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Separation of tea polyphenols using micellar electrokinetic chromatography with diode array detection

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Abstract

Micellar electrokinetic capillary chromatography has been investigated for its potential in the analysis of polyphenols in tea. Conditions have been optimised by systematically examining the effect of inorganic buffer, micelle, borate and organic solvent concentration. These conditions have then been used to analyse a range of tea types, including an instant green, Darjeeling and black Assam tea. The more fully fermented Assam proved too complex to analyse per se and was split into three fractions prior to electrophoresis. Most of the major classes of tea components were observed and identified using a diode array detector, but the theaflavins were either adsorbed or degraded by the capillary wall. © 1998 Elsevier Science B.V.

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1. Introduction

The water-soluble material of green tea (*Camellia sinensis*) consists primarily of a class of polyphenol compounds termed flavan-3-ols (catechins), together with flavonol glycosides and other discrete species such as amino acids, simple carbohydrates and caffeine. During tea processing the simple catechins of green tea undergo enzymic oxidation to produce a range of polyphenol dimers and polymers, which include theaflavins, and a highly complex class referred to as thearubigens. Thearubigens make up some 60% of the total water-soluble constituents of black tea and, together with the theaflavins, are responsible for the major organoleptic properties of colour, brightness, and astringency. Whilst the catechins are well characterised, and the theaflavins have been isolated and their structures elucidated, the complex brown pigments of the thearubigins are still poorly understood.

Polyphenols are known to have numerous physiological effects and there are numerous reports which link them with significant health benefits. Their quinone forms are well-known free radical scavengers and have been shown, both by in vitro and in vivo studies, to significantly diminish *N*-nitrosation [1]. These effects have been linked with anti-carcinogenic benefits. Their antioxidant properties have also been studied [2] and correlated to beneficial effects on coronary heart disease [3].

Traditionally, the analysis of tea infusions has been undertaken by high-performance liquid chromatography (HPLC) and, in general, has employed gradient elution to examine the whole range of

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species present. There has also been more detailed examinations of individual fractions, e.g. the flavonol glycosides [4] and higher-molecular-mass polyphenols following dialysis [5]. A series of studies has been conducted on the thearubigins by Bailey and co-workers [6–10], who distinguished different types of brown pigments according to their behaviour in HPLC systems. Whilst the broad range of species within this class could not be separated by HPLC, the general spectral characteristics of different groups of thearubigins were identified.

Several workers have reported on the analysis of polyphenols by capillary electrophoresis (CE). Most of these were separations of flavonoids (flavonols and their glycosides) in different plants or medical preparations. These were either conducted with borate buffers using solvents [11,12] or micellar systems using anionic surfactants [13–15] and organic solvents [16]. A range of polyphenols have been analysed in wine by CE [17], and methylphenols in plant material by micellar electrokinetic capillary chromatography (MEKC) using a cationic surfactant with different solvent systems [18].

This study examines the potential of MEKC for the analysis of the major chemical species in green and black teas. Together with the recently reported work of Horie et al. [19], which describes methodology for the analysis of catechins and theanine, it represents the first application of CE to tea analysis.

2. Experimental

2.1. Reagents

All reagents were of analytical-reagent grade. Sodium tetraborate and sodium hydroxide (BDH, Lutterworth, UK), sodium dihydrogenorthophosphate and orthophosphoric acid (Sherman Chemicals, Sandy, UK), together with sodium dodecyl sulphate (SDS; Sigma, Poole, UK), were all used without further purification. The water was Milli-Q (Millipore, Watford, UK) with resistivity >18 MΩ.

Tea standards were obtained from a variety of sources. Gallic acid and quercetin dihydrate were purchased from Sigma, quercetin-3-glucoside from Apin (Abingdon, UK), (+)-catechin monohydrate and epicatechin from Aldrich (Gillingham, UK). The remaining standards (epigallocatechin gallate, epicatechin gallate, epigallocatechin, theaflavin, theaflavin-3-monogallate, theaflavin-3′-monogallate and theaflavin-3-3′-digallate) were all isolated and purified in the laboratory.

2.2. Equipment

The electrophoresis system was a ^{3D}CE (Hewlett-Packard, Waldbronn, Germany) fitted with a 50-μm I.D. fused-silica capillary incorporating a ‘bubble cell’ [20] (Hewlett-Packard). Buffer pH was adjusted with an EA940 Ionalyser (Orion, Forrest Row, UK), and buffers were filtered through a 0.2-μm Minisart membrane (Sartorius, Göttingen, Germany)

2.3. Preparation of standards

Individual standards were diluted in suitable solvents to provide stock solutions of 1000 μg/ml, aliquots of which were stored frozen at −20°C. Gallic acid, catechins and theaflavin standards were prepared in acetonitrile–water (10:90). For the theaflavin standards, the solution also contained 50 μg/ml each of EDTA and ascorbic acid. Flavonol and flavonol glycosides were prepared in methanol–water (70:30). Prior to electrophoresis, aliquots were thawed and diluted in acetonitrile–water (5:95).

2.4. Preparation of tea and tea fractions

Instant green tea was prepared by dissolving 0.2 g of the powder in 100 ml of boiling water. After 5 min the solution was cooled and 5 ml of acetonitrile added. Aliquots were frozen at −20°C for up to 2 weeks.

Black Assam and Darjeeling teas were prepared from unground loose leaf. Four grams of leaf were weighed into a thermos flask, 500 ml of boiling water was added and the leaves allowed to infuse for 5 min with regular shaking every minute. After 5 min the infusion was filtered through a Büchner sintered-glass filter funnel. One hundred ml of filtrate was collected, 5 ml of acetonitrile added and then placed in cold water to cool. Infusion time was not

allowed to exceed 5 min as the theaflavins are known to degrade during heating [21]. Acetonitrile helps to keep polyphenols in solution, and prevent 'cream' formation. Aliquots of the infusion were frozen as for green tea.

Three separate fractions were isolated from black tea. Using chloroform-extracted leaf (decaffeinated), tea infusions were prepared as previously described and then extracted with ethyl acetate to provide fraction 1. The remaining water solubles were freeze dried and then extracted with methanol to provide fraction 2, leaving the residual material as fraction 3. All three fractions were either freeze dried or evaporated to provide a dry powder that was stored at −20°C. This was subsequently dissolved in acetonitrile–water (5:95) at a level of 2 mg/ml to provide a solution that was directly injected into the capillary.

2.5. Capillary electrophoresis

Running buffers were prepared from solid salts and acetonitrile, diluted in ultra-pure water and adjusted to the required pH with phosphoric acid. They were stored at ambient temperature for up to 3 days and filtered through a 0.2-μm syringe filter prior to use.

New capillaries were conditioned by flushing for 30 min with 1 *M* sodium hydroxide, 10 min with 0.1 *M* sodium hydroxide, 10 min with water, and finally 1 h with the running buffer. Existing capillaries were stored in water when not in use to avoid buffer crystallisation.

Standards or samples were injected into the capillary using a pressure of 50 mbar for 15 s. They were not filtered as it was found that individual polyphenols were retained by the filter. Electrophoresis was carried out in the positive voltage mode at 30 kV, and detection was typically conducted at a wavelength of 278 nm with spectra recorded over the range 190–600 nm. The temperature of the capillary was maintained at 25°C. The capillary was re-equilibrated between runs using a 10-min buffer flush.

Tea and tea fractions were analysed using an optimised buffer consisting of 50 m*M* monosodium dihydrogenphosphate, 50 m*M* sodium tetraborate, 20 m*M* SDS and 10% acetonitrile.

3. Results and discussion

3.1. Optimisation of the buffer

The basic buffer components employed in this study included sodium phosphate and an anionic detergent (SDS). When used above the critical micelle concentration (CMC), the surfactant provides a pseudo-stationary phase into which the analytes may partition [22,23]. Since polyphenols become negatively charged and reactive at basic pH (>7.5), and the electroosmotic flow is too low below pH 5, all work was conducted at pH 6.

Raising the phosphate buffer concentration led to improved peak resolution as a consequence of a reduced electroosmotic flow (EOF), but necessitated longer column equilibration periods, and at the highest levels led to unacceptably long analysis times. A concentration of 50 m*M* was chosen as a suitable compromise. Many of the soluble species of tea are polyhydroxylated and borate was therefore incorporated into the buffer as an additional means of achieving separation. Borate complexes vicinal hydroxyl groups on the polyphenol ring resulting in one negative charge per complex. The increased size of the complex should also facilitate its partitioning into the micellar phase, although this will be opposed to some degree by electrostatic repulsion. Introducing borate into the buffer resulted in longer migration times for most species and, up to a concentration of 50 m*M*, led to improved peak resolution. When black tea was analysed, the dominant feature was a broad unresolved band underlying most of the electropherogram. This is also observed during HPLC [8,9] analysis of black tea, and is ascribed to the polymeric thearubigen class. This class was very sensitive to the borate concentration, indicating the presence of individual species that bear both different numbers and positions of hydroxyl groups. Although the band could be shifted by raising the borate concentration, no individual species could be resolved.

The SDS concentration was particularly important for separating the similar structures of the early migrating xanthines; caffeine and theobromine. In the absence of a micellar phase they migrated together, but were easily resolved at 20 m*M* SDS. Similarly, the two pairs of catechins (Fig. 1); epi-

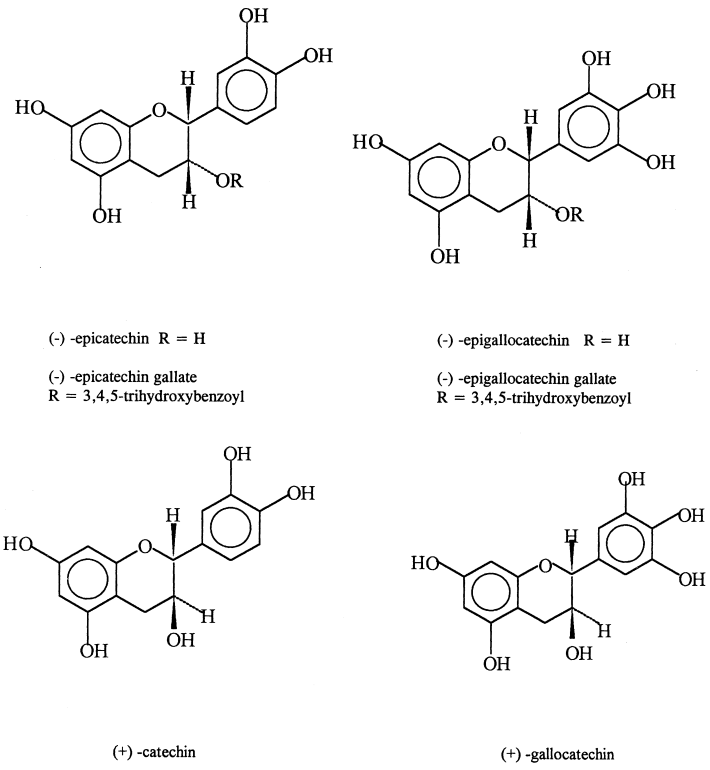

Fig. 1. Structures of the catechins.

gallocatechin (EGC)–epicatechin (EC) and epigallocatechin gallate (EGCG)–epicatechin gallate (ECG) could not be separated in the absence SDS, but were easily resolved at 10 and 20 m*M*. The order of migration of these two pairs of catechins favours EGC and EC since they lack the additional gallate ester group which provides further sites for borate complexation.

Acetonitrile was added to the running buffer in an attempt to manipulate the partitioning ratio for the more hydrophobic tea species, analogous to its use in the analysis of polyphenols by reversed-phase HPLC [24,25]. Increasing the level of acetonitrile caused a gradual reduction in the EOF, and at higher levels resulted in a general loss of peak separation due to disruption of the micellar phase. One striking effect of acetonitrile is on the shape of the caffeine peak (Fig. 2). This is very broad in a tea infusion, but much sharper in the presence of acetonitrile. As caffeine standards yield good peak shape at high concentrations, this effect is not due to overloading. Polyphenols are known to complex with caffeine via their gallate groups [26]. This association is probably the cause of the poor peak shape, and the effect of acetonitrile is to disrupt the complex by solubilising the polyphenols. Consequently, 10% acetonitrile was retained in the buffer for all further work.

3.2. Analysis of tea and tea fractions

Prior to the analysis of the various tea types, individual tea standards were analysed using the optimised buffer conditions, their spectra recorded over the range 190–600 nm and then archived in a spectral library for future reference. During this exercise no peaks were detected for either the theaflavins or gallic acid, and subsequently they were flushed through the capillary under pressure. Gallic acid was then observed, but the theaflavins yielded peaks which exhibited low absorption and lacked the theaflavin spectral characteristics. It was concluded that the theaflavins probably interacted with the

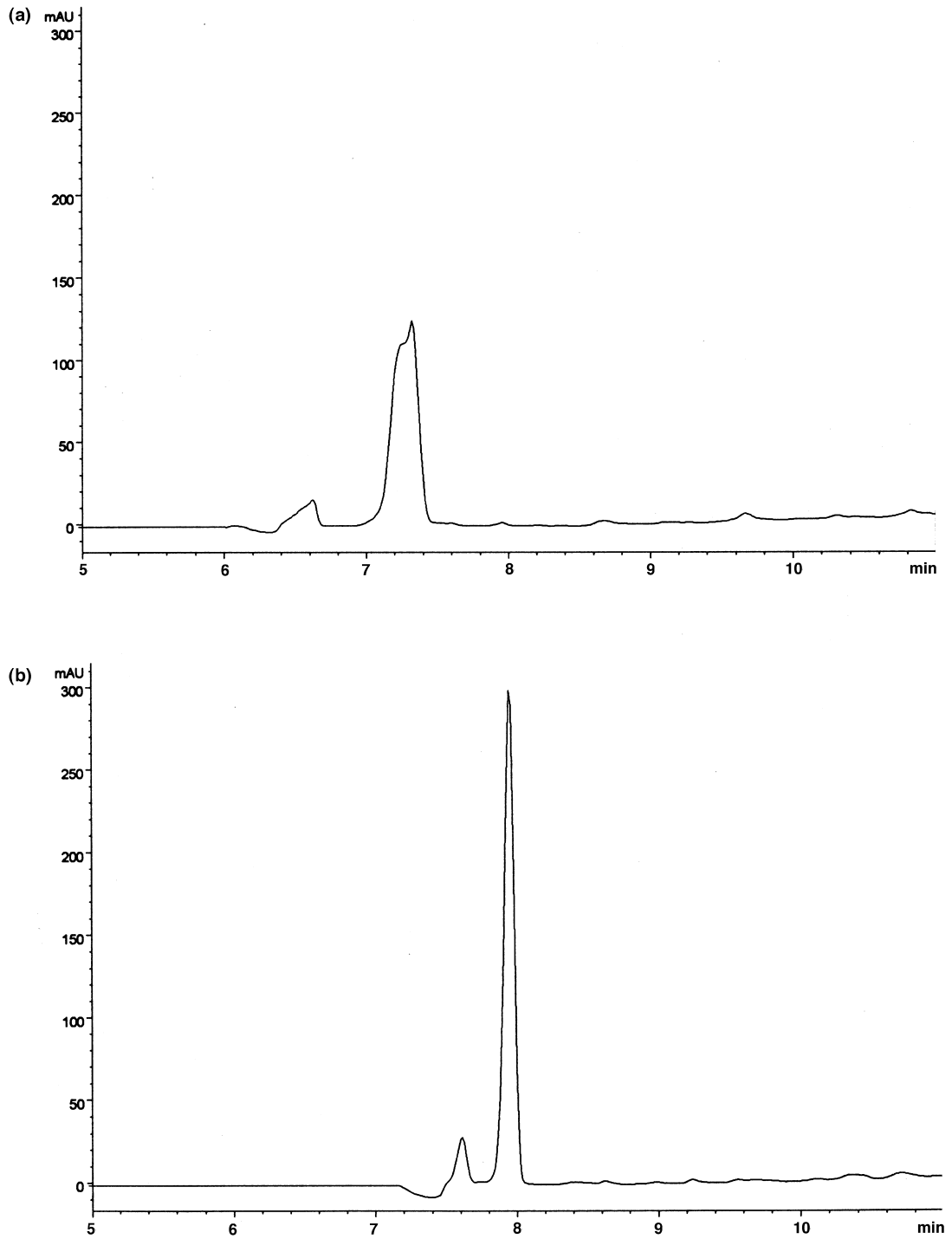

Fig. 2. Effect of acetonitrile on caffeine peak shape. (a) Caffeine with no acetonitrile in running buffer. (b) Caffeine with 10% acetonitrile in running buffer. Conditions: capillary, 64.5 cm (56 cm effective length)×50 μm I.D; running buffer, 20 m*M* SDS, 50 m*M* phosphate, 50 m*M* sodium tetraborate, pH 6.0. Voltage, 30 kV; temperature, 25°C; detection, 278 nm.

capillary wall but that gallic acid was not observed during normal electrophoresis simply because its high electrophoretic mobility in the opposite direction to the electroosmotic flow prevented it from passing the detector window.

The spectral library was used in conjunction with migration times and published data to identify components in individual tea types. The library was particularly effective for green tea where match factors were always higher than 998. For black tea, discrete peaks co-migrating with the underlying thearubigens rarely gave a match factor above 997 due to the contributing spectra of the latter.

3.2.1. Green tea

The major peaks in green tea (Fig. 3) correspond to caffeine and the galloylated catechins, EGCG and ECG. The simple catechins, EGC and EC, are present at lower levels but are still major components. The first peak in the electropherogram was identified as theobromine from the observation that its spectrum was very similar to that of caffeine, and the fact that it was only separated from it with 20 m*M* SDS, implying a similar structure.

Peak 4 appears to be a small amount of (+)-catechin; however, its spectrum is influenced by the next peak from which it is only partially resolved. Several peaks were ascribed to flavonoids due to their spectral resemblance to quercitin and quercitin-3-glucoside. These two specific compounds were not, however, identified in the electropherogram.

Most peaks in the electropherogram are observed within 18 min, however two late migrating peaks are found at 33 and 35.5 min. From their spectra, these appear to be pure, corresponding to single compounds, and both are negatively charged since they migrate against the electroosmotic flow. The first peak could not be identified, but the last had a spectrum similar to that for 5-*O*-caffeoylquinic acid as reported by Bailey et al. [10]. This is consistent with its negative charge, and it was therefore classified as a chlorogenic acid.

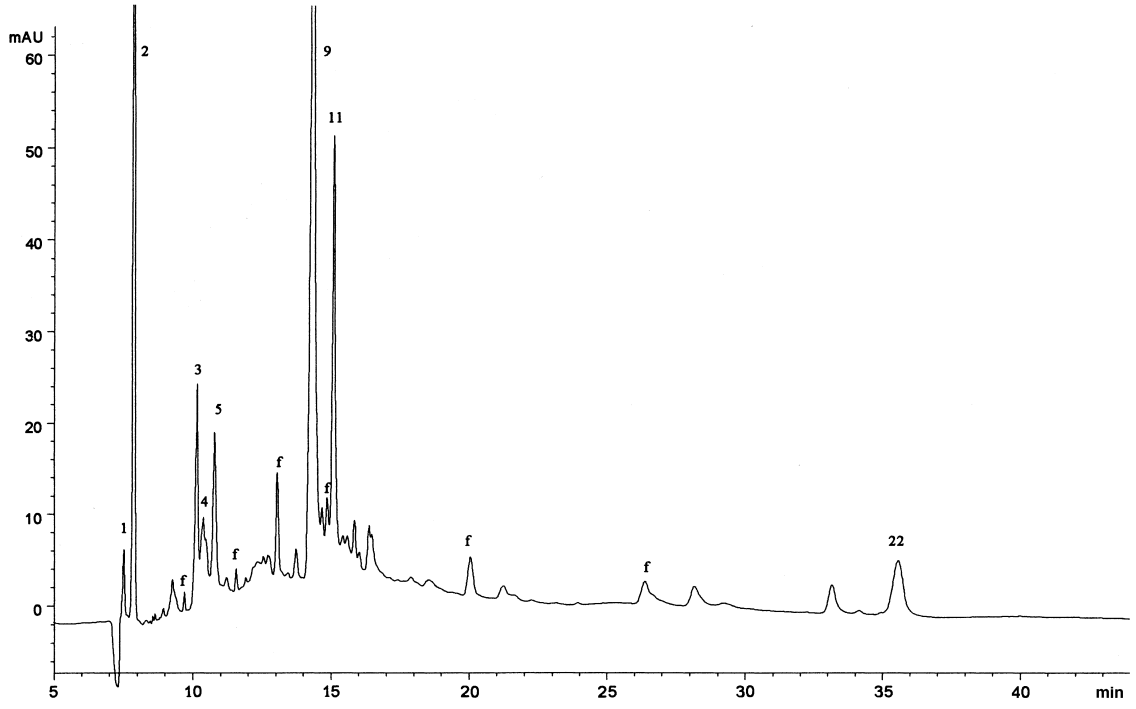

Fig. 3. Analysis of green tea. Conditions: capillary, 64.5 cm (56 cm effective length)×50 μm I.D. Running buffer, 20 m*M* SDS, 50 m*M* phosphate, 50 m*M* sodium tetraborate, 10% acetonitrile, pH 6.0. Voltage, 30 kV; temperature, 25°C; detection, 278 nm. Peaks: 1, theobromine; 2, caffeine; 3, EGC; 4, catechin; 5, EC; 9, EGCG; 11, ECG; 22, chlorogenic acid; f, diverse flavonoids and their glycosides.

3.2.2. Darjeeling tea

During the manufacture of black tea from green via the process of fermentation, the catechins are oxidised by the enzyme, polyphenoloxidase, ultimately producing the polymerised brown pigments of the thearubigen class. Darjeeling is a lightly fermented black tea, and can be expected to exhibit low, but measurable levels of catechins. From Fig. 4 it can be seen that the major catechins, the galloylated EGCG and ECG, still dominate the electropherogram, but that the simpler catechins are minor components. A very small peak was found for EC but EGC was not detected.

Diverse flavonoids were detected throughout the early part of the electropherogram, although none migrated as late as those in green tea. Most appeared before EGCG, indicating a less-efficient partitioning into the micellar phase, and a relatively slow electrophoretic mobility. A compound with similar spectral characteristics to quercitin over the wavelength range 250–450 nm was identified between EGCG and ECG. Its spectrum was attributed to a kaempferol derivative as reported by Bailey et al. [10].

A characteristic feature of Darjeeling tea is the appearance of new peaks migrating after ECG. Peaks 16 and 17 are not observed in green tea but are significant components of Darjeeling. Their spectral characteristics are very similar to that of EGCG, indeed the match factor is even slightly over 990. Spectral scanning shows peak 17 to be relatively pure, but both the shape and spectrum of peak 16 indicates the presence of two or three compounds. Since the spectra and polarity of these compounds are similar to the catechins, but their migration times are longer, they were tentatively identified as bisflavanols. Bisflavanols are formed from two catechin molecules via a carbon–carbon link at position 6′ (Fig. 5).

Peak 18 in the electropherogram appears to be a brown pigment. Its UV spectrum shows a maximum around 270 nm with a shoulder at 290 nm, and a second maximum at 400 nm which then tails into the

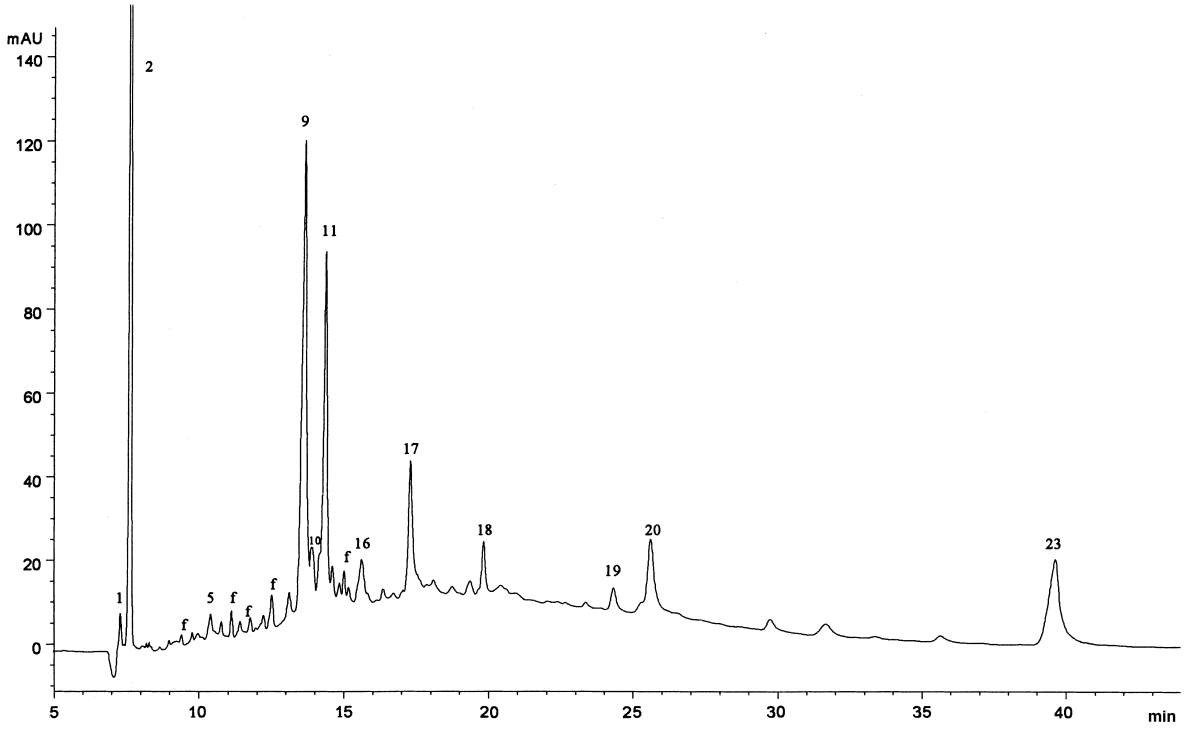

Fig. 4. Analysis of Darjeeling tea. Conditions as for Fig. 2. Peaks: 1, theobromine; 2, caffeine; 5, EC; 9, EGCG; 10, kaempferol derivative; 11, ECG; 16,17, bisflavanols; 18, Group II.3 (type 1) brown pigment; 19,20, Group II.4 (type 2) brown pigment; 23, 3-galloylquinic acid; f, diverse flavonoids and their glycosides.

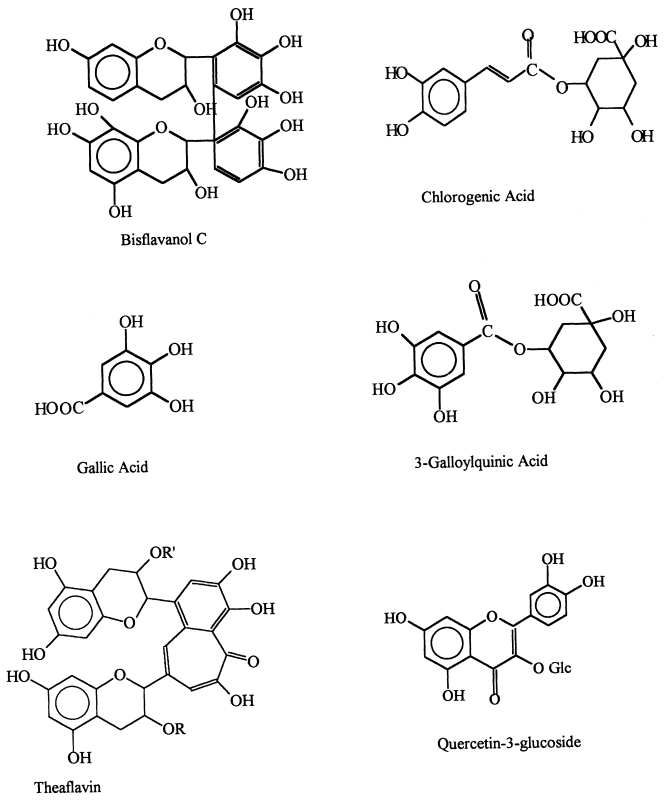

Fig. 5. Structures of tea components.

visible. This could be a Group II.3 (type I) brown pigment as defined by Bailey and co-workers [8], although it should be noted that the absorption at 270 nm is broader than reported. Peaks 19 and 20 exhibit spectra unlike those of any observed to date. They appear to be single compounds with absorption spectra that tail into the visible and could be the Group II.4 (type II) brown pigment reported by Bailey and co-workers [9]. Finally, peak 23 migrating at 39.5 min, exhibits a maxima at 300 nm and a spectra very similar to that of gallic acid, a component which, as previously discussed, is not observed under these conditions due to its high electrophoretic mobility in the opposite direction to the EOF. It was tentatively identified as 3-galloylquinic acid [27] (Fig. 5). This molecule can be considered to be formed from quinic acid esterified through the carboxylate function of gallic acid. It is negatively charged, and with a higher molecular mass than gallic acid can be expected to migrate more slowly than the latter, which is consistent with its position in the electropherogram.

3.2.3. Black tea and fractions

An electropherogram of a whole black tea infusion is shown in Fig. 6 and is characterised by the size of the underlying thearubigen band. A number of discrete species are relatively easy to identify; caffeine and theobromine migrating between 7 and 8 min, EGCG at 13 min, ECG at 14.5 min and chlorogenic acid at 33.5 min. In general, though, identification of individual compounds is complicated by the fact that many are only partially resolved and the underlying thearubigens contribute to the spectra of most peaks. In an effort to improve this situation, solvent extraction was carried out on the infusion to provide three separate fractions. These contained different classes of tea compounds

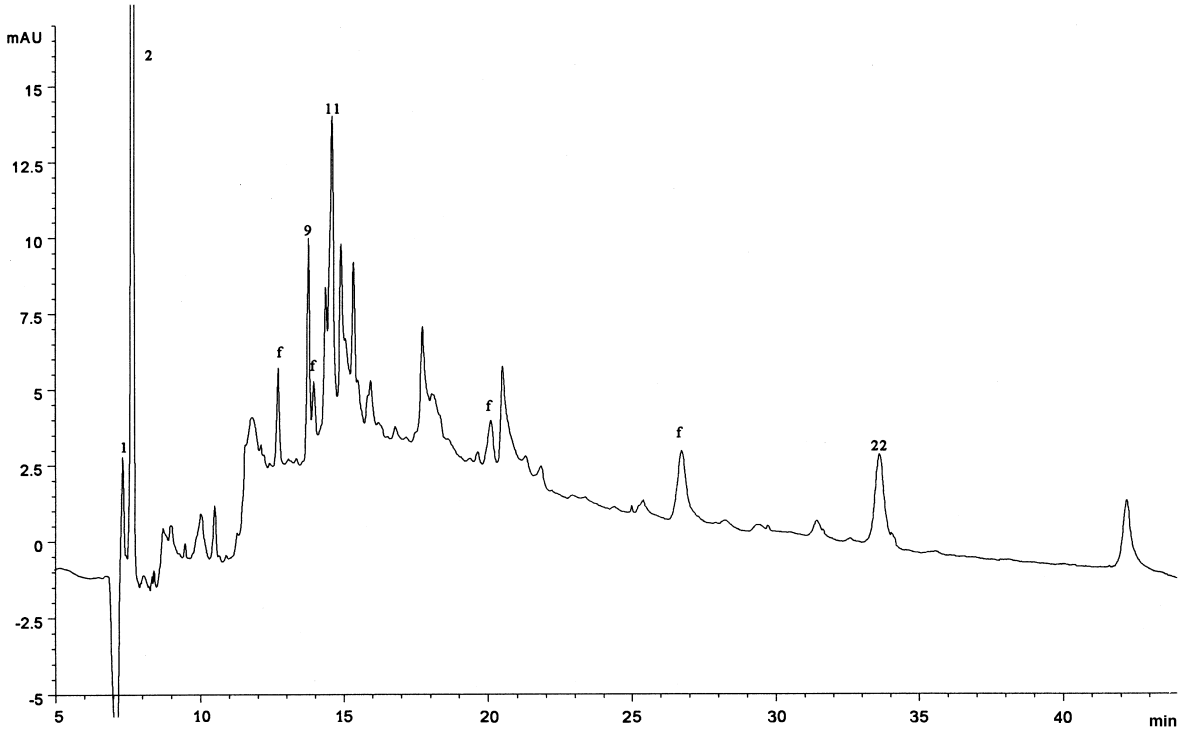

Fig. 6. Analysis of black tea. Conditions as for Fig. 2. Peaks: 1, theobromine; 2, caffeine; 9, EGCG; 11, ECG; 22, chlorogenic acid.

which could then be analysed separately to permit simplification of this highly complex infusion.

3.2.3.1. Ethyl acetate fraction

An electropherogram of the ethyl acetate fraction of black tea is presented in Fig. 7. Ethyl acetate selectively removes and concentrates the more apolar compounds from tea such as the catechins and theaflavins. As previously noted, however, the latter are not observed during analysis due possibly to an interaction between the silanol groups of the capillary wall and the benzotropolone ring.

EGCG and ECG were found at high levels, their identification simplified by the removal from the host of small peaks migrating at approximately the same time in the whole tea electropherogram. The concentration of the catechin class was such that the simpler catechins EC, EGC and even (+)-catechin, not observed in the whole tea infusion, were clearly identified showing that they are not completely degraded during processing but rather that their level is simply too low to permit detection in the whole black tea liquor. A major feature of this fraction is the greatly reduced size of the underlying thearubigen band in comparison to the whole tea. Following EGCG and ECG, the next largest peak in the electropherogram is peak 17. In common with the catechins, this species has also undergone considerable concentration during extraction. Its migration time and spectrum identify it as a bisflavanol as previously identified in Darjeeling tea.

A further significant peak appears at 23.5 min. Its spectral characteristics point to a flavonoid. It should be noted that whilst most flavonoids partition into methanol some will partition easily into ethyl acetate [28].

3.2.3.2. Methanol fraction and remaining water

The electropherograms of the methanolic fraction and the final fraction containing the water solubles, are shown in Figs. 8 and 9, respectively. Apart from two significant peaks in the water fraction (A and B),

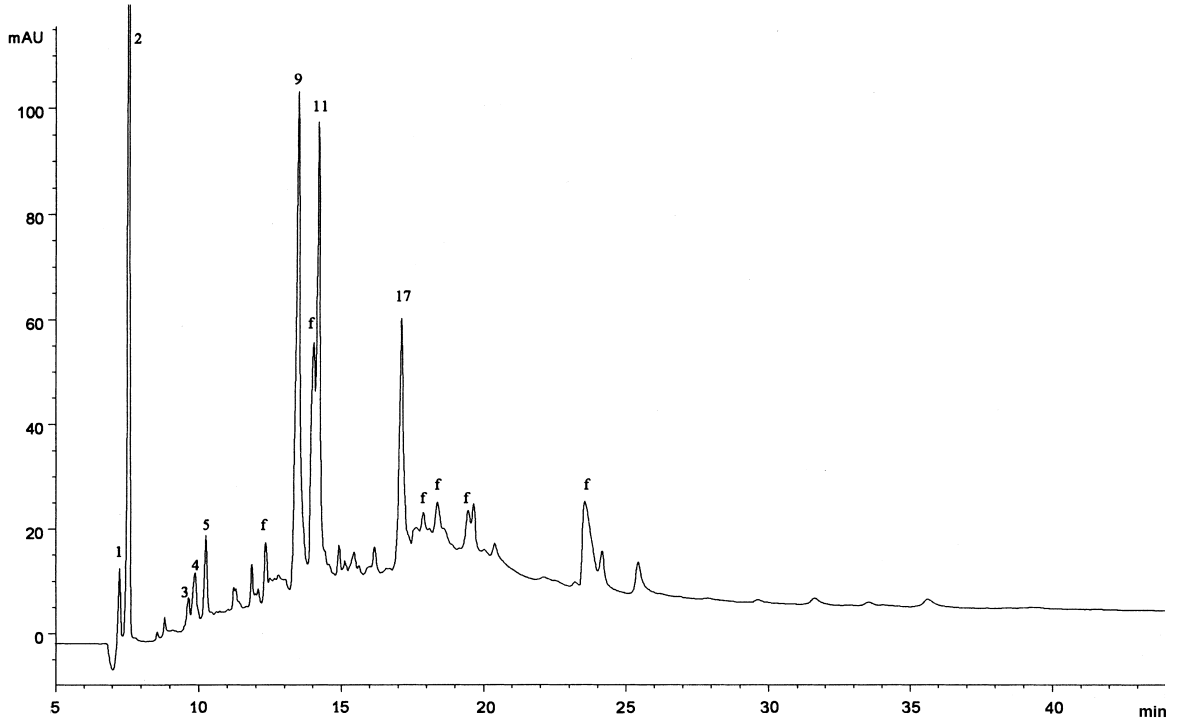

Fig. 7. Analysis of ethyl acetate fraction of black tea. Conditions as for Fig. 2. Peaks: 1, theobromine; 2, caffeine. 3, EGC; 4, (+)-catechin; 5, EC; 9, EGCG; 11, ECG; 17, bisflavanol; f, flavonoids.

the same discrete species were identified in both. It should be noted that the later peaks in the water fraction migrate significantly faster than those in the methanol, due possibly to the high level of thearubigens.

The main difference between the two fractions relates to the size and migration time window for the underlying thearubigens. In the methanol extract it rises sharply after 10 min, reaches a maxima at 14 min and then tails off after about 30 min. In the water fraction it has a much higher absorbance, a later maximum, and a long tail which extends past 35 min. Since the thearubigen band from the water fraction is shifted to longer migration times, it may carry a higher negative charge which would be consistent with its lower solubility in methanol.

Residual levels of caffeine and theobromine are found in both fractions, probably as a consequence of incomplete extraction coupled with caffeine–polyphenol complexation. The xanthines are followed by a number of small peaks migrating between 7 and 11 min, all with spectra characteristic of flavonoids. Peak 7 was tentatively identified as quercitin-3-glucoside.

There are two categories of compounds within the methanol and water fractions which resemble brown pigments. The first comprises peaks 6 and 8, and exhibits a major absorption band at 210 nm with a long tail into the visible. These compounds, lacking any major spectral features, resemble the Group I brown pigments described by Bailey and co-workers [9]. The second category comprises peaks A and B, the two species exclusive to the water fraction. The spectra of these are also similar to Bailey's Group I brown pigments, but with greater absorption at 400 nm.

A further set of significant peaks are observed to migrate around 15 min (peaks 12–15). They show high absorption at 300 nm, but could not be identified from the literature.

Peaks 21 and 22 have spectra similar to that for 5-*O*-caffeoylquinic acid as reported by Bailey et al. [10] and were therefore classified as chlorogenic acids. Peak 12 was again tentatively identified as

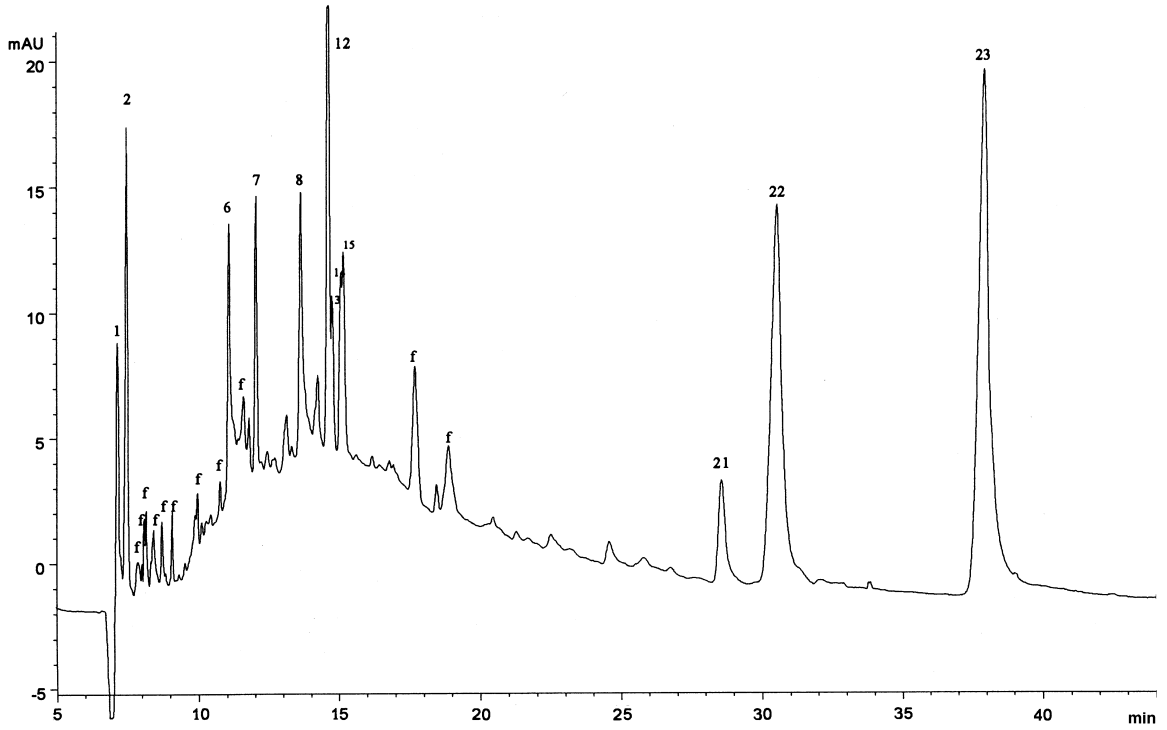

Fig. 8. Analysis of methanol fraction from black tea. Conditions as for Fig. 2. Peaks: 1, theobromine; 2, caffeine; 6,8, Group I brown pigments; 7, quercetin-3-glucoside; 21,22, chlorogenic acids; 23, 3-galloylquinic acid; f, diverse flavonoids.

3-galloylquinic acid, as previously observed in Darjeeling tea.

4. Conclusions

This study of the analysis of tea polyphenols has shown the potential of CE for the separation of individual classes and species of soluble tea components. An optimised buffer comprising 20 m*M* SDS, 50 m*M* phosphate, 50 m*M* sodium tetraborate and 10% acetonitrile at pH 6 has been developed and successfully applied to the analysis of a range of tea types. The dominant separation mechanism relies upon the partitioning of hydrophobic species between the aqueous and pseudo-stationary phase, but other interactions and mechanisms such as borate complexation, and exclusion of small anions from the micelle, provide important variables by which to manipulate the separation.

In many respects, MEKC can be considered complementary to reversed-phase HPLC for tea separations. It is capable of separating the xanthine, catechin and flavonol classes, but in common with the latter, cannot resolve the highly complex thearubigens. Low-molecular-mass acidic species such as chlorogenic acid and 3-galloylquinic acid migrate late in the electropherogram and are more easily separated than by HPLC, where they elute in a crowded section of the chromatogram close to the void volume. One particular drawback of this methodology is the adsorption of the theaflavins by the capillary wall. It is possible that metal ions adsorbed on the capillary silanol groups cause a catalysed degradation of this class, or alternatively that the aromatic benzotropolone ring is strongly retained. However, regardless of the mechanism, this class cannot be analysed by this approach and further work needs to be undertaken to address this problem by, e.g., employing physical or dynamic coating of the inner capillary wall.

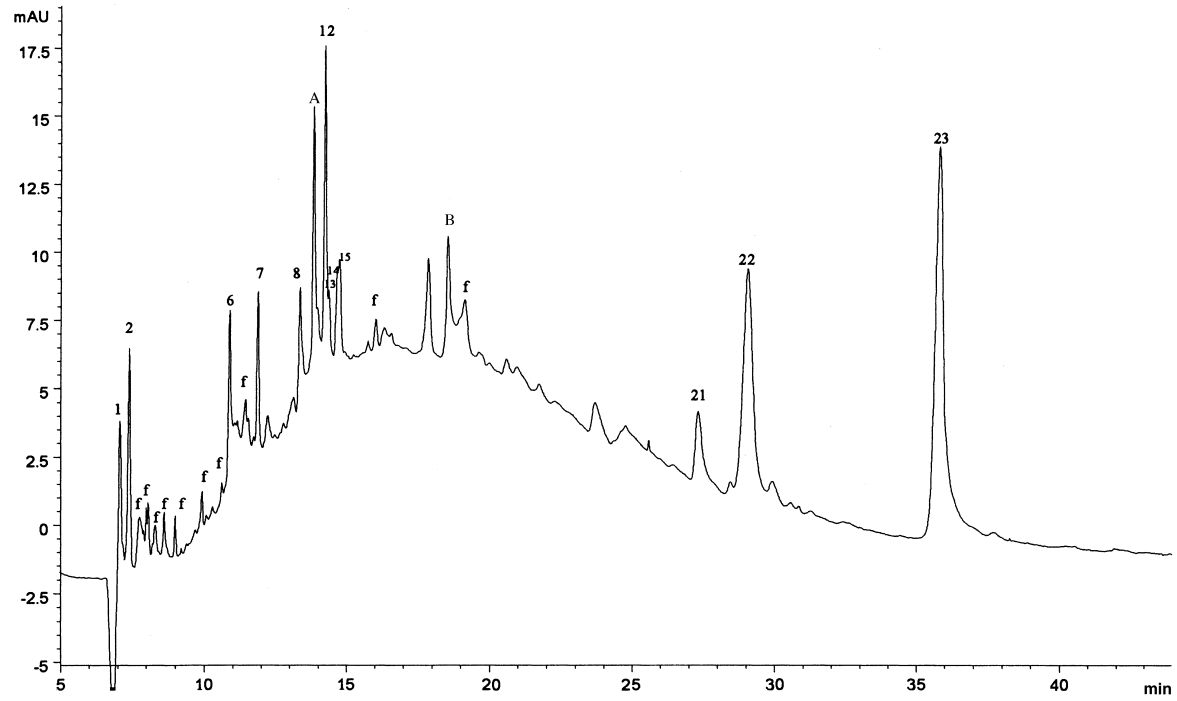

Fig. 9. Analysis of water fraction from black tea. Conditions as for Fig. 2. Peaks: 1, theobromine; 2, caffeine; 6,8, Group I brown pigments; 7, quercetin-3-glucoside; 21,22, chlorogenic acids; 23, 3-galloylquinic acid; f, diverse flavonoids. A,B, brown pigments.

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