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Counter-current chromatography of black tea infusions

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SUMMARY

Counter-current chromatography using a multilayer coil planet centrifuge, with solvent system ethyl acetate–butanol–water, permits the separation of black tea infusions into fractions which include pure S_{II} and a mixture of S_I and S_{Ia} thearubigins. Good resolution of several components of the infusion may be achieved in elution times of 1 to 2 h. The appearance of chromatograms is altered on decaffeinating the infusion. The effect of stationary phase composition is considered. Resolution of the peaks improves with butanol content.

INTRODUCTION

Black tea infusions consist of a complex mixture of polyphenols, caffeine, amino acids, proteins, carbohydrates and other components which form minor constituents. The phenolic fraction is regarded as providing most of the characteristics of tea infusions[1–3] and is derived mainly by the oxidation and coupling of naturally occurring flavanols and their gallate esters. Simple products, such as those arising from the combination of two flavanol molecules (*e.g.* bisflavanols, theaflavins) are well characterised but the most abundant polyphenolic fraction, thearubigins, is thought to consist of a complex mixture of polymeric species which has so far proved relatively intractable.

Separation of thearubigins from other components of black tea infusions may be carried out by solvent extraction and precipitation[4,5]. A broad classification of these compounds[4] is those extractable into ethyl acetate, the S_I thearubigins, and those remaining in the aqueous phase, the S_{Ia} and S_{II} thearubigins with the S_{Ia} being more soluble in diethyl ether. A commonly[4] used method for preparation of S_I thearubigins involves exhaustive extraction, with ethyl acetate, of decaffeinated infusions, evaporation of the extract followed by successive steps in which the product is dissolved in acetone and precipitated either with chloroform or diethyl ether. Apart from being lengthy, this procedure does not give rise to a product which is unequivocally that

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present in the original infusion; it is reasonable to expect the polymeric polyphenols, which are also partially oxidised, to be chemically reactive and continuing polymerisation is likely, particularly at the high concentrations present at the time of precipitation. Generally, all that may be guaranteed is that the product is free of low-molecular-weight phenols.

Chromatographic separations of thearubigins have been attempted using, for example, cellulose column chromatography[6], ion-exchange chromatography, paper electrophoresis[7], reversed-phase high-performance liquid chromatography[8] and gel filtration (*e.g.* Pharmacia LH-20)[9]. However, none has proved entirely satisfactory. The main problem is that polyphenolic components of black tea generally have high affinities for the stationary phases and become strongly adsorbed.

One solution to the problem of adsorption in a given mobile–stationary phase combination is to use liquid stationary phases permitting recovery of the components, which had not been eluted, for further separation. This may be achieved by means of counter-current chromatography (CCC); some of the earliest separations of black tea components involved the use of the Craig counter-current apparatus[4], but this technique was limited by the resolution which was possible. A new development in high-speed CCC is the multilayer coil planet centrifuge the principles of which have now been reviewed extensively[10,11].

The purpose of this work was to explore the possibility of the use of CCC for the separation of black tea infusions into polyphenolic fractions and was stimulated by the known tendency of the polymeric fractions to partition into solvents such as ethyl acetate. The use of CCC for the separation of tannins has also been documented[12].

EXPERIMENTAL

Tea infusion

Boiling water (400 ml) was added to a sample of "Lyons Red Label" tea bag tea (4 g) in a Dewar flask and the contents mixed by inverting the flask 10 times after which the mixture was left to stand for 6 min. The spent tea leaves were removed by filtering through a No. 1 sintered-glass funnel and the filtrate analysed after cooling, without delay.

Extraction of caffeine

Methanol (50 ml) was added to the hot tea infusion (200 ml) to prevent formation of "cream" and reduce the tendency for emulsion formation during subsequent extraction. The mixture was extracted with successive amounts (100 ml) of chloroform, each extract being dried (Na_2SO_4) and its absorbance measured at 276 nm (Cecil 292 UV spctrophotometer, Cecil Instruments, U.K.). Extraction was considered complete when the absorbance of the extract was <0.1. The residual chloroform and methanol were removed under reduced pressure (40°C).

Determination of partition coefficient of caffeine

An aqueous solution of caffeine was extracted twice with ethyl acetate (50 ml) in a separating funnel. The absorbance of each extract was measured at 280 nm. The procedure was repeated using a solution of caffeine in ethyl acetate and extracting with water.

Counter-current chromatography

The CCC column was a PTFE tube (*ca.* 130 m × 1.6 mm) with a total volume of *ca.* 310 ml, in an Ito multilayer coil separator–extractor (P.C. Inc., Potomac, MD, U.S.A.) running at 800 rpm. Solvent flow was metered by means of an ACS Series 750 LC pump (Applied Chromatography Services, U.K.) and column effluent monitored at 280 nm using a Cecil 292 UV monitor with an 80-μl, 1-cm path length cell.

Before use the two phases were saturated with each other and their densities measured by weight in a 100-ml volumetric flask. These were used to calculate the mass of the coil from the phase volume ratios to provide an accurate counter-balance for the centrifuge.

Prior to a chromatographic run, the column was filled with stationary phase and the mobile phase eluted from the "head" towards the "tail" of the column if the mobile phase was the more dense or *vice versa* for a less dense mobile phase. The head–tail relationship is referred to an Archimedan screw force which drives all objects in the rotating coil competitively towards the head of the coil. The column was considered to be at equilibrium when droplets of stationary phase no longer appeared in the column effluent and judged by negligible noise on the baseline of the chromatogram.

The column was regularly cleaned by passing methanol followed by aqueous NaOH (1–2%, w/w) and finally washing with water.

Two-dimensional thin-layer chromatography (TLC)

In order to identify the components in each chromatographic peak, fractions corresponding to each peak were combined and concentrated *in vacuo* (40°C). The components were separated on cellulose thin-layer plates (Polygram Cel 300, 20 cm × 20 cm, Macherey-Nagel, F.R.G.) by two-dimensional TLC with butanol–acetic acid–water (4:1:2.2) in the first direction and aqueous acetic acid (2%, v/v) in the second[4]. Phenolic components were revealed by spraying dry plates with ferric chloride (0.15%, w/w) + potassium ferricyanide (0.15%, w/w) in water. The plate was then fixed in 0.1 *M* HCl and excess reagent removed by washing in water.

RESULTS AND DISCUSSION

The distribution of a pure solute component i between two phases α and β is given by the partition (or distribution) coefficient, K, as

$$K = c_i^{\alpha}/c_i^{\beta} \tag{1}$$

where c_i^{α} and c_i^{β} denote the concentrations of that component in each of the phases, respectively. In CCC, the retention volume, V_R, of a solute is related[13] to the partition coefficient by,

$$V_R = V_s/K + V_m \tag{2}$$

where V_m and V_s are, respectively, volumes of the mobile and stationary phases and K is the ratio of solute concentration in the mobile phase to that in the stationary phase (phases α and β, respectively).

If a pure solute is dissolved in one of the phases (volume V^{α}) and solution

extracted twice with a solvent comprising the second phase (volume V^{β}), the partition coefficient may simply be calculated from the concentration of solute in each of the extracts, c_1^{β} and c_2^{β}, respectively, using,

$$K = V^{\alpha}(c_1^{\beta} - c_2^{\beta})/V^{\beta}c_2^{\beta} \tag{3}$$

The concentrations can, of course, be replaced by an appropriate measurable property of the solute, *e.g.* absorbance.

In the first instance water-saturated ethyl acetate was chosen as the stationary phase and ethyl acetate-saturated water as the mobile phase. In order to check the performance of the CCC column, a sample of caffeine (1 ml) was injected onto the column (V_s = 205 ml; V_m = 105 ml) and the position of the peak was found to correspond to K = 1.30. When a caffeine solution in water was extracted twice with ethyl acetate at 20°C, absorbances of the ethyl acetate layers at 280 nm gave K = 1.25. On the other hand, if a solution of caffeine in ethyl acetate was similarly extracted with water the value of K calculated is 0.81, the reciprocal of which, 1.23 corresponds to the values given above. The CCC experiment was, therefore, performing correctly and, in subsequent CCC runs on tea infusions containing caffeine, the position of the caffeine peak was used as a check on performance. The use of caffeine for this purpose was particularly attractive because components with K = 1 run at a retention volume equal to the column volume and caffeine in an ethyl acetate–water system would run with a similar retention volume which ever was the stationary phase.

A typical chromatogram of Lyons Red Label tea bag tea is shown in Fig. 1, component 1 being coloured (yellow) whilst component 2 was caffeine. Further resolution of the peaks was attempted by the addition of butanol to the stationary phase because this solvent has been used in the past for the selective extraction of

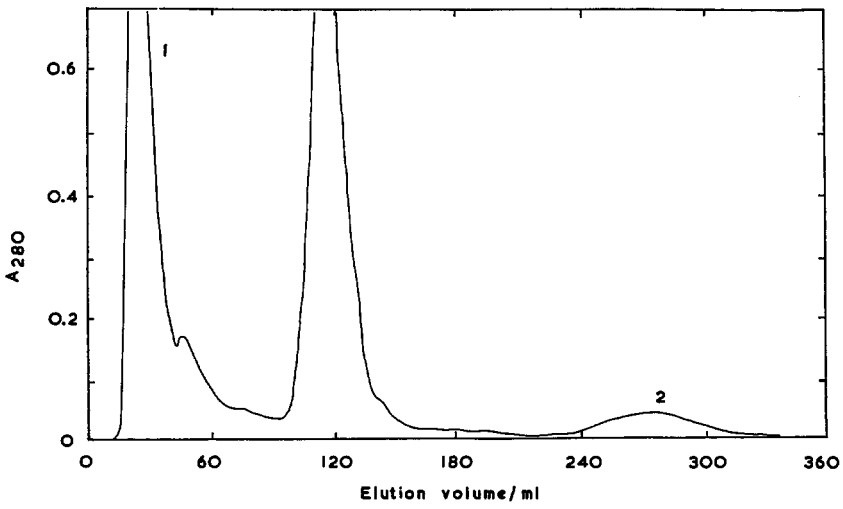

Fig. 1. Counter-current chromatogram of 1% (w/v) infusion of Lyons Red Label tea bag tea. Stationary phase: ethyl acetate-saturated water, 205 ml. Mobile phase: water-saturated ethyl acetate, 105 ml. Flow-rate: 3 ml/min.

TABLE I

EFFECT OF STATIONARY PHASE COMPOSITION ON THE VOLUMES OF MOBILE PHASE (V_m), STATIONARY PHASE (V_s), RETENTION VOLUME (V_R) OF CAFFEINE IN THE CHROMATOGRAM OF A TEA INFUSION AND THE PARTITION COEFFICIENT (K) OF CAFFEINE BETWEEN STATIONARY PHASE AND MOBILE PHASE

Chromatograms were run at 3 ml/min.

Solvent system: butanol–ethyl acetate–water (v/v/v)	V_m *(ml)*	V_s *(ml)*	V_R *(ml)*	K
0:100:100	110	200	270	1.26
10:90:100	98	212	300	1.05
20:80:100	102	208	354	0.83
30:70:100	102	208	398	0.77
40:60:100	130	180	420	0.62
50:50:100	142	168	432	0.56
80:20:100	150	160	434	0.56
100:0:100	180	130	396	0.62

individual thearubigin fractions[4,5]. The main problem with the separation illustrated in Fig. 1 is that the components tended to have too great a bias towards the mobile phase; addition of butanol to stationary phase was expected to increase its polarity and increase its affinity for the polyphenols. The effect of solvent composition of the

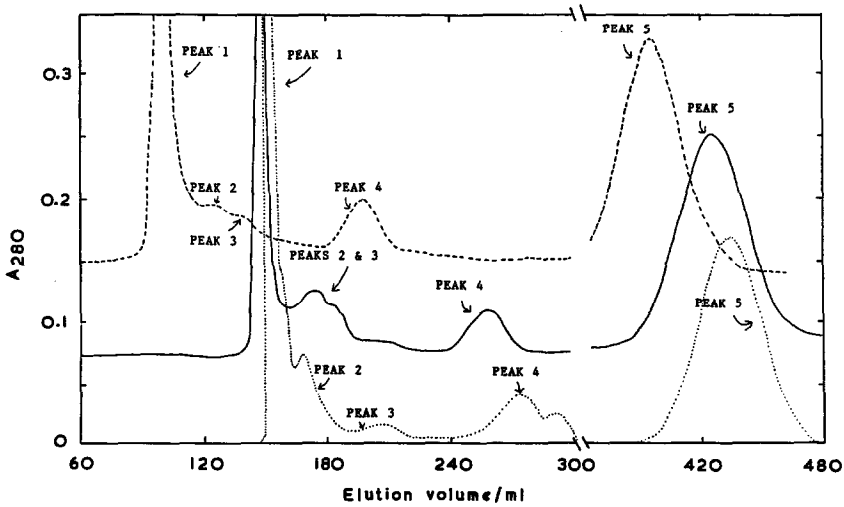

Fig. 2. Counter-current chromatogram of 1% (w/v) infusion of Lyons Red Label tea bag tea. Flow-rate: 3 ml/min. Phase compositions were as follows:

	Mobile (upper phase)	*Stationary (lower phase)*	*System composition: butanol–ethyl acetate–water (v/v/v)*
---------	102 ml	208 ml	30:70:100
———	142 ml	168 ml	50:50:100
·····	150 ml	160 ml	80:20:100

stationary phase on the performance of the CCC experiment and the calculated K values for caffeine are summarised in Table I. The tendency for the value of partition coefficient to decrease with increasing butanol content, and consequent increase of retention volume is consistent with the qualitative prediction of the polarity of the solvent mixtures but the effect is seen up to compositions, butanol–ethyl acetate–water (40:60:100, v/v/v). At higher butanol contents the K value is approximately constant. However, the mobile phase volume continues to increase; it is seen that an approximately 1:2 ratio of mobile:stationary phase for water–ethyl acetate becomes 1.4:1 for water–butanol. Chromatograms obtained at butanol–ethyl acetate–water (30:70:100, v/v/v), (50:50:100, v/v/v) and (80:20:100, v/v/v) are illustrated in Fig. 2 from which it is clear that increase in butanol content improves the resolution of the separation. The identification of the relative positions of specific peaks from one chromatogram to another shown in Fig. 2 was made with the help of chromatograms run at intermediate solvent compositions.

All phase volumes reported here correspond to those measured as the amount of stationary phase displaced by the mobile phase when the system apparently reaches equilibrium at the start of a chromatographic run. The solvent combinations used showed no obvious problems with leakage of stationary phase causing interference to the spectrophotometric measurements, but it was clear that the volume of stationary phase decreased with time during a run. Practical experience has shown that a second chromatogram obtained from the same column filling appears different from the first. It is thought that this may be due to changes in ambient temperature causing desaturation of the mobile phase with respect to stationary phase and dissolution of the latter.

It is well known that polyphenolic components of black tea infusions and particularly thearubigins form complexes with caffeine[14–16]. This binding is known to affect the distribution of caffeine between organic solvents (*e.g.* chloroform) and water and it is possible that complex formation may also affect the CCC running characteristics of the polyphenols themselves. A chromatogram of decaffeinated Lyons Red Label tea bag tea is shown in Fig. 3 which, on comparison with those in Fig. 2, shows the importance of caffeine and the potential consequences of reversible interactions on CCC data.

Identification of the components of individual fractions was carried out by means of two-dimensional TLC[4]. Peak 1 (Fig. 3) gave rise to a streak (R_F 0–0.2) in the butanol–acetic acid–water solvent and showed no mobility in acetic acid. This behaviour is characteristic of S_{II} thearubigins and no components which showed mobility in both solvents could be detected. When the stationary phase was evaporated and the product subjected to TLC, this also was mobile only in the first solvent (R_F 0.6–0.95) indicative of S_I or S_{Ia} thearubigins. Similarly, no components which ran in both solvents could be detected. It is suggested, therefore, that these two fractions consist of thearubigins. Other chromatographic peaks each contained several components which showed mobility in both solvents.

In order to explore the homogeneity of the S_I and S_{Ia} fraction it was decided to analyse the infusion by CCC using the water-rich phase as the stationary phase. Thus components which show a strong bias for the organic phase will be eluted early and it was hoped to see the S_I and S_{Ia} thearubigins close to the solvent front. Fig. 4 shows such a chromatogram with water-saturated organic phase as the mobile phase. Peak 1 was

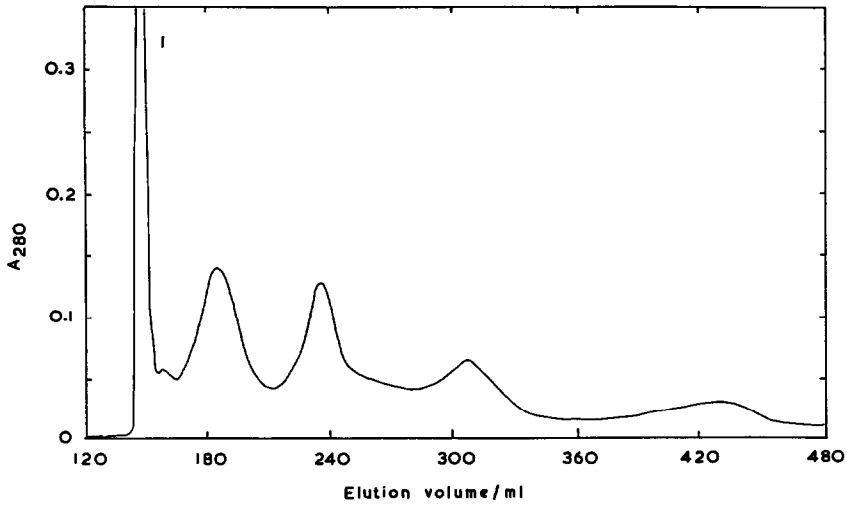

Fig. 3. Counter-current chromatogram of decaffeinated 3% (w/v) infusion of Lyons Red Label tea bag tea. Solvent system: butanol–ethyl acetate–water (50:50:100, v/v/v). Stationary phase: lower phase, 170 ml. Mobile phase: upper phase, 140 ml. Flow-rate: 3 ml/min.

identified as the thearubigin component composed of both types of S_I species whilst peak 2 was due to caffeine. It is significant that in this mode the mobile phase volume is relatively small (90 ml) which implies that components are eluted quickly and that the solvent requirement is small. It is seen that S_I and S_{Ia} thearubigins, or S_{II} thearubigins may be prepared by a single CCC run directly from black tea infusions.

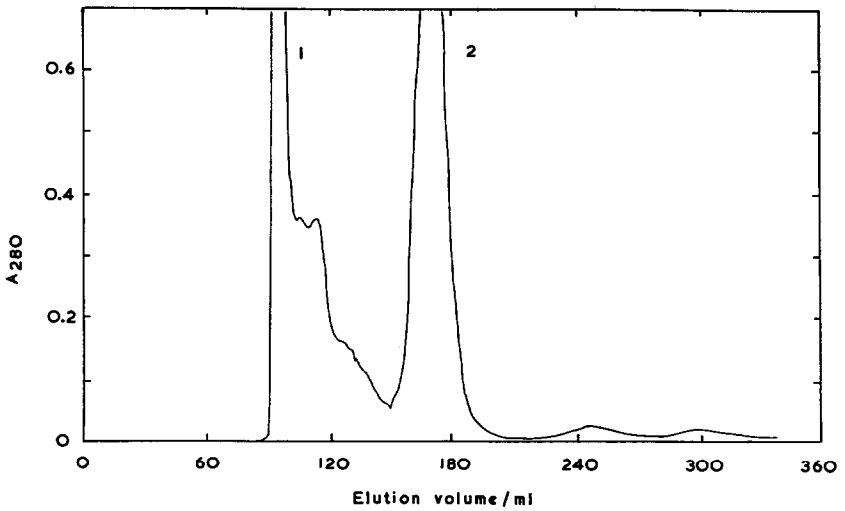

Fig. 4. Counter-current chromatogram of a 1% (w/v) infusion of Lyons Red Label tea bag tea. Solvent system: butanol–ethyl acetate–water (50:50:100, v/v/v). Stationary phase: upper phase, 220 ml. Mobile phase: lower phase, 90 ml. Flow-rate: 3 ml/min.

REFERENCES

1 E. A. H. Roberts, *J. Sci. Food Agric.*, 9 (1958) 212.
2 E. A. H. Roberts, in T. A. Geissman (Editor), *The Chemistry of Flavanoid Compounds*, Pergamon Press, Oxford, 1962, p. 468.
3 W. H. Stahl, *Adv. Food Res.*, 11 (1962) 201.
4 E. A. H. Roberts, R. A. Cartwright and M. Oldschool, *J. Sci. Food Agric.*, 8 (1957) 72.
5 A. G. Brown, W. B. Eyton, A. Holmes and W. D. Ollis, *Phytochemistry*, 8 (1969) 2333.
6 L. Vuataz and H. Brandenberger, *J. Chromatogr.*, 5 (1961) 17.
7 S. I. Ratnaike, *Ph.D. Thesis*, University of Leeds, Leeds, 1980.
8 A. Robertson and D. S. Bendall, *Phytochemistry*, 22 (1983) 883.
9 D. J. Cattell, *Ph.D. Thesis*, University of Leeds, Leeds, 1972.
10 I. A. Sutherland, D. Heywood Waddington and Y. Ito, *J. Chromatogr.*, 384 (1987) 197.
11 Y. Ito, *Trends Anal. Chem.*, 5 (1986) 142.
12 T. Okuda, T. Yoshida, T. Hatano, K. Yazaki, R. Kira and Y. Ikeda, *J. Chromatogr.*, 362 (1986) 375.
13 I. Sutherland, *Lab. Pract.*, 36 (1987) 37.
14 E. A. H. Roberts, *J. Sci. Food Agric.*, 14 (1963) 700.
15 R. F. Smith, *J. Sci. Food Agric.*, 19 (1968) 530.
16 R. L. Wickremasinghe and K. P. Perera, *Tea Quart.*, 37 (1966) 131.