PLANT PHENOLS

I. SEPARATION OF THE TEA LEAF POLYPHENOLS BY CELLULOSE COLUMN CHROMATOGRAPHY

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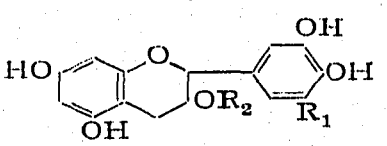
INTRODUCTION

Immediately after plucking, tea leaves are subjected to a series of operations (withering, rolling, fermentation, drying) which convert them to black tea*. The organoleptic characteristics of this manufactured tea depend to a large extent on its polyphenolic constituents, which include the following classes:

A. Flavanols and their gallic acid esters

Six compounds belonging to this class have been isolated from unfermented (green) tea and crystallized (Formulas I to IV). The work in this field has been summarized by ROBERTS[1]; for the configuration of some of these substances see HERGERT AND KURTH[2], ROBERTS[3,4], BIRCH *et al.*[5], FREUDENBERG[6], HARDEGGER *et al.*[7] and BROWN *et al.*[7a].



(I; $R_1 = R_2 = H$)
(+)-Catechin
(—)-Epicatechin

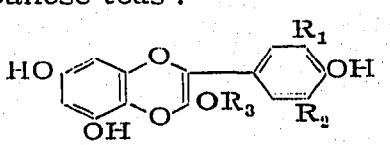
(II; $R_1 = OH$, $R_2 = H$)
(+)-Gallocatechin
(—)-Epigallocatechin

(III; $R_1 = H$, R_2 = galloyl)
(—)-Epicatechin gallate

(IV; $R_1 = OH$, R_2 = galloyl)
(—)-Epigallocatechin gallate

B. Flavonol-3-glucosides

ROBERTS *et al.*[8] have identified 3-glucosides, 3-rhamno-glucosides and 3-rhamnodiglucosides of the flavonols (V) to (VII) in green Assam tea. Numerous flavonol glucosides have also been detected in Japanese teas[9].



(V; $R_1 = R_2 = R_3 = H$)
Kaempferol

(VI; $R_1 = OH$; $R_2 = R_3 = H$)
Quercetin

(VII; $R_1 = R_2 = OH$, $R_3 = H$)
Myricetin

(Va; VIa; VIIa; R_3 = glucoside)
The corresponding 3-glucosides

* By "tea leaves" we designate the leaves as they are harvested by the pluckers and by "green tea" the manufactured tea which has been dried without fermentation.

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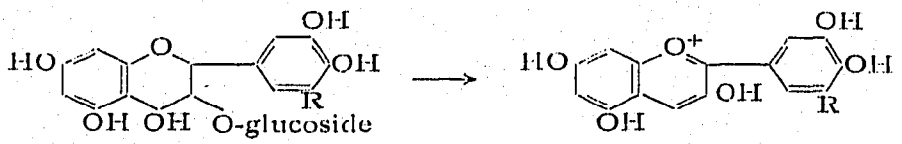
C. Leucoanthocyanins

ROBERTS *et al.*[10] have shown the presence, in green tea, of leucocyanin (VIIIa) and leucodelphinin (IXa), which, upon hydrolysis, yield cyanidin (VIIIb) and delphinidin (IXb).

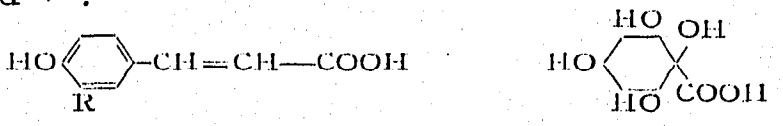


(VIIIa; R = H) Leucocyanin
(IXa; R = OH) Leucodelphinin
(VIIIb; R = H) Cyanidin
(IXb; R = OH) Delphinidin

D. Acids

Theogallin (galloyl-quinic acid)[11,12] is found in relatively large quantities, but it has not yet been obtained in crystalline form. All other phenolic acids occur in small quantities.

Gallic acid.
Chlorogenic acid (3-caffeoyl-quinic acid)[13,14].
Neochlorogenic acid (a chlorogenic acid isomer)[14].
p-Coumaryl-quinic acid[14].
m-Digallic acid[11,15].



(X; R = H) *p*-Coumaric acid
(XI; R = OH) Caffeic acid
(XII) Quinic acid

E. Oxidized and polymerized substances

These compounds, which are present only in small quantities in tea leaves, make up the major polyphenolic part of black tea. They give it its colour and, together with

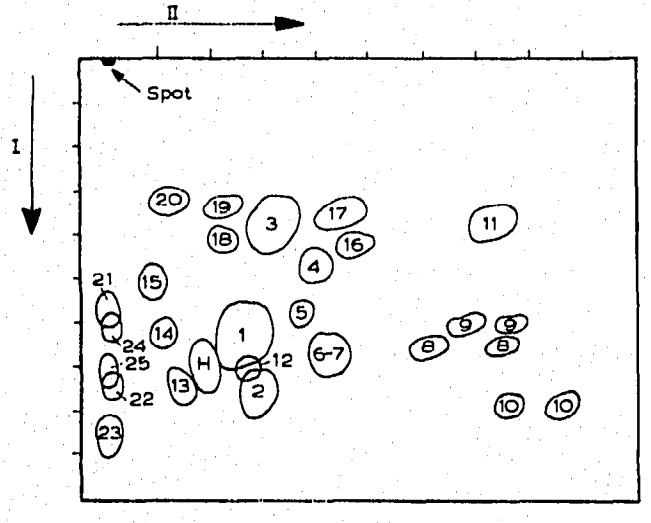


Fig. 1. Paper: Whatman No. 1. Solvent systems for first dimension: butanol–acetic acid–water (4:1:2.2); second dimension: 2% acetic acid in water.

1 (—)-Epigallocatechin gallate
2 (—)-Epicatechin gallate
3 (—)-Epigallocatechin
4 (+)-Gallocatechin
5 (—)-Epicatechin
6 (+)-Catechin
7 Gallic acid
8 Chlorogenic acid
9 Neochlorogenic acid
10 *p*-Coumaryl-quinic acid
11 Theogallin
12 *m*-Digallic acid, according to ROBERTS[11,15]
13 Kaempferol-3-glucoside (astragalin)
14 Quercetin-3-glucoside (isoquercitrin)
15 Myricetin-3-glucoside
16–20 Leucoanthocyanins
21 Myricetin
22 Quercetin
23 Kaempferol
24 Anthocyan I
25 Anthocyan II
H "Substance H"

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the volatile aroma constituents, determine its organoleptic properties. They are formed mainly from the flavanols and their gallic acid esters during the manufacture of black tea[1]. Their structure has not yet been elucidated.

Several authors[8,10,11,13–19] have identified the phenolic substances belonging to groups A to D by paper chromatography. Fig. 1 shows a two-dimensional key-chromatogram which is based on our own R_F-values except for *m*-digallic acid[11]. According to WILLIAMS[20], *cis-trans* isomerism could be responsible for the double spotting given by the derivatives of cinnamic acid in the second dimension (aqueous solvent). Table I lists the u.v. fluorescence of the substances present on the key-chromatogram, as well as the colour which develops on spraying with bis-diazotized benzidine.

TABLE I

Substance (spot in Fig. 1)	*U.v. fluorescence*	*Colour with bis-diazotized benzidine*
1– 6	violet	red-brown*
7	blue	yellow turning red-brown*
8– 9	green*	yellow
10	violet*	yellow turning brown
11	blue*	yellow
13–15	purple*	pink*
16–20	violet	brown*
21–23	yellow*	pink-brown
24	pink*	no reaction
25	salmon*	no reaction
H	violet*	yellow

* Indicates the more sensitive reactions.

Tea polyphenols are generally divided into two groups, according to their behaviour upon extraction of an aqueous solution with ethyl acetate:

(a) those passing into the organic phase,

(b) those remaining in the water layer.

The latter are separated from the other constituents of a tea extract (salts, sugars, amino acids, etc.) by precipitation as lead salts.

The composition of the tea leaves used as starting material in our study is given below (percentage calculated for dry leaf weight).

	%
Substances not extractable by 80% ethanol:	
proteins (Kjeldahl N × 6.25)	15.25
fibers (by difference)	30.25
Substances extractable by 80% ethanol:	
insoluble in water:	
pigments (chloroformic extract minus caffeine)	5.55
soluble in water:	
caffeine (according to *Manuel Suisse des denrées alimentaires*, 1939)	4.65
polyphenols soluble in ethyl acetate	26.70
polyphenols insoluble in ethyl acetate	5.20
amino acids (Kjeldahl N × 6.25)	4.15
ash	4.40
sugars (by difference)	3.85
	100.00

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The total polyphenols amount to 66% of the water-soluble substances and the polyphenols extractable with ethyl acetate amount to 83% of the total polyphenols.

The repartition of the polyphenols upon extraction with ethyl acetate depends on the conditions prevailing during extraction of the aqueous phase. Our procedure (see EXPERIMENTAL PART) gave the following results:

1. The flavanols (A) are almost completely extracted.

2. The flavonol glucosides (B) and the leucoanthocyanins (C) are distributed according to the nature of their glucosidic component. As far as the flavonol derivatives are concerned, the 3-glucosides are completely extracted while the others remain in the aqueous phase. It is probable that the same is true for the leucoanthocyanins.

3. As regards the acids (D), at pH 5.5 theogallin remains almost entirely in the aqueous solution while the others are found in both phases.

4. The same applies to the polymerized substances (E), of which only a part is extracted.

Of the polyphenols that are not extracted by the organic solvent, some precipitate completely with lead acetate at pH 5.5 (theogallin, leucoanthocyanins, polymerized substances), others at pH 8.5 (*p*-coumaryl-quinic acid, flavonol-3-glucosides), while the rest (gallic, chlorogenic and neochlorogenic acid) can be found in both precipitates.

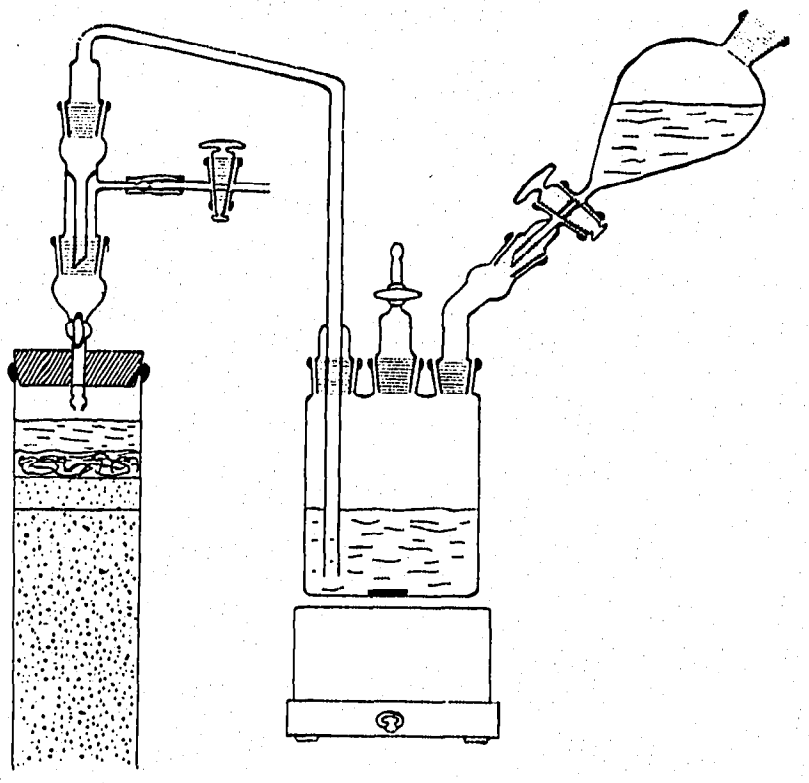

Fig. 2. Device for producing concentration gradients. This device, although not yielding linear concentration gradients as others do[25], has, however, the advantage of greater versatility. By changing the solvent volume in the mixing chamber, the gradient rate may be varied according to need.

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For studying the polyphenols of green tea, BRADFIELD *et al.*[21,22] have used partition chromatography on silica gel columns. This technique has also been applied by Russian authors[23]. Furthermore, ROUX[24] has shown that chromatography of these polyphenols from water on a collagen column yields a separation identical to that given in the second dimension of the paper chromatogram described previously.

We have now developed a technique of partition chromatography in which wet cellulose powder is used as stationary phase, and a series of solvents of increasing polarity as mobile phase; these solvents are applied by means of the device sketched in Fig. 2, to yield concentration gradients. The solvents are (in order of application):

1. Ethyl propionate–petroleum ether (9:1), saturated with water.
2. Ethyl propionate, saturated with water.
3. Ethyl acetate, half saturated with water.
4. Butanol, half saturated with water.
5. Methanol, containing 10% water.
6. Water.

The fractions obtained from this partition chromatogram are then further separated by adsorption chromatography from water on a cellulose powder column.

The topic of this first publication is a description of the separation of the tea leaf polyphenols by means of these techniques and their study.

EXPERIMENTAL PART

I. Isolation of polyphenols

(*a*) *Starting material.* Tea leaves, plucked in the fall of 1956 in Ceylon near the coast south of Colombo, were immediately cooled to 4° and shipped by air. They were received in a perfect state of freshness and were extracted 55 hours after leaving the plantation.

(*b*) *Extraction.* The tea leaves (450 g, containing 100 g solids) are homogenized with ethanol (1400 ml ethanol yield, with the leaf moisture, an 80% ethanolic solution). To avoid oxidation, 100 mg $K_2S_2O_5$ as a 10% aqueous solution are added. After disintegration, the mixture is stirred for 20 min at 40°, then filtered. The residue is extracted twice more with 750 ml of 80% ethanol (20 min stirring at 40°). The three filtrates are combined and concentrated to 500 ml in a rotating evaporator under reduced pressure. The caffeine and the pigments which partially precipitate are extracted with $CHCl_3$ using a rotating flask to avoid emulsion formation.

(*c*) *Separation of polyphenol soluble in ethyl acetate.* The aqueous phase is freed of the residual $CHCl_3$ by vacuum evaporation, then extracted six times for 5 min with one-liter volumes of ethyl acetate. The ethyl acetate extracts are combined and concentrated under reduced pressure (CO_2 atmosphere) to 100 ml. 200 ml of H_2O are added, and the remaining ethyl acetate is removed under reduced pressure. The polyphenols are freeze-dried. Yield: 26.7 g of an orange powder.

(*d*) *Separation of polyphenols insoluble in ethyl acetate.* The aqueous phase remaining after ethyl acetate extraction is freed from dissolved organic solvent by

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vacuum evaporation. A few ml ethanol are added to dissolve the sparingly soluble polyphenols. A saturated solution of $Pb(OAc)_2$ is added.

The precipitate is filtered and washed with 3 l of distilled H_2O with continuous agitation. On gradually adding Dowex 50 (H-form) to the suspension, the Pb^{++} is bound to the cation exchanger and the free polyphenols go into solution. A little ethanol is added to prevent re-precipitation. The mixture is filtered and the resin thoroughly washed with 20% ethanol. The combined filtrates are concentrated under vacuum in a rotating evaporator and the polyphenols freeze-dried. Yield: 3.6 g of a brown powder.

The filtrate and wash water from the Pb-polyphenolates are combined, concentrated under vacuum to 300 ml and adjusted to pH 8.5* with 2 *N* NH_4OH. The yellow precipitate which forms is filtered, washed with distilled H_2O, suspended in methanol and treated with Dowex 50 (H-form). After filtration and washing, the solution is concentrated under vacuum. A small amount of ethanol is added. The resulting white precipitate is discarded and the filtrate is freed from ethanol and freeze-dried. Yield: 1.6 g of a yellow powder.

The total amount of polyphenols precipitated by $Pb(OAc)_2$ is 5.2 g.

II. Adsorption chromatography with water as solvent (for ethyl acetate-soluble polyphenols only)

(*a*) *Preparation of column.* Whatman or Schleicher and Schuell ashless cellulose powder is suspended in distilled water and allowed to settle. The supernatant, containing the colloidal material, is discarded. This process is repeated until the wash-water is clear.

The glass column (68 × 3 cm) is filled with an aqueous suspension of wet cellulose corresponding to 110–130 g of dry powder. The column is packed to obtain a flow rate of 1 ml/min and washed with H_2O until the optical density at 275 mμ ($= E_{275}$) of the effluent is below 0.05 (Beckman Spectrophotometer DU).

(*b*) *Addition of substance.* 1 g ethyl acetate-soluble polyphenols in 4 ml H_2O are placed on the top of the column. After 2–3 washings with a few ml H_2O, the top of the column is fitted with a piece of cotton wool and the elution started.

(*c*) *Elution.* A drop-counter fraction collector (H. Hösli, Bischofszell, Switzerland) was employed. 4 ml fractions are collected. The elution is followed by measuring E_{275} of each fraction.

(*d*) *Analysis of fractions.* The fractions are analysed by two-dimensional paper chromatography on Whatman paper No. 1 with butanol–acetic acid–water (4:1:2.2) in the first dimension and 2% aqueous acetic acid in the second dimension. The spots are detected by their u.v. fluorescence (lamp emitting at 253.5 mμ) and by means of bis-diazotized benzidine[26].

The curve which is obtained by plotting E_{275} against the fraction number, together with the results of the paper chromatographic analysis, permit the individual

* All previous operations were carried out without pH adjustment, that is at a pH of approximately 5.5.

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fractions to be pooled into a few main fractions. These are concentrated under vacuum and freeze-dried.

III. Partition chromatography of ethyl acetate-soluble polyphenols

(*a*) *Preparation of column.* Ashless cellulose powder (Whatman or Schleicher and Schuell) was used. The dry cellulose is suspended in the organic solvent and, with vigorous stirring, distilled H_2O (half the weight of the cellulose) is added gradually. The resulting slurry is used to fill the column (68 × 3 cm). Too much water is retained when the column is filled with a suspension of cellulose in water and the latter displaced by an organic solvent. The column is washed until E_{275} of the effluent is below 0.05.

(*b*) *Deposit of substance.* 1 g ethyl acetate-soluble polyphenols are blended with 4 g cellulose powder. The mixture is moistened with a few ml H_2O and placed on the top of the column which is still covered with a small layer of solvent. The preparation is well dispersed (no air bubbles) and allowed to settle before the elution is started.

(*c*) *Elution.* Gradient elution was applied by means of the apparatus sketched in Fig. 2. Ground glass or teflon joints are essential to avoid contamination of the organic solvents. The level of the solvent container is adjusted to permit an effluent flow rate of 0.6–0.7 ml/min. 10-ml fractions are collected and the elution followed as described under IIc. In order of application, the following solvents are used:

1. Ethyl propionate–petroleum ether (9:1), saturated with water.
2. Ethyl propionate, saturated with water.
3. Ethyl acetate, half saturated with water.
4. *n*-Butanol, half saturated with water.
5. Methanol, containing 10% water.
6. Water.

Ethyl propionate (Fluka or Light) was redistilled over a Widmer fractionating column prior to use and the fraction boiling at 98.5° (with E_{275} below 0.05) was employed. The other solvents were Merck reagents for chromatography (purissimum pro analysis). Petroleum ether with the boiling range of 60–80° was used.

(*d*) *Analysis of fractions.* This is carried out as described in IId. The principal fractions are concentrated to a small volume under reduced pressure. A few ml of water are added, the remaining organic solvent is evaporated, and the substances are freeze-dried.

(*e*) *Re-chromatography of principal fractions.* The method described in IIa–d is utilized with a 60 × 1.3 cm column. The optical densities are measured at:

275 mμ for indication of flavanols,
321 mμ for indication of chloro- and neochlorogenic acids,
350 mμ for indication of flavonol glucosides,
380 mμ for indication of oxidized and polymerized substances,
500 mμ for indication of anthocyanins.

Polymerized substances remaining in the column may be eluted by adding ethanol to the water.

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IV. Partition chromatography of polyphenols precipitated as lead salts at pH 5.5

(*a*) *Preparation of column.* As described under IIIa, except that the organic solvent is ethyl acetate saturated with water.

(*b*) *Deposit of substances.* As described under IIIb.

(*c*) *Elution.* As described under IIIc, except that both E_{275} and E_{321} are determined, the latter for indicating chloro- and neochlorogenic acids. In order of application, the following solvents are used:

1. Ethyl acetate, saturated with water.
2. Butanol, half saturated with water.
3. Methanol containing 10% water.
4. Water.

(*d*) *Analysis of fractions.* As described under IId and IIId, except that detection is carried out with aqueous 1% $FeCl_3$–1% $K_3Fe(CN)_6$ (1:1).

RESULTS AND DISCUSSION

The data are shown graphically in Figs. 3, 4 and 5.

A. Recovery

1. 82% of the polyphenols applied to the adsorption column are eluted with water (see Fig. 3). The remainder, consisting to a large extent of polymerized substances, may be eluted with ethanol or methanol.

2. The elution of the partition chromatograms (see Fig. 4) is nearly quantitative.

Note: Owing to the different extinction coefficients of the various components, the quantities of the eluted substances are not proportional to the area under the elution curves.

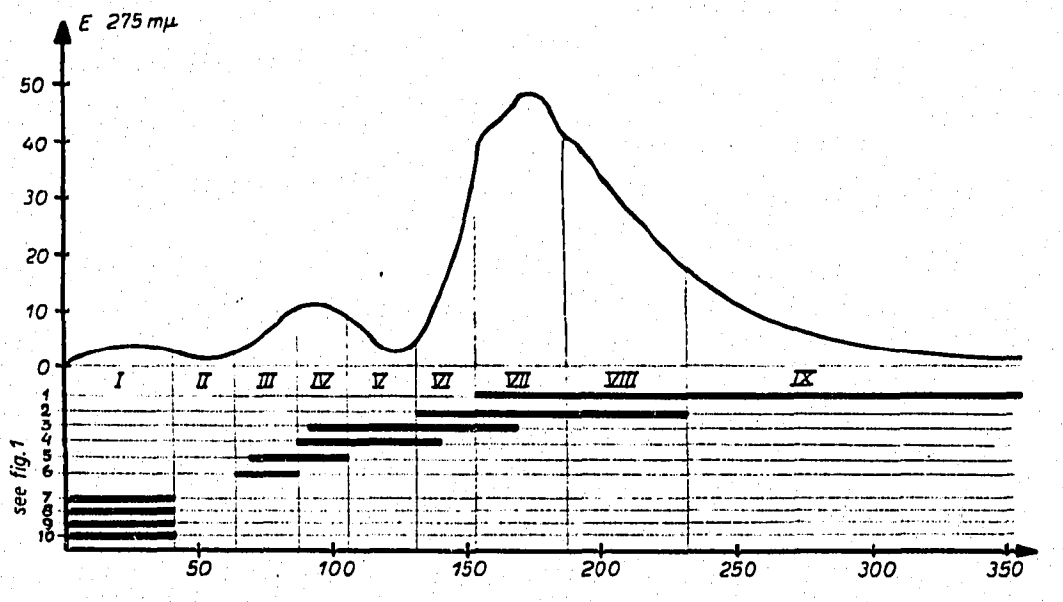

Fig. 3. 1 g polyphenols gives approximately: I–V, 160 mg; VI, 90 mg; VII, 170 mg; VIII, 255 mg; IX, 145 mg; total 820 mg.

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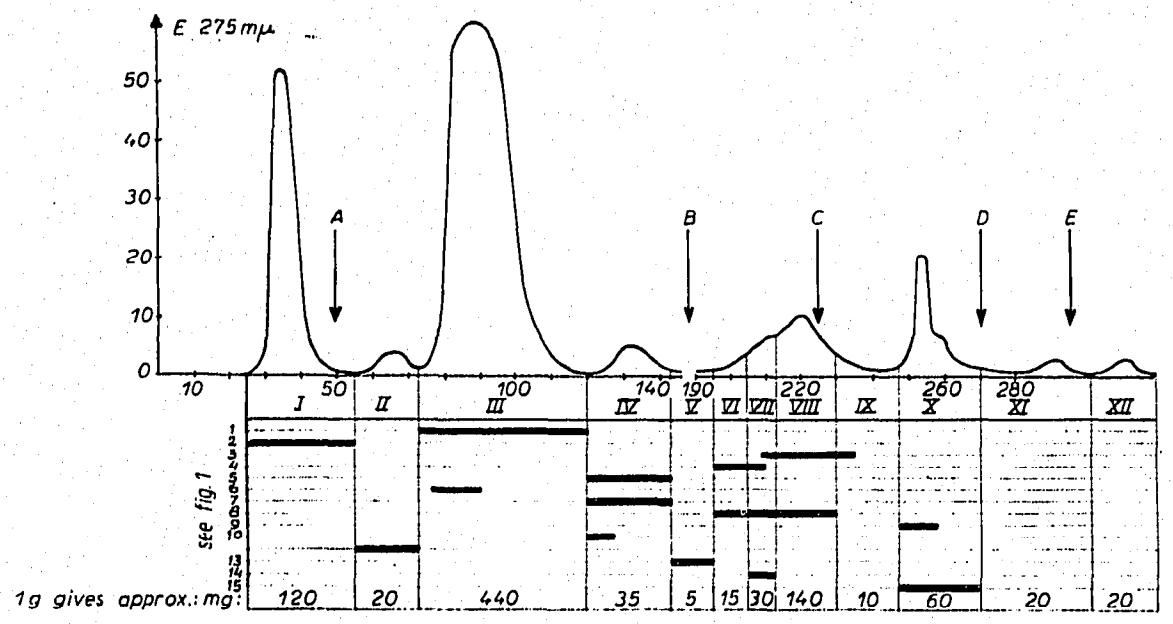

Fig. 4. Beginning of the gradient with: (A) ethyl propionate; (B) ethyl acetate; (C) *n*-butanol; (D) methanol; (E) water.

B. Flavanols

1. Table II shows the R_F values of the flavanols and their order of elution from the column.

In the adsorption chromatogram, the order of elution of (+)-gallocatechin and (—)-epicatechin does not correspond to the R_{FII} values. Inversion also occurs between (+)-catechin and (—)-epigallocatechin gallate in partition chromatography.

TABLE II

Flavanol	R_{FI}	R_{FII}	P	A
(—)-Epigallocatechin gallate	0.64	0.26	2	6
(—)-Epicatechin gallate	0.76	0.28	1	5
(—)-Epigallocatechin	0.37	0.31	6	4
(+)-Gallocatechin	0.48	0.40	5	3
(—)-Epicatechin	0.58	0.37	4	2
(+)-Catechin	0.66	0.41	3	1

R_{FI} = R_F of first dimension, R_{FII} = R_F of second dimension } of the two-dimensional paper chromatogram.
P = order of elution from partition column.
A = order of elution from adsorption column.

2. With ethyl propionate as eluent, separation of the two gallates is very difficult to achieve. Addition of petroleum ether lowers the distribution coefficient of (—)-epigallocatechin gallate more than that of (—)-epicatechin gallate, thus facilitating their separation.

In the same manner, the distribution coefficient of (+)-catechin is lowered less on addition of petroleum ether than that of (—)-epigallocatechin gallate. With ethyl propionate alone as eluent, (+)-catechin can be found in the tail fraction of the

(—)-epigallocatechin gallate; if the eluent contains 10% petroleum ether, (+)-catechin is eluted in the head fraction of the ester; with 20% petroleum ether, the two substances can even be separated. Nevertheless, we prefer an addition of only 10% and subsequent separation of the two compounds on a small adsorption column, because with higher percentages of petroleum ether elution proceeds too slowly.

3. For analytical purposes, we recommend starting with a separation by partition chromatography in order to eliminate first the two gallic acid esters which

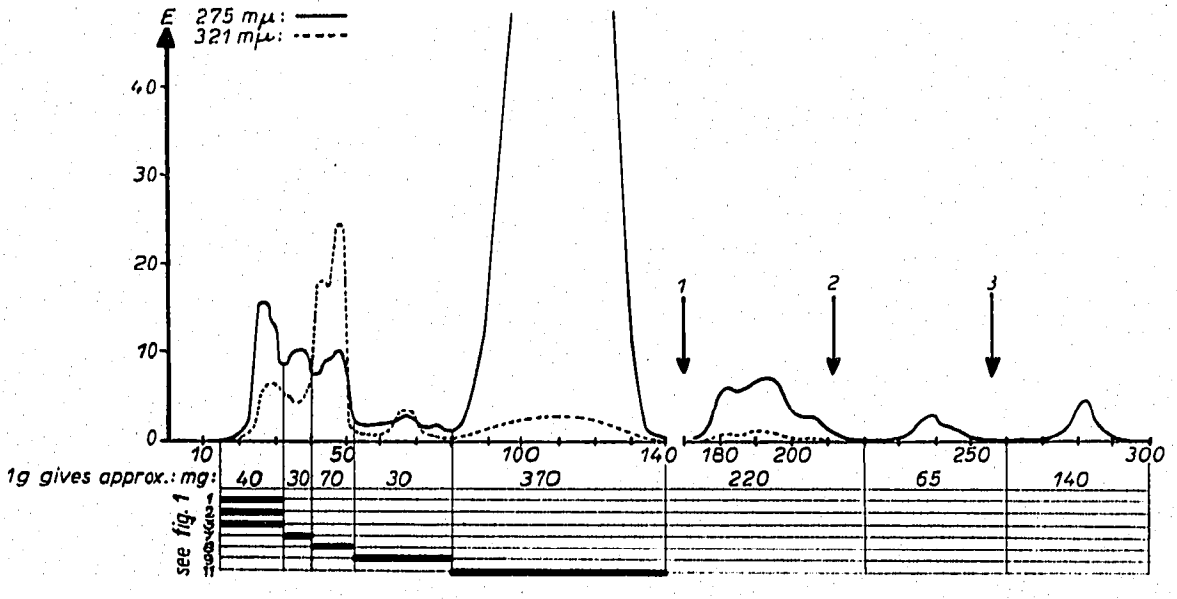

Fig. 5. Beginning of the gradient with: (1) *n*-butanol; (2) methanol; (3) water.

predominate quantitatively. Substances present in small amounts can then be detected more easily because they represent a higher relative percentage of the following fractions.

For a preparative separation, we prefer to begin with adsorption chromatography in order to eliminate a large amount of the polymerized substances which are retained on the cellulose. Then it is possible by partition chromatography to isolate from the combined main fractions III to V in a perfectly separated state:

with ethyl propionate:	(+)-catechin and (—)-epicatechin
with ethyl acetate:	(+)-gallocatechin and (—)-epigallocatechin

The combined main fractions VI to IX give:

with ethyl propionate–petroleum ether (9:1):	(—)-epicatechin gallate and (—)-epigallocatechin gallate
with ethyl acetate:	(+)-gallocatechin and (—)-epigallocatechin.

By this procedure, we have obtained the 6 flavanols in their crystalline state.

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4. Analytical data for the crystalline flavanols are reported in Table III.

5. From paper chromatography, ROBERTS AND WOOD[31] concluded that catechin and gallocatechin occur in tea leaf in the (+) form and not in the (±) form as in green tea. Our $[\alpha]_D$ values of the two compounds are a direct confirmation of this assumption.

TABLE III

a. (—)-Epigallocatechin gallate*, crystallized from H_2O.
b. (—)-Epicatechin gallate**, crystallized from MeOH–CH_2Cl_2 mixture by gradual addition of CH_2Cl_2.
c. (—)-Epigallocatechin, crystallized from H_2O.
d. (+)-Gallocatechin, crystallized from AcOEt–CH_2Cl_2 mixture by gradual addition of CH_2Cl_2.
e. (+)-Catechin***, crystallized from H_2O.
f. (—)-Epicatechin***, crystallized from H_2O.

R_{F1} and R_{F11} are given in Table II, $\lambda_{max.}$ and $\varepsilon_{max.}$ in Table IV. Infrared spectra will be published elsewhere.

	a	*b*	*c*	*d*	*e*	*f*
M.p.						
Literature	215–216°[22] 213°[23]	252–254°[22] 251°[24] 235°[23] 253°[27]	217–218°[21] 212–215°[22] 218°[23] 218°[28]	183°[29]	***	***
Found****	218°	253°	217°	186°		
Formula	$C_{22}H_{18}O_{11}$	$C_{22}H_{18}O_{10}$	$C_{15}H_{14}O_7$	$C_{15}H_{14}O_7$	***	***
Analysis						
%C calc.	57.64	59.73	58.82	58.82		
%C found	57.07	59.32	59.34	58.25		
%H calc.	3.96	4.10	4.61	4.61		
%H found	4.08	4.28	4.68	4.75		
$[\alpha]_D$						
Literature	—179°[22]	—190°[22] —177.5°[27]	—60°[21] —67.5°[28]	+13.1°[29]	+17.1°[30]	—68.2°[30]
Found*****	—185° ±2°	—190° ±2°	—59.5° ±2°	+13.8° ±2°	+13.8° ±2°	—65° ±2°

* On exposure to air and light, the crystals remain white for 10 days, in contrast to the report of BRADFIELD *et al.*[22] that they readily become brown under these conditions.

** The crystals turn light brown on exposure to air and light for a few days.

*** We did not attempt to obtain (+)-catechin and (—)-epicatechin from tea leaf in an absolutely pure state, because these two substances were available in large amount from other sources. We only compared them by paper chromatography with the pure compounds and measured their $[\alpha]_D$.

**** M.p. measured with Kofler block. Crystals dried over P_2O_5 under high vacuum at room temperature.

***** a, b, c and f: $[\alpha]_D$ determined in ethanol; d and e: $[\alpha]_D$ determined in acetone–water (1:1).

6. BRADFIELD *et al.*[22] have isolated from green tea a flavanol different from the six aformentioned, which they called gallocatechin-a-gallate. ROBERTS *et al.*[31] have shown that the substance appears when an aqueous solution of (—)-epigallocatechin gallate is heated. The transformation which takes place during heating is an

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epimerization giving rise to (—)-gallocatechin gallate (BRADFIELD's a-gallate). We did not find this substance in our leaves, which confirms the assumption of ROBERTS that this a-gallate is formed during the manufacture of green tea.

7. The results of the u.v. spectrophotometric investigation of the six flavanols are summarized in Table IV.

TABLE IV

Flavanol	*Lit. values*		*Our values in ethanol*		
	$\lambda_{max.}$	$\varepsilon_{max.}$	$\lambda_{max.}$	$\varepsilon_{max.}$	ε_{275}
(—) Epigallocatechin gallate	275 279.5	9 500[22] 9 250[22]	275	11 500	11 500
(—)-Epicatechin gallate	280	13 600[22]	279	14 000	13 500
(—)-Epigallocatechin	271	1 340[22]	271	1 450	1 275
(+)-Gallocatechin	271	1 734[29]	271	1 460	1 285
(—)-Epicatechin	280	3 300[22]	280	3 580	3 100
(+)-Catechin	280	4 061[29]	280	3 585	3 120

We did not find the double peak for (—)-epigallocatechin gallate reported by BRADFIELD *et al.*[22].

On the basis of the ε_{275} values of the different flavanols and the total density at 275 mμ corresponding to the area under the different peaks (after re-chromatography), we estimate that the following quantities of the flavanols occur in our tea leaves:

	In 1 g ethyl acetate-soluble polyphenols	*% of the dry weight of the leaf*
(—)-Epigallocatechin gallate	390 mg	10.55
(—)-Epicatechin gallate	103	2.75
(—)-Epigallocatechin	88	2.35
(+)-Gallocatechin	14	0.37
(—)-Epicatechin	24	0.63
(+)-Catechin	13	0.35
Total	632 mg	16.90

C. Acids

1. The acids present in the polyphenolic fraction soluble in ethyl acetate (gallic, chlorogenic, neochlorogenic and *p*-coumaryl-quinic acids) are all found in the first peak eluted from the adsorption chromatogram. This is not surprising, for their R_{FII} values in paper chromatograms with water (containing no acetic acid) as eluent[15] are high.

2. Gallic, chlorogenic and neochlorogenic acids are well separated by partition chromatography. The purification of chlorogenic and neochlorogenic acids from Fractions III and IV (Fig. 5), however, could not be achieved by adsorption re-chromatography. Most of the brown impurities are eluted at the same rate as these acids. A

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determination of their quantity, carried out by a new differential spectrophotometric method developed in our laboratories (to be published), indicated that they make up about 45% of Fractions III and IV respectively. A total chlorogenic acid content of approx. 0.3% of the dry leaf weight can be estimated, which include the portion present in the ethyl acetate-soluble fraction.

3. While chlorogenic, neochlorogenic and *p*-coumaryl-quinic acids yield two spots in paper chromatography with an aqueous developing agent, no double peaking could be detected in column chromatography. Chlorogenic acid yields one single peak in a column chromatogram with 2% acetic acid as eluent. Its head, center and tail give the double spotting on paper with the same solvent and it may be concluded that this separation is due to the physical structure of the paper sheet.

4. From paper chromatograms of Fraction V (Fig. 5) it can be assumed that 1 g of the polyphenols precipitated with lead acetate contains about 30% theogallin. Thus, this substance represents approx. 1% of the dry weight of tea leaves. We have not yet been able to obtain it in a crystalline state.

5. The phenolic fraction which does not precipitate with lead acetate at pH 5.5, but at pH 8.5, has been investigated by paper chromatography only. It contains mainly *p*-coumarylquinic acid, accompanied by chlorogenic, neochlorogenic, caffeic, and *p*-coumaric acids (the last two acids probably arise from hydrolysis at this high pH).

D. Anthocyans

ROBERTS *et al.*[10] have found several leucoanthocyanins in Indochina tea leaves, but they do not report having detected anthocyans. The Ceylon leaves used in our investigation contain two substances appearing together as a pink band, which, on partition chromatography, moves very slowly with ethyl acetate, but remains at the top of the column during the entire adsorption run. They may be recovered from the isolated pink section of the chromatogram by eluting the cellulose with ethanol:

	Anthocyan I	*Anthocyan II*
R_F in butanol saturated with 2 *N* HCl	0.42	0.61
R_F in butanol–acetic acid–water (4 : 1 : 2.2)	0.53	0.69
$\lambda_{max.}$ in ethanol containing 0.01% conc. HCl	510 mμ	495 mμ

Considering our mild extraction conditions, it does not seem probable that hydrolysis of leucoanthocyanins could have occurred. The two anthocyans must, therefore, have been originally present in our tea leaves. (I) might be identical with substance (P) identified by ROBERTS[10a] in black tea.

E. Flavonol glucosides

Adsorption chromatography of partition Fractions V, VII and X (Fig. 4) reveals the flavonol glucosides as yellow bands descending rather slowly. Upon concentrating the

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eluates, these glucosides crystallize. Hydrolysis with 2 *N* HCl yields the corresponding flavonols myricetin, quercetin and kaempferol, which were detected by paper chromatography.

F. Unidentified substances

1. Paper and column chromatography of tea leaf polyphenols show a number of still unidentified minor substances. For example, chlorogenic acid is often accompanied by compounds giving a blue or violet u.v. fluorescence and yellow reaction with bis-diazotized benzidine.

2. In Fraction II from partition chromatography of ethyl acetate-soluble polyphenols (Fig. 4), the so-called "Substance H" is paramount. Purification of this compound by re-chromatography is difficult, because its affinity to cellulose is almost as great as that of the accompanying polymerized substances. Therefore we have not yet obtained it in a crystalline state. Substance H yields a yellow colour with bis-diazotized benzidine (reaction of acids), a violet u.v. fluorescence (reaction of flavanols), and a violet reaction when sprayed with ferric sulfate (which might indicate a pyrocatechol structure). In ethanol, it exhibits a ε_{max} at 279 mμ. The substance is different from gallocatechin-a-gallate, *m*-digallic acid, purpurogallin, and purpurogallincarboxylic acid.

3. On concentrating Fraction IX from an adsorption chromatogram (Fig. 3) to 2 ml, a fine crystalline precipitate appears before the crystallisation of the main component of this fraction, (—)-epigallocatechin gallate. These fine crystals can be separated by centrifugation and washed free from polyphenols with ethanol. They represent approx. 0.2% of the ethyl acetate-soluble tea fraction. The white substance is not polyphenolic, contains neither N or S, does not melt up to 300° (but turns brown at about 250°), and reduces neither Fehling solution nor ammoniacal silver nitrate. The distribution coefficient ethyl acetate/water is near zero, and it is, therefore, to be expected that there is a much larger amount of this substance in the aqueous phase remaining after elimination of polyphenols.

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SUMMARY

The polyphenols of tea leaves have been studied by column chromatography on cellulose powder. Partition and adsorption techniques were applied in succession. In the partition chromatograms, the mobile phase consisted of solvent systems of progressively increasing polarity, and water was used as eluent in the adsorption chromatograms. This technique permits the isolation of nearly all polyphenolic substances of tea leaves.

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