180. METHODS FOR THE VOLUMETRIC ESTIMATION OF TEA TANNIN IN GREEN-LEAF AND BLACK TEA. A NEW ALKALINE PERMANGANATE METHOD

BY D. N. BARUA AND E. A. HOUGHTON ROBERTS

From the Tocklai Experimental Station, Indian Tea Association, Cinnamara, Assam, India

IN connexion with work in progress in this laboratory on tea fermentation, a volumetric method for the determination of tea tannin has been devised, which is free from many of the disadvantages attending other methods in common use. This method has already enabled us to confirm some of our findings on the extent of oxidation of tea tannin in tea fermentation. It also makes it possible to estimate the amount of tannin in black tea with much greater precision than was formerly possible, and it is hoped that it will facilitate any work on made tea that may be undertaken in the home country. The method may also prove to be of use to biochemists who are interested in tannins other than those obtained from tea.

In the absence of any specific volumetric reagent for tea tannins, these substances are determined in tea infusions by an oxidimetric titration of the infusion followed by a similar determination on another portion of the solution from which tannin has been quantitatively removed. The difference between the "total" and the "non-tan" titres gives the tannin titre.

The infusion is prepared by boiling 5 g. tea or 20 g. fresh green leaf with 400 ml. water for 1 hr., cooling and making up the volume of the extract to 500 ml. Fresh green leaf contains from 75 to 80 % moisture so that the above quantities are approximately equivalent to each other.

The separation into tannins and non-tans is usually effected by gelatin. The following reagents are required.

Gelatin solution. 25 g. gelatin are soaked for 1 hr. in saturated NaCl solution. The mixture is then warmed until the gelatin has dissolved and after cooling the solution is made up to 1 l. with saturated NaCl.

Acid NaCl. 25 ml. of conc. H_2SO_4 are added to 975 ml. saturated NaCl solution.

100 ml. of a tea infusion are mixed with 50 ml. of the gelatin solution, 100 ml. of acid NaCl and 20 g. of powdered kaolin. After shaking for several min. and allowing to settle, the mixture is filtered and an aliquot of the filtrate withdrawn for titration. 25 ml. of the non-tan filtrate are equivalent to 10 ml. of the original infusion.

The Lowenthal and alkaline iodine methods

Hitherto two procedures have been in common use in Tea Research laboratories for the volumetric determination of tannins, the Tocklai modification of the Lowenthal method, and Shaw's iodine method. A Lowenthal technique has been adopted as a tentative method by the Association of Official Agricultural Chemists [1936].

The Tocklai-Lowenthal method. The following reagents are required: (1) $N/25$ $KMnO_4$; (2) 1·5 g. indigo carmine dissolved in 1 l. water containing 50 ml. H_2SO_4.

A 10 ml. aliquot of the infusion is mixed with 25 ml. of the indigo-carmine solution, and the mixture diluted to 750 ml. $KMnO_4$ is then run in from a burette 1 ml. at a time with brisk shaking. As titration proceeds the blue of the indigo-carmine passes through many shades to a final yellow with a faint pink tint at the rim. This is taken as the end-point and needs considerable judgement on the part of the operator. The titre is affected to some extent by the amount of tannin in solution, the time taken in the titration and the vigour of the shaking. Repeats often differ by 0·3 ml. or more, and the variation between different operators may amount to 1 ml.

A correction in the total titre, A, must be made for the indigo-carmine oxidized. This usually amounts to 4·0 ml. For the non-tan titre, B, a slightly higher blank of 4·5 ml. is found in practice. The tentative method of the A.O.A.C. makes no mention of this variation in the blank and arrives at the tannin titre by subtracting the non-tan from the total titre. The actual tannin titre will be $(A-4·0)-(B-4·5)$.

The factor for converting ml. N $KMnO_4$ into g. tannin is 0·0416 as determined by the hide powder method. This, quite fortuitously, equals the factor for gallo-tannic acid.

The alkaline iodine method. The method was elaborated by Shaw [1930] and has been slightly modified at Tocklai. Oxidation is effected by an excess of $N/10$ I_2 in alkaline solution, and the excess I_2 determined after a period of 30 min. The same gelatin method is used for the separation of non-tans. The end-point is of course much sharper in this method than with that of Lowenthal. The main objection to the method lies in the incompleteness of the oxidation by alkaline I_2, so that the room temperature and the time during which oxidation is taking place have a distinct effect upon the final result.

In both of the above methods oxidation of the tannin to some indeterminate product takes place. Assuming tea tannin to be a mixture of catechin and gallo-catechin units [Harrison & Roberts, 1939] with a mean mol. wt. of 300, it may be calculated that in the Lowenthal method some 7·1 equiv. O_2 are consumed in the oxidation of 1 mol. tea tannin, whereas in the modified Shaw method 16·7 equiv. are used up.

For complete oxidation to CO_2, an equimolecular mixture of these two catechins would require 60 equiv. O_2 per mol. Most of the disadvantages of the two methods so far described are really due to the uncertainty of the extent of oxidation of tannin that takes place during the estimation. They may, however, be used to determine the amount of tannin in green tea leaf with fair accuracy if the conditions during the whole estimation are very carefully standardized. For manufactured (black) tea, however, they give very erroneous results, and the two methods give quite different figures for tannin contents, if the factors determined for green leaf are applied (Table 1).

Table 1. *Tannin titres for fresh and fermented tea leaf*

	Lowenthal		Alkaline I_2	
	ml. 0·04 N $KMnO_4$ per g. dry wt.	% tannin	ml. 0·04 N I_2 per g. dry wt.	% tannin
Fresh leaf	139·2	23·2	302·0	21·4
Leaf fermented 2½ hr.	88·7	14·8	247·4	17·5

It is usual to find analyses recorded for made tea, carried out by the official Lowenthal method, stating that the tannin content is from 12 to 14 %. Such a drop in the actual tannin content as a result of fermentation cannot be established. There is certainly a fall in the water-soluble tannin as a result of tea-fermentation [Harrison & Roberts, 1939] but not more than 15–20 % of the total amount of tannin in the green leaf is rendered insoluble in this way. A large decrease in the water-soluble tannin as a result of fermentation would be accompanied by a corresponding decrease in the total water-soluble solids. There is indeed a decrease here, but the average for made tea is 41 % against 45 % for green leaf, so even if the whole of the decrease in water-soluble solids were due to decreased solubility of the tannin, the fraction of the tannin thus made insoluble would not exceed 20 % of the whole.

It must be remembered that the oxidation product of green leaf tea tannin is still a tannin, and presumably this tannin, being partially oxidized, will require less $KMnO_4$ or I_2 to oxidize it. Different factors are therefore required for the tannins in made tea infusions, but even if allowance is made for the oxidation of the tannins and the proportion of these bodies which have become insoluble (by combination with leaf protein), the decrease in the Lowenthal and alkaline I_2 titres is still too great to be accounted for. Further, if tannins are incubated with tea oxidase solutions, the decrease in the tannin titre is greater than the equivalent O_2 uptake, and under such conditions none of the tannin has been rendered insoluble.

An explanation of these difficulties has been found in the condensation of the tannin bodies after oxidation. It is known that the condensation of catechins is greatly accelerated by oxidation of the molecule [Freudenberg, 1933] and from the kinetics of tea fermentation it follows that some irreversible change must occur in the primary oxidation product of tea tannin. Further, the varying liquoring characters of black tea can be very satisfactorily explained if different degrees of condensation of the oxidized tannins are assumed.

Now highly condensed products, such as the tannins in black tea, will not be so readily oxidized as their simpler parent substances in green leaf, and it will be readily seen that in both Lowenthal's and Shaw's methods, slower oxidation means less oxidation. The same quantity of tannin will have taken up less permanganate before the indigo-carmine is completely oxidized, and in the same period of incubation, less tannin will have been oxidized by the alkaline I_2. The drop in tannin content, as a result of fermentation, would therefore seem to be apparent only, and it must be concluded that the two methods in general use give very erroneous results for the tannin content of a black-tea infusion.

Reference to Table 1 shows that the apparent fall in tannin content on fermentation is not so marked when the alkaline I_2 method is used for the estimation. This is of course due to the greater number of O equivalents used up in the I_2 titration, as the actual decrease in titre (54·6 ml. 0·04 N I_2) is greater than that recorded by the Lowenthal method (50·5 ml. 0·04 N $KMnO_4$). From this result it would appear that condensed tannins are less readily oxidized by the alkaline I_2 than they are by the Lowenthal method. The example quoted in Table 1 gives the tannin changes in coarsely minced fresh leaf after $2\frac{1}{2}$ hr. fermentation. Complete oxidation of tannin to the o-quinone is not realized in this time. This point must be emphasized as otherwise it would appear that the decrease in the titre is no greater than would be expected from oxidation and combination of 15–20 % of the tannins with leaf protein. Comparison of the percentages of tannin in made tea according to these two methods, and the new alkaline $KMnO_4$ method, given in Table 4 shows that Shaw's method does give low results.

Stamm's alkaline permanganate method

From the above discussion of the methods' of tannin titration so far used in tea chemistry, it is evident that most of their drawbacks are connected with the indefinite nature of the end-products of oxidation. A method is required in which the tannins of either green leaf or black tea are oxidized to a definite end-point.

For green-leaf tannins cerimetry might prove practicable, but such a method would be of no use in estimating the tannins in black tea, where from 75 to 95 % of the tannins are already oxidized. Preliminary volumetric estimations with ceric sulphate have indicated that tea tannin is oxidized only to the quinone stage by this reagent, which implies that only 2 equiv. O are used per mol. of tea tannin.

Complete oxidation to CO_2 is desirable, and this has been realized with Stamm's alkaline permanganate method [Böttger, 1938], the second procedure, which involves back-titration with oxalic acid in an acid medium, being employed. For the theory of the method reference should be made to Böttger's monograph.

The success of the method was foreshadowed by the complete oxidation to CO_2 by alkaline $KMnO_4$ of catechol, pyrogallol, gallic acid and phloroglucinol all of which enter into the composition of one or other of the tea-tannins.

Reagents.

(1) 0.1 M $KMnO_4$ (15.803 g. per l.).
(2) 30 g. pure NaOH in 100 ml. H_2O.
(3) 0.02 M $KMnO_4$.
(4) Oxalic acid, containing about 34 g. $H_2C_2O_4, 2H_2O$ per litre.
(5) Strong H_2SO_4 containing 1 part of acid to 1 part of H_2O by weight.
(6) $MnSO_4, 4H_2O$, 50 g. per l.

Procedure. Infusions of 20 g. fresh green leaf or 5 g. made tea in 500 ml. are prepared. Solutions for non-tan estimation are prepared in the same way as for the Lowenthal method. A 20 ml. portion of the original infusion and a 25 ml. portion of the non-tan filtrate are each diluted to 100 ml., and 10 ml. and 20 ml. portions respectively of these diluted solutions are withdrawn for "total" and "non-tan" estimations.

The sample to be oxidized is treated with 20 ml. 0.1 M $KMnO_4$ and 10 ml. NaOH (2), and the mixture allowed to stand for 10 min. at room temperature (c. 30°) after which it is diluted to 250 ml.; 10 ml. H_2SO_4 (5), 20 ml. oxalic acid (4) and about 10 ml. $MnSO_4$ (6) are then added. The colour of the solution is quickly discharged on shaking, and after heating the solution to 50° the estimation is completed by titrating the residual oxalic acid with 0.02 M $KMnO_4$. The above procedure gives values, A and B, for total and non-tan titres respectively.

Reagent blanks must also be determined. The blank consumption, according to Stamm, is obtained by treating 20 ml. oxalic acid with 5 ml. H_2SO_4 and warming to 50° after which 10 ml. $MnSO_4$ and 20 ml. 0.1 M $KMnO_4$ are added; after decoloration the excess oxalic acid is titrated with 0.02 M $KMnO_4$, this final titre giving the required blank, C.

As in the Lowenthal method, the "non-tan" reagents blank is higher than the reagents blank just described. A blank non-tan filtrate is prepared using 100 ml. H_2O in place of 100 ml. infusion, and an equivalent amount of the filtrate to that used for the non-tan determination is withdrawn for titration. The non-tan blank D is generally some 0.6 ml. higher than the reagents blank C.

The final tannin titre is given by $(A–C)–(B–D)$.

Some of the stages in the above procedure require explanation. The taking of a large sample for the infusion, and the use of such a small aliquot is necessary for two reasons. Smaller samples than those used are apt to be unrepresentative, unless the green leaf is very finely minced, or the tea finely powdered. Further, the use of a much less concentrated infusion often results in incomplete precipitation of tannin by gelatin.

Considerable difficulty was experienced at first with the non-tan determinations which showed wide variations on replication. This difficulty was due to the oxidation by $KMnO_4$ of the HCl liberated on acidification after the alkaline $KMnO_4$ oxidation. If the solution is diluted to 250 ml. immediately after the incubation with alkaline $KMnO_4$ oxidation of HCl becomes a small and fairly constant factor, and the addition of oxalic acid may be postponed for 5 min. without affecting the titration values to a measurable extent. Although this factor is only of importance when titrating non-tan filtrates it is considered advisable to carry out the titration in the same way for both total and non-tan estimations.

The non-tan fraction consists mainly of glucose, caffeine and amino-acids. The first of these is known to be completely oxidized by alkaline $KMnO_4$ to CO_2. Oxidation of the other two substances has been shown to be incomplete, as higher titration figures are obtained by extending the time of incubation or increasing the temperature to 40°. This incomplete oxidation of some of the non-tan substances however, does not materially affect the result. The total contribution of caffeine and amino-acids to the non-tan titre does not exceed 1 ml. in the aliquot used. Independent measurements have shown that extreme variations in the time and temperature of incubation do not affect this titre by more than 0·2 ml. As total and non-tan titrations are carried out under almost identical conditions, the error due to this incomplete oxidation is unlikely to exceed a fraction of 0·1 ml., and may safely be neglected.

The greatest care must be exercised throughout the determination in burette and pipette measurements of $KMnO_4$, since every error is multiplied by 5 because of the change from 0·1 M to 0·02 M solution in the determination of the excess oxalic acid.

Calculation of results. The conversion of the tannin titre of a solution into a percentage by weight of water-soluble tannin in the sample requires a knowledge of the structure of tea tannins which we do not possess at present. Until such knowledge is available an empirical factor only can be employed.

There is good reason for believing that the tannins in tea consist largely of the simpler condensation products of epicatechin and gallocatechin. The possibility that the galloyl ester of catechin is also present in relatively large amounts is indicated by the recent work of Deijs [1939]. The number of equivalents of O necessary for complete oxidation to CO_2 of these substances is shown below:

	Mol. wt.	Equiv. O for complete oxidation	g. tannin per equiv. O
Catechin	290	62	4·68
Gallocatechin	306	60	5·10
Galloyl-catechin	442	86	5·14

It may be assumed therefore that each equivalent of O oxidizes 5 g. of tannin, without any very great error. On this assumption the tannin titre obtained for a 10 ml. aliquot of the fivefold diluted green leaf infusion must be multiplied by 0·625 to give the percentage water-soluble tannin in the original green leaf. Results obtained in this way have agreed quite closely with those obtained by the Lowenthal method.

For black tea infusions a different factor must be employed. The tannin molecule takes up 1 atom only (2 equiv.) of O as a result of enzymic oxidation. During fermentation such oxidation is about 85 % complete on the average, so that the uptake of O_2 amounts to 1·7 equiv./mol. of tannin. 300 g. of tannin therefore now require only 58·3 equiv. of O for complete oxidation, neglecting changes in weight of tannins due to oxidation and condensation. As 5 g. of black tea are taken for the infusion as against 20 g. fresh leaf the factor now becomes 2·57. The tannin titre multiplied by this factor gives the percentage of water-soluble tannin in black tea. It is not usual to express this percentage in terms of dry weight. For green tea, where the tannins have not been oxidized during manufacture, the factor would be 2·50.

Results. Representative results with green leaf and made tea are quoted below.

Green leaf		ml. 0·1 M KMnO$_4$
Total titre	=	18·5–6·7
Non-tan titre	=	12·0–7·5
Tannin titre	=	(18·5–6·7) – (12·0–7·5) = 7·3
% tannin (wet wt.)	=	7·3 × 0·625 = 4·56 %
Moisture content of green leaf	=	79·0 %
% tannin (dry wt.)	=	21·7 %

Made tea		ml. 0·1 M KMnO$_4$
Total titre	=	18·3–7·5
Non-tan titre	=	11·7–8·1
Tannin titre	=	(18·3–7·5) – (11·7–8·1) = 7·2 ml. KMnO$_4$
% tannin	=	7·2 × 2·57 = 18·50 %

The average water-soluble tannin content in made tea is distinctly lower than that of fresh leaf although the figures are much higher than those found for black tea by the Lowenthal method. A drop in water-soluble tannin is also observed after tea fermentation.

Thus Table 2 records percentages of tannin as found by Stamm's and Lowenthal's methods for coarsely minced samples of fresh and fermented tea leaf. Four 20 g. samples of the coarsely minced leaf were weighed out. Two were infused at once and the other two after allowing 2½ hr. for the fermentation process to take place. The figures in the above table are the means of duplicate samples; percentages are on the original wet weight basis.

Table 2. *Tannin percentages of fresh and fermented leaf*

	Alkaline KMnO$_4$	Lowenthal
Green leaf	5·08	5·41
Fermented leaf	4·22	3·23

It has already been shown that according to the Lowenthal method the fall in the KMnO$_4$ tannin titre is considerably greater than corresponds with the uptake of O_2, even when the oxidation of the tannin takes place in aqueous solution [Roberts & Sarma, 1938; Harrison & Roberts, 1939]. The following experiment shows the relative fall in tannin titre by the Lowenthal and Stamm methods when enzyme and substrate are incubated together.

The enzyme was a solution in $M/15$ phosphate buffer pH 5·8 of an arbitrary amount of the alcohol precipitate of withered tea-leaf juice [Roberts, 1940], and the substrate was tea tannin prepared according to the standard Tocklai method [Harrison & Roberts, 1939]. Mixtures of 1 ml. enzyme and 2 ml. substrate (500 mg. tannin in 50 ml. H_2O) were shaken in each of 10 Warburg vessels until

the fall in the rate of O_2 uptake indicated that oxidation of the tannin was nearly complete. The united reaction products from the Warburg vessels, after suitable dilution, were then analysed for their tannin contents by the Lowenthal and Stamm methods. 20 and 5 ml. aliquots were taken respectively for these two determinations and the corresponding amounts for non-tan estimations. The initial tannin content of the enzyme-substrate mixture was determined on another solution containing 20 ml. of the tannin solution and 10 ml. enzyme, diluted to 200 ml.

Table 3

	Stamm ml. 0·1 M KMnO$_4$	Lowenthal ml. 0·04 N KMnO$_4$
Initial tannin titre	7·9	9·4
Final tannin titre	7·7	6·4

The calculated difference in tannin titre for alkaline KMnO$_4$ is 0·26 ml., so the observed difference agrees very well with that predicted.

DISCUSSION

The foregoing results show that Stamm's alkaline KMnO$_4$ method may be adapted to the determination of tannins both in green leaf and in made tea.

The method, although tedious in comparison with that of Lowenthal, is capable of greater precision; the end-point is sharp and does not vary with the personal factor and the conditions under which the determination is carried out. For rapid methods of estimation of unoxidized tannin where great accuracy is not called for, the Lowenthal is more convenient, but for the estimation of tannin in fermented tea the Stamm procedure is the only method which gives results approaching accuracy. The Lowenthal titre varies so much with the conditions of fermentation that it can give no reliable measure of the tannin content. Cases, particularly those where slowly fermenting varieties of tea are concerned, are frequently met where one tea, which possesses the highest Lowenthal titre of several under investigation, actually has the lowest true tannin content.

In conclusion it may be of interest to record percentages of tannin for two samples of black tea, as determined by the three methods here reviewed. The first tea was unusually high in tannin, and only some 70 % of its tannin had been oxidized during fermentation. A longer period of fermentation would have accentuated the differences found by these methods. The second tea had been fermented for 5¾ hr. at 90°, and must be considered distinctly overfermented.

Table 4

	Tannin %		
Sample	Lowenthal	Shaw	Stamm
A	16·0	18·9	20·2
B	13·8	15·0	18·0

The above method applies not only to tea tannin but has been successfully used in the estimation of tannins in other plant tissues.

SUMMARY

Current methods for the volumetric estimation of tannins are reviewed. Errors in such determinations are mainly due to the arbitrary nature of the end-point which corresponds with no definite end-product of oxidation.

For unoxidized tannins these methods give fairly accurate results but they are completely unreliable when oxidized tannins are being estimated, as the titration is carried to a different end-point owing to decreases in the rate of oxidation of the tannin bodies as a result of condensations following oxidation.

The Stamm alkaline $KMnO_4$ method gives a much more accurate result for oxidized tannins, as in this method the tannins are oxidized completely to CO_2.

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REFERENCES

Association of Official Agricultural Chemists (1936). Official and Tentative Methods of Analysis, 4th ed. p. 196.

Böttger (1938). Newer Methods of Volumetric Chemical Analysis. London.

Deijs (1939). *Rec. Trav. chim. Pays-Bas*, **58**, 805.

Freudenberg (1933). Tannin, Cellulose, Lignin. Berlin.

Harrison & Roberts (1939). *Biochem. J.* **33**, 1408.

Roberts (1940). *Biochem. J.* **34**, 500.

—— & Sarma (1938). *Biochem. J.* **32**, 1819.

Shaw (1930). Tannin Principles of Tea, United Planters' Association of South India, Madras.