A Study of the Polyphenols in Tea Leaf by Paper Chromatography

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Earlier work on the isolation and characterization of the components of the so-called tea tannin, reviewed by Harrison & Roberts (1939), relied mainly on the classical methods of selective extraction and precipitation. The development of partition chromatography as a preparative method has enabled much further progress to be made in the elucidation of the nature of these polyphenols. Bradfield and coworkers have isolated seven catechins in Ceylon green tea, namely L-gallocatechin, DL-gallocatechin, L-*epi*catechin, catechin, L-gallocatechin gallate, L-*epi*catechin gallate and a galloyl ester of gallocatechin (Bradfield, 1946; Bradfield, Penney & Wright, 1947; Bradfield & Penney, 1948). Later, Bradfield & Bate-Smith (1950) suggested that L-gallocatechin and L-gallocatechin gallate might more accurately be identified as L-*epi*gallocatechin and L-*epi*gallocatechin gallate respectively; whilst the galloyl ester of gallocatechin, i.e. the 'substance 2*a*' of Bradfield & Penney (1948), might be the galloyl ester of L-gallocatechin. Further evidence in support and extension of these views will be considered in this communication.

The success of preparative partition chromatography suggested that useful information might be obtained by applying the methods of paper chromatography (Consden, Gordon & Martin, 1944) to a study of the catechins and other polyphenols in tea, and this communication describes the provisional identification of the main polyphenols in tea leaf. The effect of high temperatures during extraction is found not only to affect the relative proportions of some of the various polyphenols but may also result in the production of artifacts, not necessarily present in the natural state. The behaviour of individual polyphenols on enzymic oxidation is discussed. The presence of a large number of so far unidentified anthoxanthins is recorded.

METHODS AND MATERIALS

Sources of polyphenols

Fresh juice. A fine mince of plucked tea shoots is squeezed by hand through fine cloth. The resultant juice is clarified by centrifuging for 5 min., rapidly heated to 100° to prevent enzymic oxidation, and then cooled to room temperature.

Reconstituted juice. It is not always convenient to examine leaf at the time of plucking, and it has been found that identical chromatograms are obtained if the leaf is dried in a current of air heated to 90°, powdered, and subsequently ground with three times its weight of water. The 'reconstituted' juice is then obtained by squeezing through cloth and centrifuging as above. In the case of manufactured tea the product is powdered and mixed with twice its weight of water, after which the same procedure is followed.

Catechin preparations. Mixed tea catechins obtained by the method described by Roberts & Wood (1950) are referred to as precipitated catechins. Other catechin preparations have been obtained essentially by the method described by Bradfield *et al.* (1947). The final product, referred to subsequently as amorphous tea catechin, is obtained by removing the ethyl acetate *in vacuo* in a current of CO_2, taking up the product in the minimum of acetone, and evaporating to dryness.

Chromatographic technique

Apparatus. For two-dimensional analysis cabinets similar to that described by Dent (1948) are used, the one for *n*-butanol-acetic acid being lined with aluminium sheet. In both cabinets the solvent trough is of stainless steel. Sheets of Whatman no. 1 paper have been used throughout.

Quantities required for chromatography. 35 μl. Juice (fresh or reconstituted) represents a suitable quantity for analysis. This quantity is measured out in two portions, allowing the first portion to dry before adding the second. With catechin preparations 20 μl. of a solution containing 100 mg./ml. is a suitable amount.

Solvents. Phenol, saturated with water, is used as the first solvent. For the second solvent the non-aqueous phase of a mixture of *n*-butanol (40%), acetic acid (10%), and water (50%) was first employed, but temperature fluctuations often resulted in separation into two phases. This was avoided by making up a mixture of *n*-butanol (80%) and acetic acid (20%), and adding water just short of the amount required for saturation. Percentages are v/v. A reproducible pattern of spots, and no separation of the solvent into two phases, has resulted.

Spray fluids. For general use $FeCl_3$ (0·1%, w/v) in water or $FeNH_4(SO_4)_2.12H_2O$ (0·2%, w/v) have been found of most value, since they differentiate between catechol and pyrogallol groupings, the former giving a green colour, the latter a dark grey-blue. When both groupings are present in the same molecule, e.g. L-*epi*catechin gallate, the colour is also dark grey-blue. The spots do not always develop their full intensity on spraying, particularly if the paper still contains traces of acetic acid. By holding an opened bottle of ammonia close to each spot full intensities are developed. The spots for catechin and *epi*catechin, if subjected to this treatment, turn dark blue and are then indistinguishable from the other catechins. Some of the anthoxanthins give characteristic, although much weaker, colours with ferric salts. KCN (1%, w/v) has been used to detect gallic acid,

giving an immediate pink colour which fades rapidly. Catechin gallates give an orange-pink which fades, and then gives place to various shades of buff, brown and orange. To detect anthoxanthins the dry chromatogram (before spraying) is exposed to ammonia vapour; the anthoxanthins give temporary yellow colours.

R_F values. R_F values vary considerably with temperature, and it is impracticable to maintain a constant temperature in our laboratory throughout the year. Individual catechins, however, are readily recognized by their position in the overall pattern of spots. R_F values as such are not measured, but they are roughly indicated in Fig. 1, representing average conditions (25°).

RESULTS

Chromatograms of fresh juice and of catechin preparations

A typical two-way chromatogram of fresh juice is illustrated in Fig. 1, the size of the ovals indicating the relative intensities of the spots. In this particular case spraying with $FeCl_3$ reveals nine components, two of which, 9 and 10, give green spots, while the remainder give dark blue-grey spots. After a few days spots 2 and 3 fade to a brown colour.

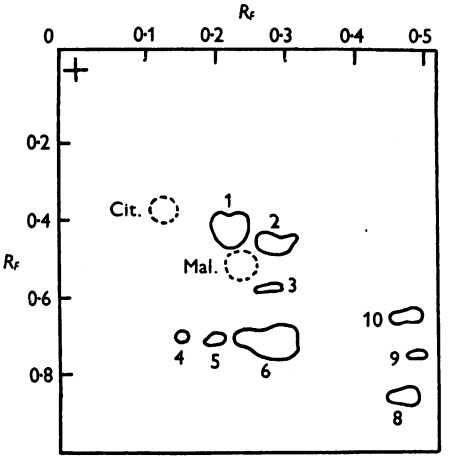

Fig. 1. Chromatogram of tea-leaf juice, showing positions of major polyphenolic components (spots 1–6 and 8–10), and citric and malic acids. The chromatogram was run first from left to right with phenol as solvent, and then downwards with *n*-butanol-acetic acid. R_F values are approximate only. Colour reagent $FeCl_3$. Size of spot roughly proportional to colour intensity.

The fading of spot 2 is of particular value, owing to the frequent merging of spots 1 and 2 in chromatograms. In such cases only part of the spot fades to brown, so that evidence for both components is available when separation is poor.

The chromatogram (Fig. 2) for precipitated catechins, prepared from the same batch of leaf, is not dissimilar save that spot 1 is absent, a new spot (no. 7) appears and the relative intensities of some of the other spots are affected. The relative proportions of the ten components in precipitated and amorphous preparations and in fresh juice are indicated in Table 1.

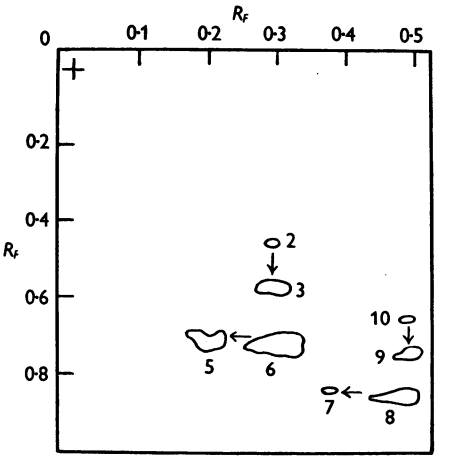

Fig. 2. Chromatogram of amorphous or precipitated catechin preparation. The arrows indicate the direction of epimeric changes caused by heating the solutions. Conditions of chromatography as in Fig. 1.

The fact that spot 1 is either faint or absent altogether in chromatograms of catechin preparations indicates that this component is not extractable from aqueous solution by ethyl acetate. Spots 5 and 8 are relatively more intense in catechin preparations

Table 1. *Comparison of fresh juice with crude catechin preparations*

(Relative colour intensities after chromatography and colour development with $FeCl_3$. Spot numbers refer to Figs. 1 and 2 and colour intensity is indicated by the scale + + + + very strong to (+) trace.)

Spot no.	Fresh juice	Amorphous catechin	Precipitated catechin
1	+ + +	(+)	.
2	(+)	(+)	.
3	+(+)	+(+)	+(+)
4	+	+	+
5	+(+)	+ +(+)	+ + +(+)
6	+ + +(+)	+ + + +	+ + + +
7	.	(+)	+
8	+(+)	+ +(+)	+ +(+)
9	+	+(+)	+(+)
10	+	+	+

than in the original juice, suggesting that selective extraction and precipitation may be operative. The appearance of the new spot 7 suggests that the treatment given during preparation may result in the production of substances not present in the original juice.

In the preparation of precipitated catechins the green leaf is refluxed for 1 hr., and in the procedure of Bradfield *et al.* (1947), which forms the first stage of our method of preparing amorphous catechins, there are several stages involving refluxing or boiling. These preparations therefore differ from fresh juice in that they have been maintained at a relatively high temperature for an appreciable period, and not for the short time required for inactivation of enzymes in the fresh juice. To test the effect of such high temperatures chromatograms were run with fresh juice and fresh juice refluxed for 1 hr. Typical results of such an experiment are shown in Table 2. Spots 2 and 6 show a definite decrease, spots 3 and 9 show an increase in intensity and spots 5 and 7 make their appearance.

Table 2. *Effect of heat treatment on the chromatographic behaviour of fresh juice*

(The spot numbers refer to the spots indicated in Figs. 1 and 2. Relative colour intensities ($FeCl_3$) are indicated in the same way as in Table 1.)

Spot no.	Juice unboiled	Juice boiled	Juice from untreated mince	Juice from autoclaved mince
1	+ +	+ +	+ + +	+ +(+)
2	+ +(+)	+(+)	+ +(+)	+(+)
3	+(+)	+ +(+)	(+)	+(+)
4	(+)	+	+	+
5	.	+(+)	.	+(+)
6	+ + +(+)	+ + +	+ + + +	+ + +
7	.	(+)	.	(+)
8	+	+	+ +	+
9	(+)	+	(+)	+
10	+(+)	+(+)	+(+)	+

It is not possible to express slight variations adequately by the method of estimation adopted, and in this particular case there is no apparent change in the intensity of spots 8 and 10. In other experiments decreases in one or both of these spots have been observed (cf. also Table 3). In such cases, however, changes in some of the other components have been less definite. Greater effects are observed after autoclaving the mince at 130° before expressing the juice, and as a result of such treatment significant decreases are observed in the intensities of spots 2, 6, 8 and 10. Spots 3 and 9 show increased intensity, whilst spots 5 and 7 appear on the chromatogram as a result of the autoclaving treatment (Table 2).

Epimerization

During the earlier stages of this work the only catechin available was Merck's catechin. Addition of this substance to tea-leaf juice intensified spot 9 in the resultant chromatogram. It seemed reasonable to deduce that spot 10, which like 9 also gives a green colour on spraying with $FeCl_3$, was due to L-*epi*catechin. This was confirmed later by a comparison of R_F values in *n*-butanol-acetic acid with those obtained by Bradfield & Bate-Smith (1950), and by addition of authentic specimens of L-*epi*catechin to the expressed juice, which resulted in intensification of spot 10.

The changes in the relative intensities of spots 9 and 10 as a result of boiling appear, therefore, to be due to epimerization, L-*epi*catechin being converted into catechin. Confirmation was provided by refluxing a solution of catechin for 2 hr.; this treatment resulted in the appearance of a second green spot, in the position expected for L-*epi*catechin, on a one-way chromatogram run with *n*-butanol-acetic acid and sprayed with $FeCl_3$. This also indicates that the epimerization is reversible.

The possibility therefore arises that spots 2, 3 and 5–8 may represent three further pairs of epimers. Grouping into pairs was facilitated by the observation that some sources of leaf were lacking in certain catechins. On one occasion juice expressed from the leaf of one clone of tea (14/5/18) was found to contain no catechins corresponding with spots 2 and 3. Leaf was also available from an unidentified *Camellia* from northern Burma, which although botanically distinct, bears many resemblances to tea, and can be crossed with it. The juice expressed from leaves from this source gave a chromatogram consisting of spots 1, 8 and 10 together with others corresponding to hitherto unidentified polyphenols.

The effects of refluxing juice from these two sources are given in Table 3.

Table 3. *The effect of boiling juice lacking in certain catechins on its chromatographic behaviour*

(The spot numbers refer to Figs. 1 and 2. Colour intensity after spraying with $FeCl_3$ is indicated by the same convention as in Table 1.)

	Clone 14/5/18		*Camellia* sp.	
Spot no.	Unboiled	Boiled	Unboiled	Boiled
1	+ + +	+ + +	+ + + +	+ + + +
2	.	.	.	.
3	.	.	.	.
4	.	.	.	.
5	.	+(+)	.	.
6	+ + + +	+ + +	.	.
7	.	(+)	.	(+)
8	+(+)	+	+ +	+
9	(+)	(+)	.	(+)
10	+	(+)	+	(+)

From the results in Tables 2 and 3 it will be seen that spot 3 only increases in intensity, as a result of boiling, if spot 2 is originally present. Further evidence for the pairing of spots 2 and 3 is their similar behaviour in fading to a brown colour after a few days. An increase in the intensity of spot 5 (or its appearance) only takes place if the catechin corresponding to spot 6 is present in the unboiled juice. Finally, the appearance of spot 7, after boiling a juice containing catechins corresponding to spots 8 and 10, indicates that spots 7 and 8 form the

third pair, as spot 10 has already been shown to be paired with spot 9.

The relationships between the four pairs of catechins are shown diagrammatically in Fig. 2; an arrow linking each pair indicates the direction of the change taking place on boiling. It is noteworthy that each pair of catechins is represented by spots very close to each other on the chromatogram, which is consistent with the differences in chemical composition being in each case relatively small.

Identification of spots on the chromatogram

In view of the isolation of seven catechins in tea leaf (Bradfield *et al.* 1947; Bradfield & Penney, 1948) it appeared likely that these catechins were responsible for seven of the ten spots under consideration. As Bradfield (1946) found that the substance originally described as L-gallocatechin gallate was always the most abundant, it seemed that it might be spot 6 on our chromatograms.

If juice is inoculated with the spores of *Aspergillus niger* a vigorous growth of the mould develops on the surface of the juice. According to Deijs & Dijkman (1936). *A. niger* contains a powerful tannase capable of eliminating gallic acid from such galloyl esters as are found in tea, so that one would expect a marked decrease in the content of galloyl esters and a corresponding increase in gallic acid, all of which changes should be detectable on the chromatogram. Autoclaved juice is more suitable for such an experiment than fresh juice as it contains appreciable amounts of the polyphenols corresponding with spots 5 and 7.

In one experiment on autoclaved juice suitable growth of the mould was attained 3 days after inoculation. A chromatogram of the inoculated juice was then run and compared with that of the control (Table 4). The result of such treatment was a marked decrease in the strength of spots 5–8 and a considerable increase in that of spot 4, which latter is thus indicated as gallic acid. It follows that spots 5–8 are probably galloyl esters. As spot 6 has already been provisionally identified, spot 8 probably corresponds with L-*epi*catechin gallate, a known component of the tea leaf, while spots 5 and 7 normally absent from unboiled tea juice are probably galloyl esters formed from 6 and 8 respectively, on boiling. This leaves spots 2 and 3 unidentified, and by elimination these may be L-*epi*gallocatechin and DL-gallocatechin respectively. The *epi* structure is allotted to the catechin of lower R_F in *n*-butanol-acetic acid by analogy with the identification of L-*epi*catechin with spot 10.

Subsequent to these preliminary identifications, authentic specimens of the following catechins were received: L-*epi*gallocatechin, DL-gallocatechin, L-*epi*gallocatechin gallate, gallocatechin-a gallate, L-*epi*catechin gallate and L-*epi*catechin. The nomenclature is that as revised by Bradfield & Bate-Smith (1950).

By addition of suitable amounts of these substances to tea juice, intensifications of the appropriate spots occurred, enabling more positive identifications to be made. In some cases where the original spot is very strong, juice from fermented tea proved more suitable, as on fermentation several of the spots decrease very considerably in intensity.

Table 4. *Effect of incubation of juice with* Aspergillus niger *on its chromatographic behaviour*

(Spot numbers as in Figs. 1 and 2. Colour intensity after spraying with $FeCl_3$ as in Table 1.)

Spot no.	Control	Incubated with *A. niger*
1	+ + +	+ + + +
2	+	+(+)
3	+ +	+ +
4	+ +	+ + + +
5	+ + +	+(+)
6	+ + +	+
7	+	(+)
8	+ +	(+)
9	+(+)	+(+)
10	+(+)	+(+)

These experiments support the provisional identifications of spots 2, 3, 6 and 8, and identify spot 5 with gallocatechin-a gallate. Provisional identifications of spots 9 and 10 have already been made. The possible identification of spot 7 as catechin-a gallate is treated in the discussion.

The provisional identification of spot 4 with gallic acid follows from the results of inoculation with *Aspergillus niger*. Its acidic nature is indicated by the results of spraying with bromocresol green (Lugg & Overell, 1947) while spraying with potassium cyanide gives the characteristic fading pink colour reaction for gallic acid. Confirmation is afforded by adding gallic acid to juice when the resultant chromatogram has spot 4 greatly intensified.

The identity of spot 1 has not yet been established, but the following considerations lead to its tentative identification with *m*-digallic acid. Spraying the chromatogram with bromocresol green gives two intensely yellow spots corresponding with spots 1 and 4 (other organic acids are also shown up). Subsequent spraying with ferric chloride changes these two yellow spots to a dark blue-grey. Spot 1, therefore, is an organic acid containing a pyrogallol group.

An aqueous solution containing no other polyphenols apart from traces of gallic acid can be obtained by exhaustive extraction of a green leaf infusion with ethyl acetate. The aqueous layer on concentration and chromatography has a strong spot 1 and also contains a little gallic acid. This solution on hydrolysis with hydrochloric acid gives gallic acid as the only polyphenolic product as

judged by paper chromatography of the hydrolysate. No characteristic dark-red precipitate is formed, such as is obtained when catechins are similarly treated. A more concentrated solution was obtained by precipitation with lead acetate, decomposition of the lead salt with N-sulphuric acid, and subsequent concentration. This solution gave no pine-shaving test for phloroglucinol, and no precipitate with bromine water. On the other hand, it gave a precipitate with gelatin. Judging by its persistence in fermented tea as discussed later, it is oxidized only slowly by tea oxidase.

The fact that spot 1 is an acid, and gives gallic acid on hydrolysis, suggests its possible identity with *m*-digallic acid. This is in accordance with the results of the tests with the pine shaving, bromine water and gelatin, and its failure to be extracted from aqueous solution by ethyl acetate. Further work in connexion with the identification of this polyphenolic acid is in progress.

Provisional identifications of the ten most frequently occurring spots are given in Table 5.

Table 5. *Provisional identification of polyphenols on chromatograms*

Spot no.	Polyphenol
1	*m*-Digallic acid (?)
2	L-*epi*Gallocatechin
3	DL-Gallocatechin
4	Gallic acid
5	Gallocatechin-a gallate
6	L-*epi*Gallocatechin gallate
7	Catechin-a gallate (?)
8	L-*epi*Catechin gallate
9	Catechin
10	L-*epi*Catechin

Other substances appearing on the chromatograms

Polyphenols. The ten components discussed in detail above appear to be those which most generally occur in teas grown in north-east India. In addition, however, other spots, the positions of which are given in Fig. 3, giving dark blue-grey colours with ferric chloride are observed from time to time. Normally they occur in traces only, although towards the end of the year spot 14 is sometimes present as a major component. So far no progress has been made in their identification.

Anthoxanthins. Any spot which gives a temporary yellow colour with ammonia vapour, and subsequently a faint green, buff or olive colour on spraying with ferric chloride, is referred to as an anthoxanthin. As many as fourteen anthoxanthins have been differentiated on one chromatogram. The more commonly occurring spots are shown in Fig. 4; on spraying with ferric chloride spots *A* 6, *A* 7 and *A* 8 give green colours while the remainder give buff or brownish olive colours. Spots *A* 1 and *A* 2 appear to be characteristic of a particular botanical type, as they occur only in bushes of China type or in bushes which have originated from such a type by hybridization. So far little progress has been made in the identification of the anthoxanthins. Judging by the R_F values in *n*-butanol-acetic acid given by Bate-Smith & Westall (1950) no spot corresponding to kaempferol is present on any of our chromatograms, although Oshima & Ka (1936) claim to have established its presence in tea. Quercitrin has been added to tea juice and travels to a position (*A* 11) not normally occupied by any natural anthoxanthin, although some sources show a faint spot in this position. It does not therefore appear to be of general occurrence in north-east Indian teas, although its presence has been reported by Deuss (1923). Spot *A* 4 has been identified as rutin.

Organic acids. After spraying with ferric chloride the paper on drying is a very faint brown in colour, due presumably to deposition of ferric hydroxide. There are, however, a number of clear patches where the paper retains its original white colour. If the chromatogram is first sprayed with bromocresol green these clear patches show up as yellow spots on a blue to green background. The clear patches may therefore be ascribed to free acids which presumably prevent precipitation of ferric hydroxide. Identification of all such patches has not yet been successful, but two of them correspond to citric and malic acids and are marked as Cit. and Mal. in Fig. 1.

Variation in chemical composition between individual bushes

Owing to the extensive hybridization that has occurred in the past it is difficult to obtain even a small plot of reasonably homogeneous tea bushes. Studies of the variation in chemical composition within the species are therefore best made on individual bushes, or on clones raised from cuttings of individual bushes. From the material available some twenty bushes and clones (referred to by their code numbers) have been chosen representing a wide range of botanical types.

Chromatograms have been run on reconstituted juices from these sources and typical results are given in Table 6. Each source has been examined several times throughout the season; the results quoted represent samples taken at about the same season in the year. 2/2/23, 14/4/23, 16/10/22 and 107/6 are bushes having much the same pattern of polyphenols as the average, although minor differences are apparent. 107/6 is unusual in containing trace amounts of both galloyl esters corresponding to spots 5 and 7.

Distinct abnormalities are shown by the other two sources. In 14/3/32 spot 9 (catechin) and spot 3 (DL-gallocatechin) are more abundant than spots 10 and 2 (the corresponding epimers), which is the reverse of what is normally found, while spot 5 (gallo-

Table 6. *Relative proportions of polyphenols in leaf from individual bushes as judged by chromatography*

(Sheets were sprayed with $FeCl_3$. Colour intensity is indicated by the same convention as in Table 1. Spot numbers as in Figs. 1 and 2.)

Bush no. ...	2/2/23	14/3/32	14/4/23	14/6/28	16/10/22	107/6
Date (1950)	29 May	29 May	6 June	31 May	29 May	30 June
Spot no.						
1	+ + +(+)	+ + + +	+ + +	+	+ + +(+)	+ + + +
2	+ +(+)	+(+)	+ + +	+ + +(+)	+ + +	+ +
3	(+)	+ +	+	+	+(+)	+
4	+	+(+)	(+)	+	+	(+)
5	(+)?	+ +	.	.	(+)?	(+)
6	+ + + +	+ + +(+)	+ + + +	+ + + +	+ + + +	+ + + +
7	.	.	.	.	.	(+)
8	+ +(+)	+ +	+ +	+ +	+(+)	+ +(+)
9	(+)	+(+)	+	+	+	(+)
10	+ +	+	+ +(+)	+ +	+(+)	+(+)

catechin-a gallate), which is normally absent or present in trace amounts only, is present in appreciable amounts. In 14/6/28 the *m*-digallic acid spot (1) is unusually weak. Apart from these two particular cases there are no major deviations from the normal distribution of polyphenols in the sources so far investigated.

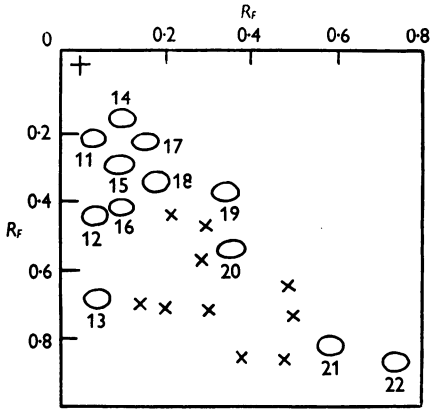

Fig. 3. Map showing location of unidentified polyphenols (11–22) occurring in tea-leaf juice. The crosses indicate positions occupied by identified polyphenols (cf. Figs. 1 and 2). Colour reagent $FeCl_3$.

Rather more variation appears amongst the minor, uncharacterized polyphenols. Fig. 3 shows the positions of twelve of these unidentified spots in relation to the identified catechins. Of these no. 20 gives a greenish-grey colour with ferric chloride, while all the remainder give dark blue-grey colours. None of these substances appears to be of general occurrence in tea as on many chromatograms they are entirely absent.

Considering spots 11–18 as a group, different sources of leaf differ markedly in the number and intensity of the spots on their chromatograms. The pattern for any given source is, on the whole, reproducible throughout the season. Bush 2/5/23 shows traces only of from two to four spots in this group, one of which is usually no. 14. Bushes 14/3/32 and 14/5/18 show as many as six spots, some of appreciable strength. The predominant spot in bushes 14/5/18, 16/10/22 and 107/6 is no. 14. In bush 124/30/33 spots 11 and 14 are of equal intensity, while in bush 14/3/32 spot 16 is usually rather stronger than spot 14. Bushes 2/2/23, 14/4/23 and 14/6/28 contain traces only of spots in this group. The overall intensity of these spots is increased in December, i.e. at the end of the growing season, when spot no. 14 in bush 14/3/32 attains medium strong intensity.

Spots 19 and 20 are of much more sporadic occurrence, and cannot be associated with any particular source.

Spots 21 and 22 are only very rarely observed. Both are present on several chromatograms of bush 14/4/23, and no. 21 alone sometimes occurs in bush 14/3/32.

Polyphenol distribution in the tea shoot

To determine the variation in polyphenol distribution from one part of the shoot to another, shoots of three leaves and a bud were divided into the following five portions: (1) bud plus first leaf, (2) second leaf, (3) third leaf, (4) upper stem, down to the point of attachment of the second leaf, (5) lower stem, below the second leaf. Chromatograms (Table 7) were compared of these five portions, and of hard, senile leaves gathered from below the plucking surface of the bush. Progressing from the youngest leaves to the more mature leaves, the two gallocatechins both increase in strength, whereas *m*-digallic acid, gallic acid, L-*epi*gallocatechin gallate and L-*epi*catechin gallate all decrease in strength. With respect to these six components the upper stem is intermediate between the third leaf and the senile leaf, while the lower stem is merely a weaker edition of the upper stem. Catechin and L-*epi*catechin appear to be most abundant in the second leaf and the upper stem, decreasing slightly in both younger and older leaves.

Table 7. *Comparison of chromatograms made on juice from different parts of the tea shoot*

(Chromatograms sprayed with $FeCl_3$ and colour intensities indicated as in Table 1. Spot numbers as in Figs. 1 and 2.)

Spot no.	Bud and 1st leaf	2nd leaf	3rd leaf	Upper stem	Lower stem	Senile leaf
1	+ + +	+ + (+)	+ +	+ (+)	+	(+)
2	+ +	+ + +	+ + (+)	+ + +	+ +	+ + + (+)
3	+	+ (+)	+ (+)	+ +	+	+ +
4	+ +	+	+	(+)	(+)	(+)
5	.	.	.	.	.	.
6	+ + + +	+ + + (+)	+ + +	+ + +	+ +	+ + (+)
7	.	.	.	.	.	.
8	+ +	+ +	+ (+)	+	.	+
9	(+)	+	(+)	+	(+)	(+)
10	+	+ +	+ (+)	+ +	+	+ (+)

Table 8. *Chromatograms of juice at successive stages of enzymic oxidation*

(Spot numbers as in Figs. 1 and 2. Colour reagent $FeCl_3$. Colour scale as in Table 1.)

Spot no.	Percentage oxidation				
	0	10	45	70	83
1	+ +	+ +	+ +	+ +	+ (+)
2	+ +	+ +	.	.	.
3	(+)	(+)	(+)	.	.
4	(+)	+	+ (+)	+ +	+
5	.	.	.	.	.
6	+ + +	+ + +	+	.	.
7	.	.	.	.	.
8	+ +	+ +	+ +	+ (+)	+
9	+	+	+	(+)	(+)
10	+ +	+ +	+ +	+ (+)	+

Table 9. *Chromatograms of teas fermented for different lengths of time*

(Colour reagent $FeCl_3$. Spot numbers as in Figs. 1 and 2 and colour scale as in Table 1.)

Spot no.	Withered leaf	Time fermented (hr.)			
		2	3	4	5
1	+ + + +	+ + + +	+ + + +	+ + +	+ + (+)
2	+ + +	.	.	.	.
3	+ +	(+)	.	.	.
4	+	+ + (+)	+ + +	+ + +	+ +
5	.	.	.	.	.
6	+ + + +	+ +	+	(+)	(+)
7	.	.	.	.	.
8	+ (+)	+ (+)	+	(+)	(+)
9	(+)	+	(+)	.	.
10	+ (+)	+ +	+	(+)	(+)

Effect of fermentation

Fresh juice. Uncentrifuged juice (with the oxidase-containing chloroplasts still in suspension) is measured into a series of Warburg vessels and oxygen uptakes recorded in the usual way. At intervals one vessel is removed and the contents immersed in boiling water to stop fermentation. The last vessel is allowed to oxidize to completion, so that from the oxygen uptakes the percentage oxidation in each vessel can be calculated. Chromatograms are then run of the contents of selected vessels with the results shown in Table 8. L-*epi*Gallocatechin is the first catechin to become completely oxidized followed by DL-gallocatechin and L-*epi*gallocatechin gallate, while L-*epi*catechin gallate, L-*epi*catechin and catechin remain unaffected until more than half the total oxygen consumed in fermentation has been taken up. *m*-Digallic acid appears to be almost unaffected throughout fermentation while gallic acid shows a marked increase in the earlier stages of fermentation.

Fermenting tea leaf. Samples of fermented tea leaf were fired at varying time intervals from the start of rolling, and chromatograms run on 'reconstituted juices' from these samples. The effect of varying time of fermentation on the chromatogram is shown in Table 9. As with juice, the two gallocatechins and L-*epi*gallocatechin gallate are the first to be oxidized. *m*-Digallic acid is practically unaffected while gallic

acid increases considerably. After 4 hr. the chromatogram shows catechins in only trace amounts, but both gallic and *m*-digallic acids remain strong. Both acids decrease appreciably in strength if fermentation is further prolonged.

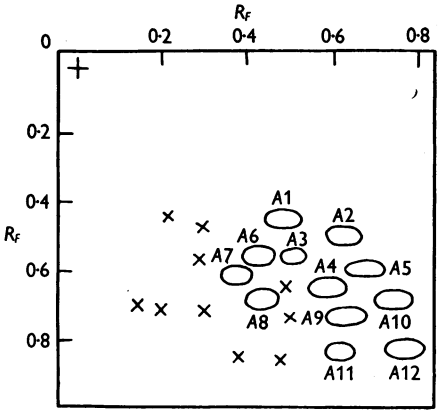

Fig. 4. Map showing location of the chief anthoxanthins found in tea-leaf juice. Crosses indicate positions of identified polyphenols (1–10). Colour reagent, NH_3 vapour.

Associated with a decrease in the intensities of the individual spots, there is a marked increase in the intensity, after spraying with ferric chloride, of a rather diffuse area at and near the origin. This diffuse area is probably to be identified with the various condensation products (cf. Harrison & Roberts, 1939) formed from the initial oxidation products of the catechins. In the earlier stages of fermentation three well defined spots corresponding with spots 11–13 of Fig. 3 are invariably encountered, whether or not they are present in the original green leaf. The significance of their apparent production during fermentation is not clear.

Chromatograms of fermented teas have never shown any detectable changes in the spots for anthoxanthins, suggesting that these substances undergo little or no chemical change during fermentation.

DISCUSSION

Our results indicate that partial epimerization of L-*epi*catechin to catechin takes place as a result of moderately long periods of boiling tea-leaf juice or infusion, as in the preparation of catechins from tea leaf. The presumption is that spots 2 and 3 on our chromatograms form a similar pair of epimers, and the gain in strength of spot 3 at the expense of spot 2, as a result of boiling, is due to an epimeric change. The conversion of one gallocatechin into another on boiling is strong supporting evidence for the view advanced by Bradfield & Bate-Smith (1950) that the L-gallocatechin and DL-gallocatechin isolated from tea by Bradfield *et al.* (1947) are in fact diastereoisomers.

L-*epi*Gallocatechin gallate and gallocatechin-a gallate form another pair of interconvertible substances, and, as already suggested by Bradfield & Bate-Smith (1950), the latter substance may possibly be the galloyl ester of gallocatechin and not *epi*-gallocatechin. The R_F values of these two substances, however, are almost identical in *n*-butanol-acetic acid, but are appreciably different in phenol. As the epimeric pairs so far considered have almost identical R_F values in phenol but appreciably different values in *n*-butanol-acetic acid, there is some uncertainty in characterizing the two gallocatechin gallates as simple epimers. The retention of the name gallocatechin-a gallate therefore serves to indicate that the evidence is not yet sufficiently precise to conclude that this substance differs from L-*epi*gallocatechin gallate only in the configuration of the substituted hydroxyl group in the 3-position.

The formation of the catechin corresponding to spot 7 from L-*epi*catechin gallate on boiling, and the relative positions of spots 7 and 8 on the chromatogram, suggest that this catechin bears the same relation to L-*epi*catechin gallate that gallocatechin-a gallate does to L-*epi*gallocatechin gallate. We propose therefore to refer to this substance as catechin-a gallate. The existence of this substance does not appear to have been established before.

It follows from the above results that the extract used by Bradfield (1946) in his approximate analysis of the catechins of Ceylon green tea does not give a true picture of the catechins and other polyphenols in the green leaf. The method of extraction leads to a considerable modification of the relative proportions of the epimers of the simple catechins, and the transformation products of galloyl esters, represented on the chromatograms by spots 5 and 7, are normally artifacts, as these substances are only rarely detectable in fresh tea-leaf juice. Further, an important polyphenol, possibly identical with *m*-digallic acid, is not appreciably extracted from the infusion by ethyl acetate, so that its occurrence in tea was not recognized.

We may conclude that the so-called tea tannin is a mixture of eight catechins, traces of gallic acid and appreciable amounts of what is possibly *m*-digallic acid, together with a few other polyphenols normally occurring in trace amounts only. There is no evidence for the presence of simple polymers of catechins, as suggested by Harrison & Roberts (1939), so that this early suggestion is now withdrawn. This conclusion has an important consequence when considering analytical methods for the determination of tannin in tea. Not all of the substances concerned are precipitated by gelatin, even in the presence of acid and salt, so that the estimation of tea tannin by Löwenthal's method, as

described by the Association of Official Agricultural Chemists (1945), and by Barua & Roberts (1940), is fundamentally wrong. We have been unable to find any non-phenolic component of the tea leaf oxidizable by potassium permanganate under the conditions of the Löwenthal titration so that the non-tan titre may probably be taken as a measure of the polyphenols not precipitated by gelatin. There is apparently, therefore, no point in carrying out the gelatin precipitation, and the total potassium permanganate titre is considered a reasonably good estimate of total polyphenols (including catechins) in unoxidized tea leaf. Procedures involving oxidation by alkaline iodine and alkaline potassium permanganate, also described by Barua & Roberts (1940), cannot give precise values for total polyphenols, whether the non-tan separation is carried out or not, as under alkaline conditions, substances other than polyphenols (glucose, etc.) are oxidized by iodine and potassium permanganate. On account of the complications introduced by condensation during fermentation, the alkaline potassium permanganate method of Barua & Roberts (1940) still gives the best estimate of total polyphenols in fermented tea, but it is now emphasized that the titre is only strictly proportional to these polyphenols so long as a constant proportion of the polyphenols is precipitated on treatment with gelatin. For most teas this appears to be approximately the case (unpublished observations), but it is emphasized that all oxidimetric methods of titration for polyphenols in tea give results which need to be interpreted with considerable care.

The successive oxidation of substrates during fermentation, as indicated by chromatography, is a useful confirmation of earlier manometric work (Roberts & Wood, 1950). The appearance of gallic acid indicates that the galloyl esters are hydrolysed as well as oxidized during fermentation. Neither gallic acid nor *m*-digallic acid are oxidized rapidly by the tea-oxidase, hence their persistence in fermented tea.

The establishment of the presence of these two acids in fermented tea shows that complete oxidation of polyphenols cannot normally be realized in fermentation. After catechins have been completely oxidized a slow uptake of oxygen will persist for many hours owing to the slow oxidation of these polyphenolic acids, and it will be difficult to state just when oxidation is complete. The estimation of the percentage oxidation of substrates in partly fermented tea by the residual uptake method (Roberts, 1941) is therefore a rather less precise method than was originally thought, although it still remains a useful technique.

SUMMARY

1. Chromatograms of tea-leaf juice show both epimers of catechin and gallocatechin together with their galloyl esters. Gallic acid and a polyphenolic acid believed to be *m*-digallic acid are also present.

2. The same chromatogram sometimes reveals traces of other polyphenols, while anthoxanthins and organic acids are also detected.

3. The boiling of a tea-leaf infusion results in epimerization of the simple catechins and a transformation, which may be epimerization, of the galloyl esters. Estimations of the relative abundance of catechins in tea must therefore be carried out on freshly expressed juice.

4. The variations in the relative proportions of the catechins and polyphenolic acids in different individual bushes and in different parts of the shoot are studied.

5. During fermentation the various catechins are oxidized successively rather than simultaneously. Gallic acid is liberated during fermentation. The chromatogram of fully fermented tea shows no catechins, but considerable amounts of gallic and *m*-digallic acids.

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