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SEPARATION OF DERIVATIZED BLACK TEA THEARUBIGINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

SI thearubigins were extracted from black tea infusions and converted into acetyl and methyl derivatives. Both derivatives gave rise to discrete components upon thin-layer chromatography on silica and were eluted in high yield from silica high-performance liquid chromatographic columns using mixtures of chloroform and methanol. Acetyl derivatives tended to undergo time-dependent changes in chromatographic behaviour, whilst methyl derivatives appeared to be stable.

INTRODUCTION

Thearubigins play an important role in the quality of black tea infusions. Together with theaflavins, they determine the strength and colour of the infusion[1], play a role in its mouthfeel[2] presumably due to their astringency[3] and are involved in the quality-related ability of the infusion to form "tea cream", a precipitate of complexes of caffeine with theaflavins and thearubigins[4–6]. Thearubigins are the most abundant phenolic fraction of black tea.

Thearubigins is the name originally assigned to all the acidic brown pigments of black tea[1]. A broad classification of these compounds is those extractable into ethyl acetate, the SI thearubigins, and those remaining in the aqueous phase, the SIa and SII thearubigins with the SIa group being more soluble in diethyl ether[7]. Other separations based on extractability have also been carried out[8–11]. Other separation attempts include the use of cellulose column chromatography[9], Toyopearl chromatography[12], ion exchange and paper electrophoresis[13], reversed-phase high-performance liquid chromatography (HPLC)[14] and gel chromatography media, *e.g.*, Pharmacia LH20[15]. However, none has proved entirely satisfactory.

One problem with the chromatographic systems involving a stationary phase is the high affinity of the thearubigins for the stationary phase; this can potentially be controlled by derivatizing the phenolic groups. The purpose of this work was to investigate the potential for normal phase HPLC of derivatized SI thearubigins.

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EXPERIMENTAL

Extraction of SI thearubigins

An infusion of black tea leaf (25 g) in boiling water (1 l) was maintained at 95°C for 5 min. The infusion was filtered (glass fibre paper) and freeze dried. The tea solids (50 g) obtained from several pooled freeze-dried extracts were dissolved with stirring in hot aqueous methanol (1 l, 25% methanol). The presence of methanol was necessary to prevent the formation of tea cream and to reduce the tendency for emulsion formation on addition of chloroform. The solution was extracted exhaustively with chloroform to remove caffeine in a continuous liquid–liquid extraction apparatus. To test for the satisfactory removal of caffeine, a further extraction of the whole of the aqueous phase was carried out in a separating funnel containing chloroform (1 l), the extract was dried over anhydrous $MgSO_4$ and its absorbance measured at 276 nm in 1-cm silica cells. This procedure was repeated unil the absorbance of the extract was <0.1. Chloroform remaining in the aqueous phase was removed under reduced presure at 30°C. The solution was extracted repeatedly with an equal volume of water-saturated ethyl acetate until no more colour was extracted. The ethyl acetate extracts were pooled, dried over $MgSO_4$ and evaporated to dryness under reduced pressure at 30°C. To ensure complete removal of ethyl acetate, the solids were dissolved in acetone and evaporated to dryness as before. These solids were dissolved in the minimum volume of acetone (1 volume) and the solution added dropwise with stirring to chloroform (10 volumes) cooled in ice. The precipitate was removed by centrifugation (15 000 *g*, 10 min), redissolved in the minimum volume of acetone and reprecipitated in chloroform as before. The precipitate was dissolved in the minimum volume of acetone (1 volume) and precipitated three times by adding to peroxide-free diethyl ether (5, 10 and 15 volumes) with stirring in ice. To ensure complete removal of diethyl ether, the final precipitate was dissolved in acetone and evaporated to dryness under reduced pressure at 30°C to give the SI thearubigin fraction.

The product was shown to be free from low-molecular-weight impurities by thin-layer chromatography (TLC) and gas chromatography (GC). The TLC method was a variation of the paper chromatographic method of Roberts *et al.*[7] and Ratnaike[13] in which the paper was replaced with a 0.1-mm cellulose thin layer on a polymer film backing (Polygram Cel 300, 20 cm × 20 cm; Macherey-Nagel, F.R.G.) sheets. These sheets gave better resolution, shorter development time and easier handling than the papers. The components were separated by two-dimensional chromatography with butanol–acetic acid–water (4:1:2.2) in the first direction and aqueous acetic acid (2%) in the second. The components were visualized by spraying with an aqueous solution of a mixture of ferric chloride (0.3%, w/w) and potassium ferricyanide (0.3%, w/w). The plates were fixed in dilute acid (HCl) and excess of ferricyanide was removed by washing in water. The GC method was as used for the analysis of tea flavanols as their trimethylsilyl derivatives[16].

Acetylation of thearubigins

The acetylation procedure was a modification of that used by Cattell[15]. The sample (0.1 g) was dissolved in dry pyridine (1 ml), acetic anhydride (6 ml) added and the mixture warmed at 30°C for 1 h. Water (10 ml) was added with cooling and the precipitate filtered off. The product was dissolved in chloroform (5 ml) and the

solution extracted with aqueous $NaHCO_3$ (10%, w/v; 2 × 5 ml) and water (2 × 5 ml) before being evaporated to dryness.

The degree of acetylation was assessed by carrying out the reaction using half the amounts of reagents given above and adding the reaction mixture to water (20 ml) followed by pyridine (50 ml). The resulting solution was titrated with 0.5 *M* NaOH to pH 9.2 using a pH meter, and a blank titration was carried out on a solution omitting the thearubigin sample. In order to check the efficiency of acetylation, a sample of (−)epicatechin (Sigma) was treated similarly.

Methylation of thearubigins

The methylation of thearubigins using diazomethane has been described by Ratnaike[13]. Diazomethane was prepared as a solution in diethyl ether from N-methyl-N-nitrosotoluene-*p*-sulphonamide according to the method described by Vogel[17]. The thearubigin sample (20 mg) was dissolved in methanol (2 ml) in an ice-bath and excess of diazomethane solution was added. The presence of the excess of reagent was demonstrated by adding a few drops of glacial acetic acid to an aliquot of the reaction mixture, when gas was immediately evolved. The reaction mixture was immediately evaporated to dryness under reduced pressure at 30°C.

Separation of derivatized thearubigins

A preliminary choice of the solvent system for HPLC separations on silica was made by means of TLC (silica gel G $60F_{254}$, 0.25 mm, activated at 110°C for 30 min). Components were located under UV light. High-performance liquid chromatographic separations (ACS liquid chromatography system LC750 with two reciprocating pumps and a decilinear programmer; Applied Chromatography Systems, Macclesfield, U.K.) were carried out on a Partisil 10 column (HPLC Technology, Macclesfield, U.K.; particle size 10 μm, 25 cm × 0.46 cm) at 1 ml min^{-1} with the column effluent monitored spectrophotometrically (1-cm path length cell, ACS 750-11 monitor at 254 nm or Cecil CE212A spectrophotometer at 330 nm). The recovery of samples from the column was measured by comparing the UV spectrum of the sample applied to the column with that of the eluted mixture, at the same dilutions.

RESULTS AND DISCUSSION

When subjeced to two-dimensional TLC analysis on cellulose the thearubigin preparation was found as a streak (R_F 0.3–0.9) in butanol–acetic acid–water as the solvent, and no evidence of components showing mobility in both solvents was seen at any level of application of the sample. This is consistent with the chromatographic behaviour of SI thearubigins[18]. Similarly, no evidence of the presence of monomeric tea polyphenols was seen from GC analysis of trimethylsilyl derivatives of the thearubigin sample.

The acetyl derivative of the thearubigin preparation formed readily and it was found that 20 ± 2% (mean of three determinations ± standard deviation) of the mass of the original thearubigin was due to OH groups which became acetylated. Acetylation of (−)-epicatechin indicated that 28.3 ± 2.3% of its mass was due to OH groups, compared with the expected value of 29.3% corresponding to five OH groups in the molecule. Whilst it is not possible to use this result to predict the degree of

acetylation per monomer in thearubigins, it shows that a substantial number of OH groups had been derivatized; there would have been approximately three acetyl groups per monomer unit if the thearubigin consisted of catechin repeating units.

A characteristic of thearubigins is their lack of mobility when examined by TLC on silica and no resolution of components on cellulose. The behaviour of thearubigins, acetylated thearubigins and acetylated instant green tea (mixture of flavanols) on silica is shown in Table I for a wide range of solvents. In all cases, derivatization has allowed chromatograms to be obtained, with particularly high mobility in ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1) and chloroform–methanol (3:2). Also, the appearance of the chromatograms was different from those of the low-molecular-weight components of green tea, indicating that the spots observed were not due to the low-molecular-weight precursors of thearubigins present in green tea, which can be released by hydrolysis of thearubigins during the acetylation procedure. Of the two solvents capable of moving all the sample away from the origin, that which did not

TABLE I

TLC ANALYSIS OF ACETYLATED GREEN TEA (ACETYLATED MIXTURE OF GREEN TEA FLAVANOLS), SI THEARUBIGINS AND ACETYLATED SI THEARUBIGINS ON SILICA ($60F_{254}$) USING A VARIETY OF SOLVENTS

All components identified under UV ligh. Mobility shown as R_F value; those values underlined refer to coloured (yellow/brown) spots.

Solvent	R_F		
	Acetylated green tea	*Thearubigins*	*Acetylated thearubigins*
Benzene–ethyl formate–formic acid (72:24:1)	0, 0.050, 0.09, 0.14, 0.18	0 + short dark streak	0, 0.10, 0.92 + short dark streak
Toluene–ethyl formate–formic acid (50:40:10)	0.43, 0.46, 0.52, 0.54	0 + short dark streak	0, 0.35, 0.41 + short dark streak
Methyl ethyl ketone	0.73, 0.81 + pale streak	0 + long dark streak	0, 0.47, 0.49, 0.91, + long pale streak
Benzene–ethanol (75:25)	0.26, 0.67, 0.77, 0.81, 0.88	0 + short dark streak	0, 0.63, 0.64, 0.71 + long pale streak
Cyclohexane–acetone (20:25)	0, 0.34, 0.50, 0.53, 0.56, 0.58, 0.61 + pale streak	0 + short dark streak	0, 0.53, 0.58, 0.92 + long pale streak
Cyclohexane–chloroform–pyridine (10:30:25)	0.80, 0.85, 0.88 + pale streak	0	0, 0.69 + long pale streak
Cyclohexane–chloroform–acetic acid (20:25:5)	0, 0.04, 0.07, 0.12, 0.15, 0.17, 0.24	0	0, 0.12, 0.25 + short dark streak
Chloroform–ethyl acetate–formic acid (25:20:5)	0.57, 0.61, 0.66, 0.69, 0.73	0	0, 0.60, 0.75, 0.98 + long dark streak
Ethyl acetate–methyl ethyl ketone–formic acid–water (50:30:10:10)	0.99	0 + long dark streak	0.97
Benzene–ethyl acetate (75:25)	0, 0.16, 0.24, 0.33, + pale streak		0, 0.05, 0.08, 0.21, 0.97
Chloroform–methanol (30:20)	0.99	0 + short dark streak	0.96 + pale streak

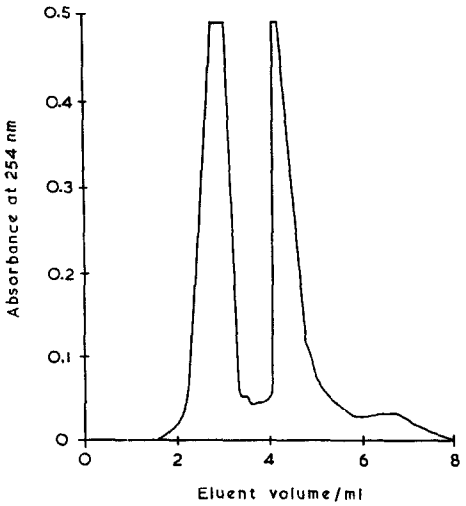

Fig. 1. Separation of acetylated SI thearubigins by HPLC on Partisil 10 (25 cm × 0.46 cm) with chloroform–methanol (4:6) at 1 ml min^{-1}.

contain acid was preferred because the risk of possible modification of thearubigin was avoided and HPLC analysis was, therefore, attempted using chloroform–methanol mixtures. The best separation shown in Fig. 1 was achieved using chloroform–methanol (4:6, v/v). When the UV spectrum (250–700 nm) of the sample for analysis was compared with that of the pooled fractions eluted from the column, the absorbances at any wavelength were within ±5% (from three sets of experiments) and

TABLE II

TLC ANALYSIS OF METHYLATED SI THEARUBIGINS ON SILICA ($60F_{254}$)

All components identified under UV light. Mobility shown as R_F value; those values underlined refer to coloured (yellow/brown) spots.

Solvent	R_F
Ethyl acetate	0 + long dark streak
Ethyl acetate–methyl ethyl ketone (25:15)	0, 0.03, 0.05, 0.15 + long dark streak
Ethyl acetate–methyl ethyl ketone–formic acid–water (25:15:5:5)	0.12, 0.20, 0.28, 0.37, 0.45, 0.49, 0.54, 0.60, 0.64, 0.95
Ethyl acetate–methyl ethyl ketone–water (25:15:5)	0, 0.03, 0.05, 0.09, 0.12, 0.15, 0.18, 0.34, 0.94 + long dark streak and early spots overlapped
Ethyl acetate–methyl ethyl ketone–formic acid (25:15:5)	0, 0.04, 0.06, 0.09, 0.13, 0.94 + long pale streak and early spots overlapped
Ethyl acetate–methyl ethyl ketone–methanol (25:15:5)	0, 0.04, 0.12, 0.19, 0.29, 0.39, 0.95 + long dark streak
Ethyl acetate–methyl ethyl ketone–methanol (25:15:10)	0, 0.17, 0.25, 0.39, 0.46, 0.56, 0.66, 0.73, 0.94 + long dark streak and early spots overlapped
Ethyl acetate–methyl ethyl ketone–methanol (25:15:10)	0, 0.11, 0.40, 0.52, 0.60, 0.66, 0.75, 0.96 + long dark streak and early spots overlapped
Chloroform	0, 0.94
Methanol	0, 0.57, 0.62, 0.66, 0.79 + long pale streak

it is concluded that the chromatogram represents an high proportion of the thearubigin applied. A feature of the acetyl derivatives was their apparent instablity. When left to stand over a period of hours, samples of acetylated thearubigin showed a progressive increase in colour remaining at the point of application in TLC experiments when analysed using any of the solvents given in Table I. Similarly, the slowest migrating peak in the chromatogram in Fig. 1 becomes more prominent with ageing of the sample and it is likely that acetylation does not afford complete protection of the sample.

The analysis of methylated thearubigins by TLC is summarized in Table II. As was the case for acetylation, methylation allows components of thearubigins to be resolved and, in general, it was possible to separate the mixture into a greater number of spots than was the case for acetylated samples. The best separation by HPLC was obtained using a chloroform–methanol gradient as follows: initially chloroform–methanol (9:1) was used for 5 min followed by an increasing concentration of methanol at 1% min^{-1} for 10 min, 2% min^{-1} for 5 min, 5% min^{-1} for 5 min and finally 10% min^{-1} until the solvent was pure methanol. A typical chromatogram for methylated thearubigins from a sample of Lipton's yellow label tea bags is illustrated in Fig. 2 where up to fourteen components may be discerned. Thearubigins from an instant tea of SriLankan origin showed a similar chromatogram but the peaks appeared to be of different sizes. Table III shows a comparison of the positions and intensities of the peaks measured from two separate experiments for each of the methylated thearubigins from the two teas, with the errors shown as standard deviations. When the UV spectrum of the methylated sample for analysis was

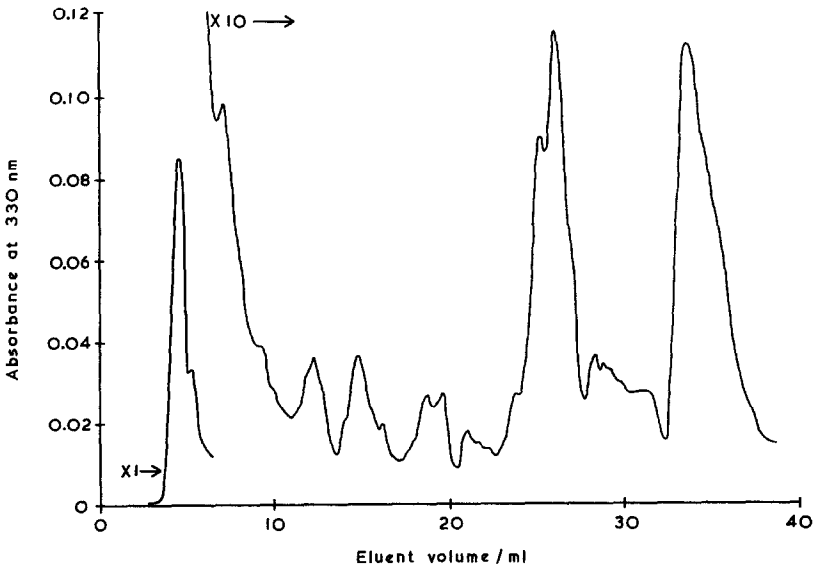

Fig. 2. Separation of methylated SI thearubigins by HPLC on Partisil 10 (25 cm × 0.46 cm) at 1 ml min^{-1} using the following gradient system: chloroform–methanol (9:1) for 5 min followed by gradient elution increasing the concentration of methanol at 1% min^{-1} for 10 min, 2% min^{-1} for 5 min, 5% min^{-1} for 5 min and finally at 10% min^{-1} until the solvent was pure methanol.

TABLE III

COMPARISON OF RETENTION VOLUMES AND PEAK HEIGHTS OF COMPONENTS PRESENT IN METHYLATED SI THEARUBIGINS FROM TWO BLACK TEAS

Teas: 1 = Liptons yellow label tea bags; 2 = SriLankan instant tea. All analyses were carried out on a Partisil 10 column (25 cm × 0.46 cm) at 1 ml min^{-1} using the following gradient system: chloroform–methanol (9:1) for 5 min followed by gradient elution increasing the concentration of methanol at 1% min^{-1} for 10 min, 2% min^{-1} for 5 min, 5% min^{-1} for 5 min and finally at 10% min^{-1} until the solvent was pure methanol.

Tea 1		*Tea 2*	
Elution volume (ml)	*Absorbance at 330 nm*	*Elution volume (ml)*	*Absorbance at 330 nm*
4.9 ± 0.5	0.084 ± 0.003	4.6 ± 0.2	0.046 ± 0.004
5.4 ± 0.3	0.034 ± 0.004	6.2 ± 0.2	0.024 ± 0.002
7.3 ± 0.1	0.013 ± 0.001	7.1 ± 0.1	0.022 ± 0.002
9.7 ± 0.2	0.007 ± 0.001	9.6 ± 0.4	0.014 ± 0.002
11.9 ± 0.3	0.007 ± 0.001	10.8 ± 0.5	0.011 ± 0.001
14.3 ± 0.3	0.007 ± 0.001	14.0 ± 0.3	0.010 ± 0.001
		15.3 ± 0.3	0.007 ± 0.003
17.5 ± 0.1	0.006 ± 0.001	17.7 ± 0.1	0.008 ± 0.001
18.4	0.011	18.8 ± 0.2	0.011 ± 0.001
19.7	0.007	20.0 ± 0.1	0.014 ± 0.002
		21.7 ± 0.4	0.013 ± 0.001
23.7 ± 0.1	0.010 ± 0.003		
25.0 ± 0.1	0.009 ± 0.003	24.4 ± 0.5	0.011 ± 0.001
26.0	0.015	26.8	0.012
27.8 ± 0.1	0.010 ± 0.004		
		29.7	0.020
		30.7 ± 0.3	0.016 ± 0.002
32.7 ± 0.4	0.010 ± 0.002	32.2	0.013

compared with that of the pooled fractions eluted from the column, the absorbances at any wavelength were within ±10% (from three sets of measurements) and the chromatograms observed therefore represent a large proportion of the thearubigin. It was noticed, however, that a small amount of sample was retained irreversibly, reducing the life of columns and therefore necessitating the use of the minimum amount of derivatized thearubigin sample. This led generally to the need for high sensitivity in detection. It is now appropriate to consider these separations as a means of identification of different tea varieties or blends.

In general, HPLC of methylated thearubigins with a methanol–chloroform solvent gradient was able to achieve better separation than TLC with any of the solvents tried. When chloroform or methanol was used as TLC solvents some of the sample always remained at the origin. If mobility in TLC is indicative of mobility under HPLC conditions, then the components at the origin probably represent less than 10% of the absorbance of the sample at 330 nm.

CONCLUSION

Thearubigins are regarded as polymeric polyphenols and a reason for their high affinity towards silica which prevents its use for their analysis is likely to include polar interactions with OH groups on the polymer. This is supported by the observations made here that acetylation or methylation of these groups causes thearubigins to become mobile on silica in a variety of solvents. We report here the first partial separation of thearubigins as their derivatives by HPLC which should now form the basis of a renewed attempt to characterize these polymers.

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