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ESTIMATION OF THEAFLAVINS IN TEA BY GAS–LIQUID CHROMATOGRAPHY OF THEIR TRIMETHYLSILYL ETHERS

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SUMMARY

A method for separating and estimating the theaflavins of tea by temperature-programmed gas–liquid chromatography of their trimethylsilyl ethers is described. 6-in. columns of 3 % OV-1 on Chromosorb W separate theaflavin from its two monogallates and its digallate, and used with a 1,3-distearin standard, give results reproducible to within ± 5 %. Fractions suitable for analysis are obtained by chromatography of ethyl acetate extracts of whole tea solubles on Sephadex LH-20. This method gives lower results than ROBERTS' spectrophotometric method: chromatograms of acetate extracts before and after bicarbonate extraction suggest that this is a result of incomplete extraction of thearubigins in the ROBERTS' method.

INTRODUCTION

There are two limitations to the ROBERTS' spectrophotometric method for estimation of theaflavins in black tea extracts[1,2]. The first and fundamental limitation arises from the method's failure to separate the mixture of theaflavins obtained by solvent extraction into its individual components. The method can therefore only give a figure for total theaflavins, which is of limited value if (for example) one wishes to estimate and compare the rates of formation of individual theaflavins during fermentation with the rates of disappearance of their precursor flavanols[3]. Even this overall figure can be misleading, for it is calculated on the basis of mean extinction coefficients which are tacitly assumed to be independent of the composition of the theaflavin fraction[1,2]. Recent investigations show this assumption to be false: although the molar extinction coefficients (ε) of the theaflavins are closely similar, their gram concentration extinction coefficients ($E_{1\,cm}^{0.01\%}$) differ considerably (Table I). The second limitation arises from the method used to separate the theaflavins from SI thearubigins also present in the ethyl acetate extract of the tea infusion[1,2]. The technique of extracting the thearubigins with dilute bicarbonate solution carries the risks of incomplete extraction if the period of shaking is too short, and of theaflavin autoxidation if it is too long. Unless automated, the method is operator-sensitive and often leads to poor reproducibility.

The aim of this investigation was to develop a more specific and reproducible method which would make individual theaflavin levels accessible. We wish to report

a method based on the separation of theaflavins by gas–liquid chromatography (GLC) of their trimethylsilyl ethers.

TABLE I

EXTINCTION COEFFICIENTS OF THE THEAFLAVINS IN METHANOL SOLUTION

	377 nm		*459 nm*	
	$E^{0.01\%}_{1\ cm}$	ε	$E^{0.01\%}_{1\ cm}$	ε
TF1	1.91	10 800	0.720	4060
TF2A	1.39	10 004	0.546	3910
TF2B	1.45	10 038	0.570	4081
TF3	1.18	10 231	0.407	3540

EXPERIMENTAL

(1) GLC equipment and conditions

A Pye Series 104 dual column instrument fitted with flame ionisation detectors was used. Glass columns (lengths 6 in.–7 ft., I.D. 4 mm) were filled with the required packings and conditioned overnight at 10° above their operating temperature. Flow rates: air, 500 ml/min; H_2, 60 ml/min; carrier (argon), 60–120 ml/min, depending on column. Temperature programme for compensated 6-in. column of 3 % OV-1 on 80–100 mesh Chromosorb W: 260° isothermal for 10 min, then at 48°/min to 300°, isothermal hold.

Columns. 3 % OV-1 on acid-washed HMDS treated Chromosorb W (80–100 mesh; 6 in., 12 in., 3 ft., 7 ft.). 1 % OV-1 on acid-washed DMCS treated Chromosorb G (80–100 mesh; 3 ft., 7 ft.). 3 % OV-1 on Diatomite CQ (100–120 mesh, 5 ft.).

(2) Theaflavin standards

Pure theaflavins were obtained from black tea solids by ethyl acetate extraction, chromatography on Sephadex LH-20, and final separation on columns of silica[4]. Theaflavin monogallate isomers were synthesised by ferricyanide co-oxidation of (−)-epigallocatechin gallate with (−)-epicatechin (TF2A) and (−)-epigallocatechin with (−)-epicatechin gallate (TF2B)[3].

(3) Extraction of theaflavins from tea samples

(a) Instant tea solids (1.0 g) were dispersed in warm distilled water (100 ml) and the well-shaken dispersion extracted with ethyl acetate (100 ml). The acetate extract was shaken for 30 or 60 sec with freshly-prepared 2.5 % sodium bicarbonate solution (100 ml) and samples of the acetate solution (4 ml) taken for spectrophotometric estimation of theaflavins[1,2]. The remaining extract was filtered through anhydrous sodium sulphate (wash) and evaporated to dryness at 30° under reduced pressure. The solids so obtained were dissolved in dry redistilled pyridine (2 ml) and derivatised as described below (4a).

(b) Instant tea solids (1.0 g) were dispersed in warm distilled water (100 ml) and the dispersion extracted with ethyl acetate (4 × 100 ml). The combined extracts were dried (Na_2SO_4), evaporated to dryness (30°, reduced pressure) and the solids

taken up in a minimum of 60 % aqueous acetone. This solution was applied to a column (50 × 2 cm I.D.) of Sephadex LH-20 preswollen in 60 % aqueous acetone, eluted with 60 % aqueous acetone, and the theaflavin band (elution volume $\simeq 5\ V_0$) collected[5]. The acetone was distilled off at room temperature under reduced pressure: the theaflavins were extracted from the aqueous solution with 3 volumes of ethyl acetate. The combined extracts were evaporated to dryness (30°, reduced pressure), the solids dissolved in dry redistilled pyridine (1.0 ml) and derivatised as described in (4a).

(4) Derivatisation

(a) The dry pyridine solution obtained from (3a) or (3b) was transferred to a septum-sealed tube containing a known weight of 1,3-distearin (Applied Science Laboratories), bis(trimethylsilyl)acetamide (BSA, 2.0 ml) was injected through the septum, and the reaction mixture analysed at regular intervals.

(b) A mixture of pure theaflavins (10 mg of each) and 1,3-distearin (5 mg) was dissolved in dry pyridine (1 ml) in a septum-sealed tube, BSA (2.0 ml) was injected through the septum, and the mixture analysed at regular intervals.

RESULTS

(1) Separation of trimethylsilyl (TMS) derivatives of theaflavins

TMS derivatives of theaflavin (TF1), a mixture of the two monogallates (TF2) and the digallate (TF3)[3] were prepared by overnight reaction with BSA in dry pyridine. Separation was first attempted on a 3-ft. column of 1 % OV-1 on 80–100 mesh Chromosorb G at 310° with a flow rate of 100 ml/min. The TF3 peak preceded the TF2 peak (Fig. 1): in view of the higher molecular weight of TF3, this elution order is anomalous, and was thought to arise from decomposition of the TF3 derivative

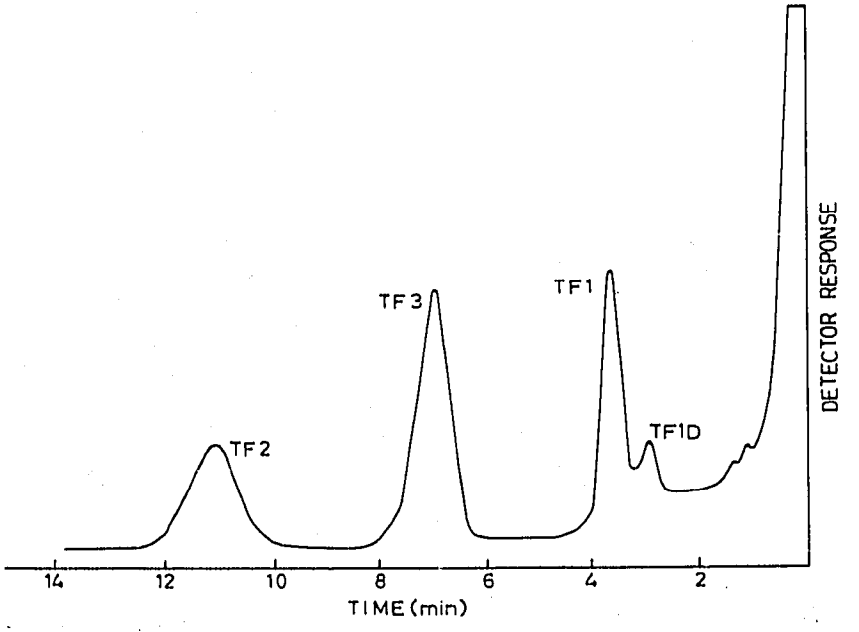

Fig. 1. Separation of theaflavin TMS derivatives. Column, 3 ft. of 1 % OV-1 on 80–100 mesh Chromosorb G; temperature, 310°; flow rate, 100 ml/min.

to one of lower molecular weight. Support for this suggestion came from experiments with TF1. The TF1 peak had a minor pre-peak (Fig. 1): material corresponding to the combined peaks was trapped from the stream-split column effluent in a cooled capillary, taken up in carbon tetrachloride and re-injected. The pre-peak was enhanced at the expense of the (formerly) main second peak (Fig. 2), and the mass spectrum of the trapped material differed considerably from that of the derivative before chromatography[3]. Attempts to trap the TF3 peak for mass spectral comparison with the unchromatographed derivative were unsuccessful.

Columns prepared from extremely inert acid-washed hexamethyldisilazane-treated Chromosorb W still gave two peaks for TF1 under essentially similar conditions of flow rate and temperature; accordingly column length and temperature were

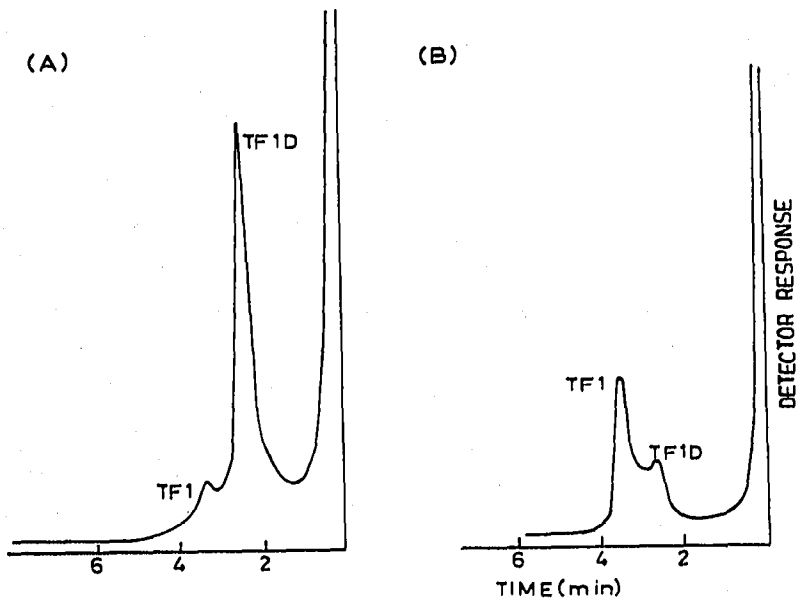

Fig. 2. Decomposition of TF1-TMS derivative during chromatography. (A) re-injected trapped material; (B) original derivative.

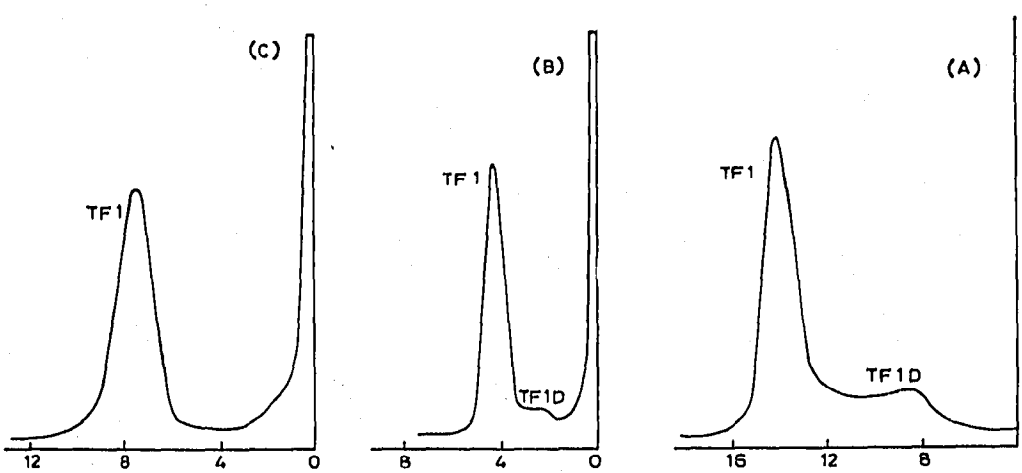

Fig. 3. Chromatograms of TF1-TMS derivative on columns of 3 % OV-1 on 80–100 mesh Chromosorb W (acid washed and HMDS-treated). Length, temperature and flow rate: (A) 3 ft., 280°, 120 ml/min; (B) 1 ft., 280°, 120 ml/min; (C) 6 in., 260°, 100 ml/min.

reduced in an effort to minimise decomposition. Reducing the column length from 3 ft. to 1 ft. resulted in a marked improvement (Fig. 3). By reducing the column length to an effective 6 in. from the point of injection and lowering the temperature to 260°, decomposition of the TF1 derivative was apparently halted. Separation of TF1, TF2 and TF3 on this compensated 6-in. column was achieved in 25 min by temperature-programming from 260° to 300° at 48°/min after the emergence of TF1 (10 min). Under these conditions, the elution order was normal (TF1, 2, 3) and only a small TF3 decomposition peak (TF3D, Fig. 4) was observed. 1,3-Distearin was found to be a suitable internal standard under these conditions.

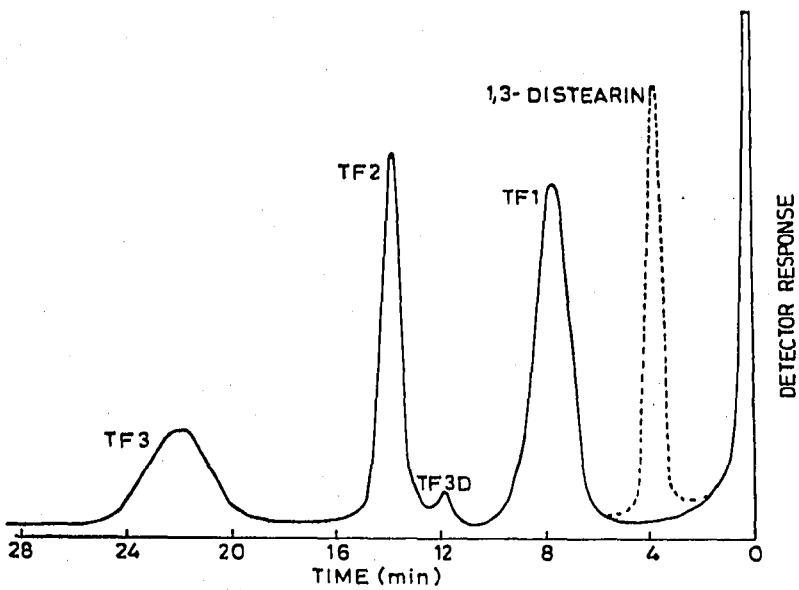

Fig. 4. Separation of theaflavin TMS derivatives on temperature-programmed 6-in. column.

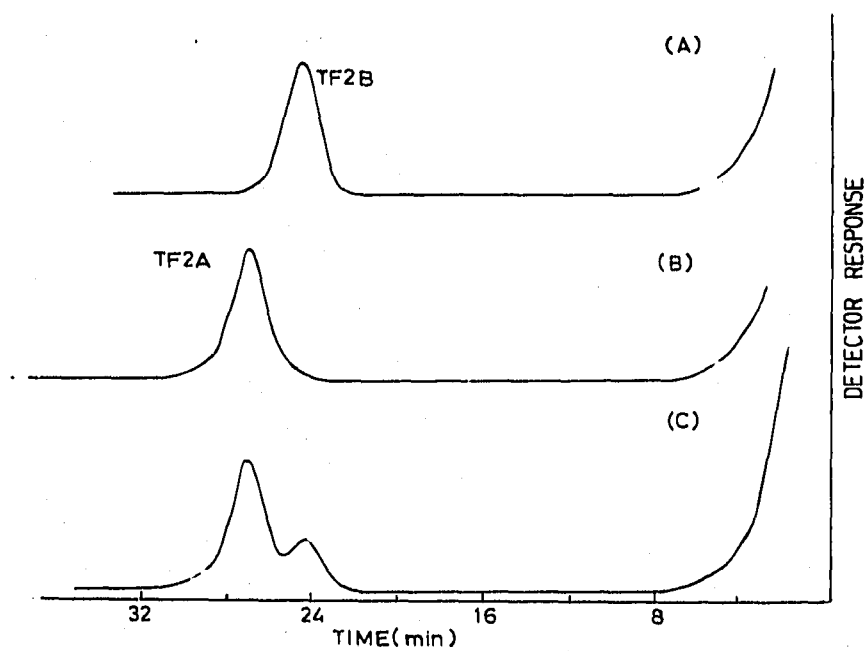

Fig. 5. Separation of TF2 (monogallate) isomers. Column 7 ft. of 1 % OV-1 on Chromosorb G; temperature, 330°; flow rate, 100 ml/min. (A) and (B): synthetic isomers; (C) naturally-occurring mixture TF2.

Separation of the two isomeric theaflavin monogallates TF2A and B was only possible on 7-ft. columns at 320–330°. Under these conditions their apparent decomposition products were well-separated in 25 min. The two isomers in TF2 were originally identified[3] by comparison with synthetic standards (Fig. 5).

(2) *Estimation of theaflavins*

(a) *Derivatisation conditions and detector constants.* The trimethylsilylation of a mixture of pure theaflavins and 1,3-distearin by BSA in pyridine was monitored by analysing 5-μl aliquots at regular intervals on the temperature-programmed 6-in. column. Equilibrium was reached after 4 h at room temperature, and peak areas remained constant for at least a further 20 h (Fig. 6). The trimethylsilylation of samples obtained from whole tea solubles (EXPERIMENTAL 3b) showed essentially similar kinetics. Calibration data obtained from standard mixtures of theaflavins and 1,3-distearin derivatised overnight are presented in Table II. Plots of weight of theaflavin per weight of distearin (W_t/W_s) against the corresponding peak area ratio (A_t/A_s) were linear, least-mean-squares refined values of the slopes (W_tA_s/W_sA_t) being taken as the detector constants (F).

(b) *Estimation of theaflavins in whole tea extracts.* Two freeze-dried black tea extracts (F4 and F8) were prepared for analysis by extraction with ethyl acetate and

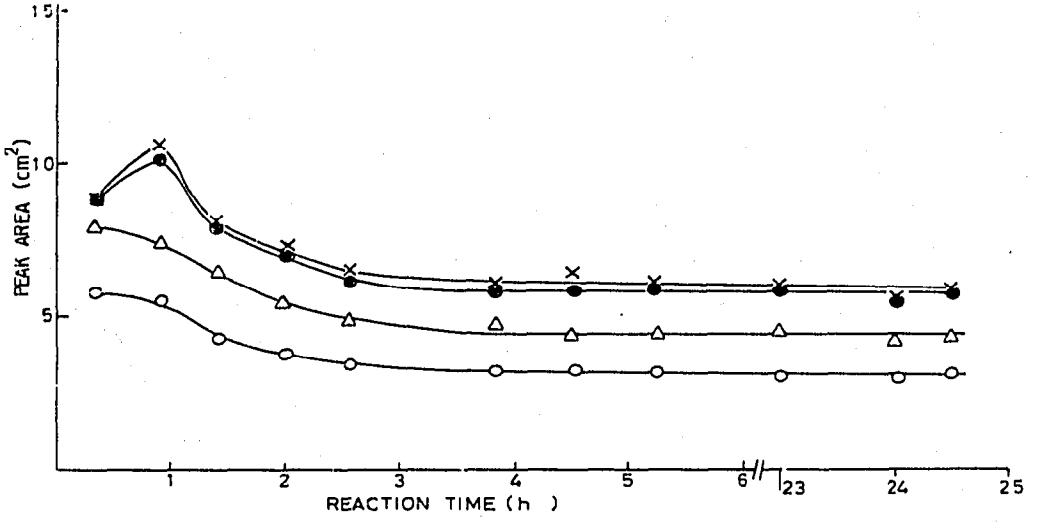

Fig. 6. Rate of derivatisation of theaflavins by BSA in pyridine. ○ = 1,3-distearin (internal standard); △ = theaflavin digallate, TF3; ● = mixture of theaflavin monogallate isomers, TF2; × = theaflavin, TF1.

TABLE II

THEAFLAVIN DETECTOR CONSTANTS ON A 1,3-DISTEARIN STANDARD

	F	*Standard error*
TF1	0.934	±1.8%
TF2	0.761	±3.8%
TF3	1.189	±2.8%

chromatography on Sephadex LH-20 (EXPERIMENTAL 3b), derivatised overnight, and analysed on the temperature-programmed 6-in. column (Fig. 7). The results are presented in Table III, together with figures for total theaflavins obtained by the ROBERTS' spectrophotometric method (extraction as in EXPERIMENTAL 3a)[1,2].

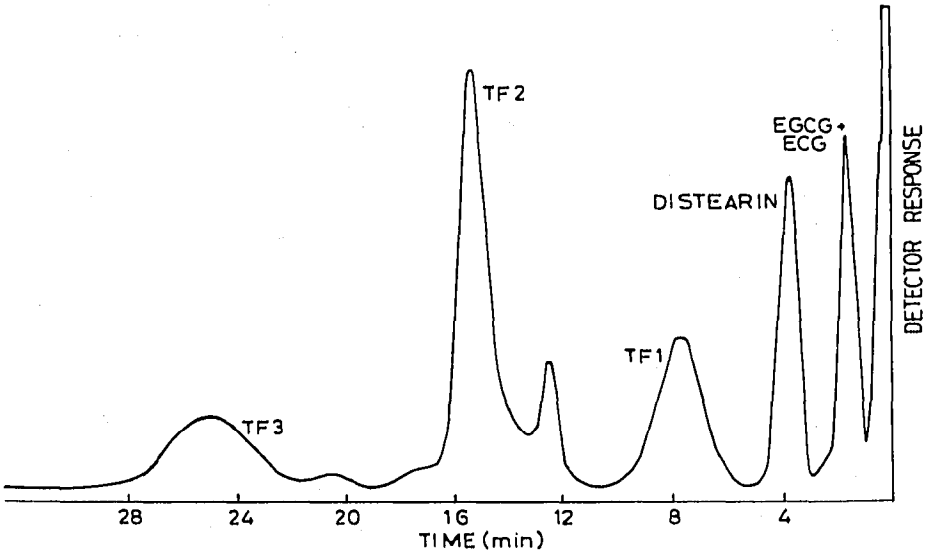

Fig. 7. Chromatogram of whole tea theaflavin fraction from Sephadex LH-20 column.

TABLE III

THEAFLAVIN LEVELS IN BLACK TEA EXTRACTS

	F4	*Mean*	*F8*	*Mean*
TF1	0.230, 0.225, 0.235, 0.219, 0.241	0.230	0.527, 0.531, 0.522, 0.487	0.517
TF2	0.946, 0.994, 0.972, 0.968, 0.970	0.970	1.526, 1.582, 1.610, 1.528	1.562
TF3	0.896, 0.884, 0.912, 0.889, 0.894	0.895	1.749, 1.828, 1.822, 1.862	1.815
Total	(GLC)	2.095		3.894
Total	(ROBERTS)	2.50		4.64

The fact that the spectrophotometric method gives higher results in both cases suggests that bicarbonate extraction of SI thearubigins from the ethyl acetate extracts is incomplete, and that the unextracted thearubigins make a significant contribution to light absorption at 380 and 460 nm (refs. 1, 2). Chromatograms of ethyl acetate-extracted solids before and after bicarbonate extraction support this hypothesis; they show that the whole acetate extract contains at least three major species in addition to the flavanols and theaflavins, and that these are incompletely removed by even a 1-min bicarbonate extraction (Fig. 8). Chromatography on Sephadex LH-20 removes these materials much more efficiently (Fig. 7). Preliminary experiments with separated SI thearubigins, prepared as described by ROBERTS[6], show that at least one of the non-theaflavin GLC peaks may be due to thearubigins. TF4, the

co-oxidation product of (−)-epicatechin and gallic acid[3], cannot be estimated by the short-column GLC method since its TMS derivative is not resolved from those of EGCG and ECG. However, investigations to be reported later suggest that it is only a trace constituent of the theaflavin fraction.

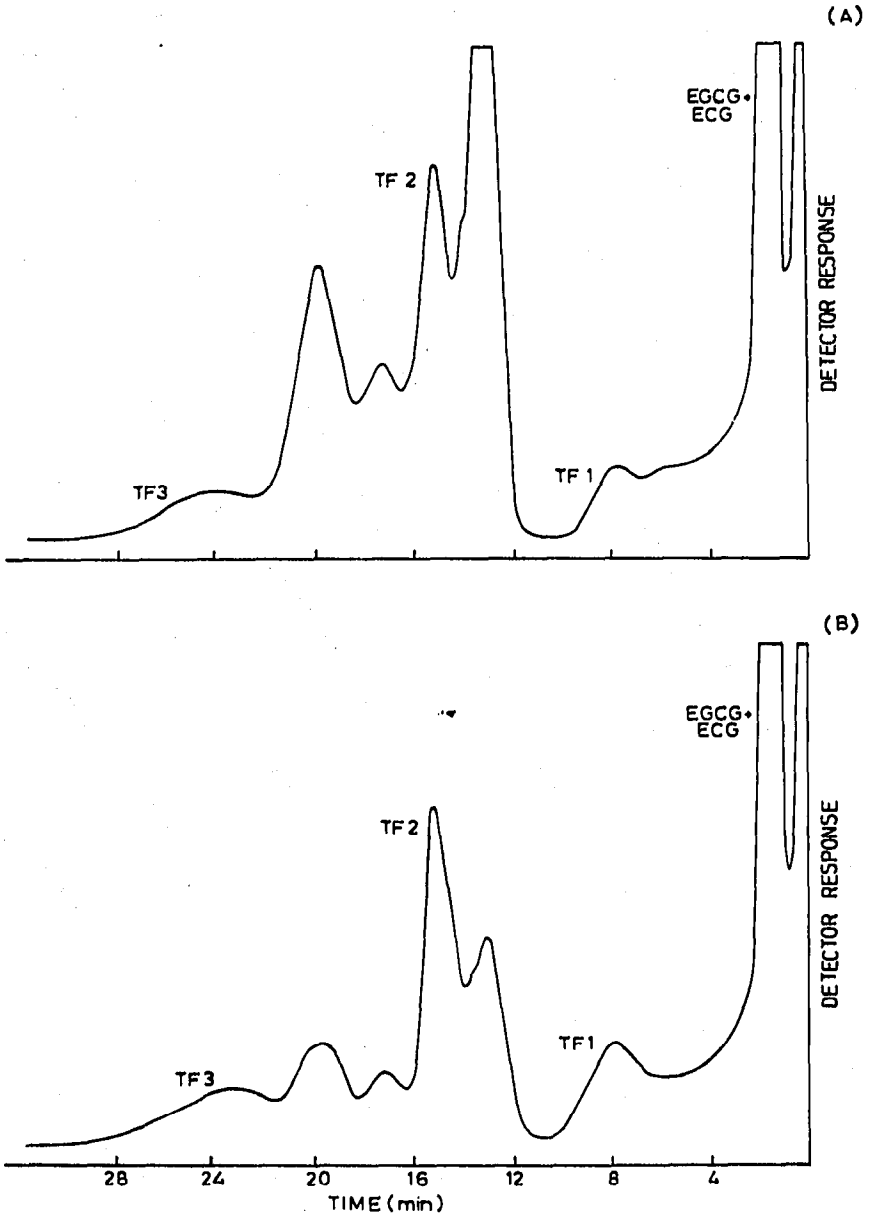

Fig. 8. Chromatograms of ethyl acetate extract of aqueous whole tea solution. (A) before bicarbonate extraction; (B) after 1 min bicarbonate extraction.

CONCLUSIONS

The trimethylsilyl derivatives of theaflavin (TF1) and its digallate (TF3)[3] can be separated without decomposition from those of the isomeric monogallates (TF2) on twin 6-in. columns of 3 % OV-1 on 80–100 mesh Chromosorb W under the following

conditions: Carrier (argon) flow rate, 100 ml/min; temperature, 260° for 10 min, programme at 48°/min to 300°, isothermal hold. Elution is complete in 25 min.

TMS derivatives of the two isomeric monogallates are not resolved under these conditions: their decomposition products can be separated at 320–330° on 7-ft. columns of 1 % OV-1 on Chromosorb G (80–100 mesh) or 3 % OV-1 on Chromosorb W (80–100 mesh).

Trimethylsilylation of theaflavins by BSA in pyridine reaches equilibrium within 4 h at room temperature; this equilibrium is maintained for at least 20 h. Detector constants with respect to a 1,3-distearin standard are reproducible to better than ±5 %.

Fractions suitable for derivatisation and analysis can be prepared from whole tea solubles by extraction with ethyl acetate followed by chromatography on Sephadex LH-20. Initial tests of this procedure show the results to be reproducible to within ±5 %.

The ROBERTS' spectrophotometric method gives total theaflavin levels some 15–25 % higher than those obtained by the GLC method. Chromatography of ethyl acetate extracts of tea before and after bicarbonate extraction suggests that extraction of SI thearubigins from solutions in ethyl acetate is incomplete, and that unextracted thearubigins are estimated as theaflavins in the spectrophotometric method.

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