.04	0.0130	+30

acid, if the latter is present in amounts of the same order as *l*-ascorbic acid, can be largely eliminated. When, however, the reductic acid is higher, the error increases. This is seen in Table 3 in which the approximate figures given are calculated by the summation of the individual colour values.

Purified reductone. The solubility of the 2:4-dinitrophenylhydrazine derivative of this substance in the concentration of H_2SO_4 employed by Roe & Kuether is low. By using reasonably dilute solutions of reductone, however, this difficulty can be overcome, although under these conditions the derivative does not produce its maximum colour. By carrying out the Roe & Kuether reaction with such dilute solutions for 15 min. at 25° , about 50% of the final colour developed in 3 hr. at 37° is generated (Table 4). When, however, *l*-ascorbic acid was added to solutions of reductone and the ratio of

l-ascorbic acid to reductone was 4:1 or more, it was found that *l*-ascorbic acid could be determined with a maximum error of the order of 6% by deducting the value of 'ascorbic acid' obtained at 25° from the final value at 37° (Table 5). Under these conditions the colour generated by the ascorbic acid at 25° roughly compensates for the incomplete reductone reaction.

Heated alkaline glucose solutions. Roe & Oesterling (1944) have already observed that when a 1% solution of glucose containing 0.5% NaHCO_3 was boiled for 30 min., a value for 'ascorbic acid' was obtained by their method which was higher than the direct indophenol titration, but when the heating was continued for 90 min. both methods gave results of a similar order. In view of these observations we decided to compare the colour reaction produced after 15 min. at 25° and after 3 hr. at 37° by alkaline glucose solutions submitted to various temperatures. The results are given in Table 6. It will be seen that in most cases the values for 'ascorbic acid' obtained by the Roe & Kuether method show fair agreement with those obtained by direct indophenol titration. Although by deducting the figures given by the reaction for 15 min. at 25° the result would still be misleading, the error would be minimized. Even then the corrected figures would indicate the presence of 'ascorbic acid' to the extent of 34% in the NaHCO_3 and Na_2CO_3 solutions, and 55% in the NaOH solutions, of the direct indophenol titra-

Table 4. *The production of colour by purified reductone after 15 min. at 25° and 3 hr. at 37°*

Reductone (mg.)	'Ascorbic acid' found		'Ascorbic acid' at 25°
	at 25° (mg.)	at 37° (mg.)	'Ascorbic acid' at 37° (%)
0.005	0.0016	0.0035	46
0.005	0.0017	0.0035	49
0.0025	0.0007	0.0015	47
0.0025	0.0008	0.0016	50
		Average	48

Table 5. *Recovery of l-ascorbic acid in the presence of purified reductone*

<i>l</i> -Ascorbic acid added (mg.)	Reductone added (mg.)	'Ascorbic acid' found		<i>l</i> -Ascorbic acid after correction (mg.)	Error	
		at 25° (mg.)	at 37° (mg.)		Before correction (%)	After correction (%)
0.020	0.005	0.0021	0.0234	0.0213	+17	+6½
0.010	0.0025	0.0011	0.0118	0.0107	+18	+7
0.020	0.0025	0.0012	0.0208	0.0196	+4	-2
0.020	0.0025	0.0014	0.0211	0.0197	+5½	-1½

Table 6. *The production of colour by heat-treated alkaline glucose solutions after 15 min. at 25° and 3 hr. at 37°*

Solution	Heat treatment	'Ascorbic acid' found		'Ascorbic acid' at 25°	'Ascorbic acid' by direct indophenol titration
		at 25° (mg./100 ml.)	at 37° (mg./100 ml.)	'Ascorbic acid' at 37° (%)	(mg./100 ml.)
NaHCO_3 (0.5%)	At boiling-point for 30 min.	10.0	14.5	69	15.6
		11.9	18.0	66	18.4
	At 90° for 30 min.	1.08	1.55	70	1.8
	In boiling water-bath for 30 min.	3.5	5.5	63	4.4
Na_2CO_3 (0.5%)	At boiling-point for 30 min.	23.0	33.1	70	40.0
		27.0	38.2	71	44.0
	At 90° for 30 min.	12.3	19.0	65	20.0
	In boiling water-bath for 30 min.	19.0	27.5	70	28.7
0.3N-NaOH	At 90° for ½ min.	10.8	23.5	46	25.0
		9.9	21.9	45	24.4
0.02N-NaOH	At 90° for 10 min.	5.3	11.75	45	11.3
		7.1	15.0	47	16.4

tion and of the uncorrected Roe & Kuether values. As has been pointed out by Snow & Zilva (1944), if their correction formula is applied to the Lugg method the error arising from the presence of the products formed in the NaOH-treated glucose solutions is also not eliminated but is greatly reduced. The application of the Lugg method would, as will be shown later, in this case be more suitable than that of Roe & Kuether although the latter offers additional qualitative information. It is of interest to note that, whilst Lugg's method indicates a difference between the heat-treated alkaline solutions of glucose prepared with 0.3N- and 0.02N-NaOH (Snow & Zilva, 1944), the behaviour of each of these solutions in the colour reaction is the same. Results obtained by us from the application of the corrected Lugg method to glucose solutions treated with NaHCO_3 and Na_2CO_3 , such as were used in the colour reaction experiments, showed that the error due to the products obtained can be reduced to 6%. Table 7 gives the recovery of *l*-ascorbic acid added to the variously treated alkaline glucose solutions. It will be seen that the colour obtained is additive and that, providing the ratio of alkaline glucose to ascorbic acid is less than one to five, the maximum error is 6%. This is in agreement with the expected error calculated by summation.

DISCUSSION

The main fact observed in these experiments is a differential rate of reaction of the primary oxidation products of *l*-ascorbic acid, reductic acid, purified reductone and the products of the heat treatment of alkaline solutions of glucose with 2:4-dinitrophenylhydrazine. After 15 min. at 25°, using the Roe & Kuether reaction, *l*-ascorbic acid, reductic acid, purified reductone, heat-treated NaHCO_3 and Na_2CO_3 solutions of glucose and heat-treated NaOH solutions of glucose generate 2.5, 92, 48, 66 and 45% respectively of the colour given in 3 hr. at 37°. It therefore follows that in a known mixture of any one of the above and *l*-ascorbic acid a correction can be applied for the interfering substance. In an unknown solution, however, the error due to these substances can be reduced but not eliminated, which is more or less the case with Lugg's method if the correction of Snow & Zilva (1944) is applied. In view of this it would be of interest to compare the two procedures. Lugg's method is based on the fact that *l*-ascorbic acid and other indophenol-reducing substances combine with HCHO at different rates according to the pH of the reacting solution and thus lose their capacity for reducing the indicator.

Table 7. Recovery of *l*-ascorbic acid in the presence of the products obtained from the heat treatment of alkaline glucose solutions

‘Ascorbic acid’ obtained by direct indophenol titration from heat-treated alkaline glucose solutions		‘Ascorbic acid’ found		<i>l</i> -Ascorbic acid after correction (mg.)	Error	
					Before correction (%)	After correction (%)
<i>l</i> -Ascorbic acid added (mg.)	Solution	‘Ascorbic acid’ (mg.)	at 25° (mg.)	at 37° (mg.)		
0.020	NaHCO_3	0.0024	0.0022	0.0229	+14½	+3½
0.020	Na_2CO_3	0.0033	0.0030	0.0238	+19	+4
0.020	0.3N-NaOH	0.0040	0.0026	0.0235	+17½	+4½
0.020	0.02N-NaOH	0.0042	0.0031	0.0245	+22½	+7

Analogues of l-ascorbic acid. *d*-Araboascorbic acid and *d*-glucoascorbic acid when submitted to the Roe & Kuether procedure for 15 min. at 25° and for 3 hr. at 37° gave colours similar to *l*-ascorbic acid, the intensity corresponding approximately to that given by equimolar amounts of ascorbic acid.

2,3-Diketo-l-gulonic acid. Solutions of this substance gave the reaction, the intensity of the colour generated at both temperatures being similar to that given by equimolar quantities of ascorbic acid.

The following common ketonic substances do not interfere with Roe & Kuether's method, since they give a yellow coloration with 2:4-dinitrophenylhydrazine on solution in 85% H_2SO_4 : pyruvic acid, acetoacetic ester, acetone, cyclohexanone, salicyl aldehyde, formaldehyde, methyl hexyl ketone, vanillin and *p*-hydroxyphenyl pyruvic acid.

At pH 1.5 *l*-ascorbic acid combines only very slowly with HCHO, whilst at pH 3.5 the combination is rapid. Pure reductone, reductic acid and heat-treated alkaline solutions of glucose, however, react at a much slower rate than *l*-ascorbic acid at pH 3.5. In comparing the two methods useful information can be obtained if the following four possibilities be considered. The symbols used in the case of the Lugg method are:

v_1 = titre after reaction at pH 1.5 with HCHO,
 v_2 = titre after reaction at pH 3.5 with HCHO.

In the colorimetric procedure the limit chosen for the percentage of colour generated in 15 min. at 25° is such that, in the presence of the above substances, the final error in the assessment of ascorbic acid would be within 5%.

When $v_2 < v_1/6$ and the colour reaction at $25^\circ < 5\%$ of the final colour. Both the formalin and the 2:4-dinitrophenylhydrazine methods indicate the absence of interference from the above substances. Hence the figures given for *l*-ascorbic acid are in all probability reliable. It should be borne in mind, however, that neither method corrects for the analogues of *l*-ascorbic acid and that any 2:3-diketo-*l*-gulonic acid present will interfere in the colorimetric method.

When $v_2 > v_1/6$ and the colour reaction at $25^\circ > 5\%$ of the final colour. Both methods in this case suggest that one or more of the substances, such as reductic acid, purified reductone and the products resulting from the heat treatment of alkaline solutions of glucose, may be present. It therefore remains to consider the limitations of each method under these conditions. Snow & Zilva (1944) have shown that, when purified reductone is present, the error due to this substance is 5% or less if their correction is applied in the Lugg procedure. When the reaction is carried out at pH 2.0 instead of 3.5 (cf. Mapson, 1943 *a, b*), the error is 7%. The interference due to the reducing substances produced by the heat treatment of NaOH solutions of glucose can also be diminished by the corrected Lugg procedure, the errors in this case being 8 and 22% when the NaOH concentrations are 0.02 and 0.3N respectively. When, however, our differential colorimetric procedure is used, only about 50% of the interfering substance can be eliminated. In the case of heat-treated NaHCO_3 and Na_2CO_3 solutions of glucose we have found that the error due to these substances can be reduced to 6% by the corrected Lugg method

whilst the Roe & Kuether method, after correction, still gives an error of 30%. Thus it will be seen that in all the above cases, the Lugg procedure gives a more reliable estimate of the *l*-ascorbic acid present. This is not the case if reductic acid is the interfering substance, since it has been pointed out by Snow & Zilva (1944) that corrections to the Lugg procedure applied for this substance in unknown solutions are unreliable. The colorimetric method, on the other hand, reduces the error to below 6% if the reductic acid is present in amounts equal to *l*-ascorbic acid or less.

When $v_2 > v_1/6$ and the colour reaction at $25^\circ < 5\%$ of the final colour. In this case interference is indicated only by the Lugg method. No evidence is obtained concerning the identity of these interfering substances. It is, however, clear that reductic acid, purified reductone or the products obtained by the heat treatment of alkaline glucose solutions are not the cause of interference since the reaction with 2:4-dinitrophenylhydrazine for 15 min. at 25° is very small.

When $v_2 < v_1/6$ and the colour reaction at $25^\circ > 5\%$ of the final colour. This condition is the opposite to that given in the preceding case and the interference in the colorimetric method cannot be due to purified reductone, reductic acid or the products obtained by the heat treatment of alkaline solutions of glucose because, if these were present, relatively higher titres with indophenol would be obtained after reaction with HCHO at pH 3.5. With the evidence so far available, it is impossible to state definitely the cause of the interference under these conditions in the colorimetric method.

Table 8. *A comparison of the results obtained by the Roe & Kuether and by the Lugg method applied to substances of unknown composition*

Substance	Ascorbic acid		Indophenol titration method		'Ascorbic acid' at 25° in colorimetric method (%)	Relative titration values obtained with Lugg's method at pH 1.5 and 3.5	Remarks
	Colorimetric method (mg./100 g.)	Lugg's corrected method (mg./100 g.)	Before H_2S (mg./100 g.)	After H_2S (mg./100 g.)			
	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)	(mg./100 g.)			
Chocolate	21.4	22.4	23.8	26.1	<5	$v_2 < v_1/6$	No significant amount of reductic acid, reductone or the products from heat-treated alkaline glucose is indicated
(containing added ascorbic acid)	21.2	21.4	21.9	24.4			
	21.8	22.8	23.2	25.1			
Canned potatoes:							
Liquor	11.0	10.4	11.5	11.5	<5	$v_2 < v_1/6$	"
Tissue	8.1	7.5	8.6	9.2			
Stored dried cabbage	22.1	34.1	25.8	50.0	>5	$v_2 > v_1/6$	Both methods indicate the presence of interfering substances similar in character to the above
Stored dried soup	6.6	7.9	11.5	20.7	>5	$v_2 > v_1/6$	
Canned meat and vegetables:							
Potato	2.0	2.5	3.5	3.9	<5	$v_2 > v_1/6$	Interference in the Lugg method due to substances other than the above
Meat	1.8	2.8	1.9	4.0	"	"	
Gravy	1.9	3.5	4.8	5.4	"	"	
Bean	1.7	1.6	2.4	2.8	"	"	
Carrot	1.9	2.2	3.0	3.4	"	"	

To illustrate the value of a comparison of the two methods we introduce Table 8, in which the analytical results of a number of substances, which on theoretical grounds might be expected to contain some interfering substances, are given. It will be seen in the first place that the values obtained by direct indophenol titration either before or after H_2S treatment are consistently higher than those obtained by the formalin and 2:4-dinitrophenylhydrazine methods; in some cases the difference is very great. Values obtained before and after treatment with H_2S are given because it is not easy in these cases to say which is correct, since any increase after treatment may be due to the presence of dehydroascorbic acid or to the formation of indophenol-reducing substances other than ascorbic acid. The second and most important conclusion from this experiment is that the comparison of the two methods throws some light on the character of the interfering substances. Thus in the case of the stored dried cabbage the interference is in all probability due to the presence of one or of a mixture of substances such as reductone, reductic acid and products similar to those obtained by heating alkaline glucose solutions, whilst in the case of the meat and vegetable ration there is an indication that an indophenol-reducing substance or substances not previously investigated are present.

From the above considerations it may generally be concluded that each one of the methods is likely to yield more accurate results than the direct titration in acid solution, and if used in conjunction with one another some qualitative indication of the type of interfering substances may be obtained. In spite of this far greater specificity obtained with the Lugg and with the Roe & Kuether methods with solutions or extracts of unknown composition, caution must be used in the interpretation of the results until

further knowledge is available, even if the figures should agree with those given by direct indophenol titration in acid solution.

SUMMARY

1. *d*-Glucoscorbic acid, *d*-araboscorbic acid and 2:3-diketo-*l*-gulonic acid cannot be distinguished from *l*-ascorbic acid by the Roe & Kuether method.

2. *l*-Ascorbic acid, reductic acid, purified reductone, heat-treated $NaHCO_3$ and Na_2CO_3 solutions of glucose and heat-treated $NaOH$ solutions of glucose generate after 15 min. at 25° 2.5, 92, 48, 66 and 45 % respectively of the colour given by these substances in 3 hr. at 37° , i.e. the conditions of the Roe & Kuether method.

3. Corrections can therefore be made for any one of the above products when they are present with *l*-ascorbic acid, providing that the analogues of ascorbic acid and diketogulonic acid are absent. In unknown solutions, however, the error due to these substances can be reduced but not eliminated.

4. When Roe & Kuether's method is used in conjunction with Lugg's method an indication of the types of interfering substances may be obtained.

5. Caution is urged in the interpretation of the results obtained by Lugg's and by Roe & Kuether's methods.

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