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Isolation and high-performance liquid chromatographic analysis of thearubigin fractions from black tea

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ABSTRACT

Fractions consisting of black tea pigments were isolated and analysed by reversed-phase HPLC. The fractions were shown to be mixtures of the same pigments that had been observed previously, on the HPLC chromatograms of black tea liquor. The thearubigin fractions isolated by Roberts (Roberts' SI and SII thearubigins) were shown to be mixtures of pigments previously classified as group I, II, and III pigments (pigments excluded from, resolved by, and remaining unresolved by HPLC, respectively), showing that the pigments in these three groups were similar to those in groups previously designated as thearubigins by Roberts. The SI and SII fractions were distinguishable by HPLC, and SI-like and SII-like fractions could be isolated by methods different from those of Roberts (*e.g.*, a Sep-Pak C_{18} cartridge). Fractions enriched in group I pigments were isolated using a Bond-Elut SCX cation-exchange cartridge (and on a larger scale on a column of Amberlite CG-120 cation-exchange resin). These fractions were shown by paper chromatography and HPLC to be mixtures of "anthocyanidin" pigments of various molecular masses. HPLC and mass spectrometry showed that an anthocyanidin, designated by Roberts as tricetinidin, was present in the mixture.

INTRODUCTION

The reversed-phase HPLC of black tea liquor was described in previous publications [1–3]. The black tea pigments were classified into three groups, group I being excluded from the columns, group II being resolved by the columns and group III remaining unresolved [2]. The group II (resolved) pigments were classified, by their photodiode-array spectra, into two types [2]. Type I resolved pigments, being yellow, showed an absorption maximum at, or just below, 400 nm, and type II resolved pigments, being brown, showed absorption that tailed off into the visible region. In subsequent publications, the isolation and analysis of a fraction of group III pigments, designated the theafulvins, was described [4,5], and mixtures of pigments from groups I, II and III have been produced by the chemical oxidation of individual catechins [6]. ^{13}C NMR spectroscopy showed the theafulvins to be flavanol polymers with different inter-monomer linkages to proanthocyanidin polymers [4]. In this paper a description is given of the isolation of further pigment fractions and their analysis by the new HPLC methods. This was done to explore methods for isolating new pigment fractions from black tea, and to apply the new HPLC methods comparatively to the analysis of these fractions and of fractions previously designated as thearubigins.

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To place our work within the older literature, it was decided to compare the HPLC of black tea liquor with that of Roberts' SI and SII thearubigin fractions [7]. Roberts *et al.* [8] separated thearubigins into ethyl acetate-soluble (SI) thearubigins and butanol-soluble (SII) thearubigins. The HPLC of the SI and SII thearubigin fractions was of great interest for two main reasons: (1) the chromatograms would help to identify thearubigins on tea chromatograms, and (2), if distinctive enough, the chromatograms would be useful for classifying other thearubigin fractions as SI-like or SII-like. The SI and SII fractions were isolated by Roberts' method [8] and analysed by HPLC. Also, other pigment fractions from black tea, "polar" and "non-polar" fractions, isolated using a Sep-Pak C_{18} cartridge by a method of Wellum and Kirby [9], were analysed by HPLC. The fractions were distinguishable by HPLC, the former being SII-like and the latter SI-like.

The group I pigments were of interest because they are excluded from all the HPLC columns. The exclusion could be due to molecular size, the material being either of high molecular mass or in a highly associated form, or to the charge carried. Four approaches for their isolation were explored: (1) exclusion from a Sep-Pak C_{18} cartridge, (2) exclusion from a column of Sephadex LH-20, (3) selective adsorption on a column of cellulose and (4) concentration by cation exchange, using cartridges and a column of resin. The fractions were analysed using our HPLC methods in conjunction with photodiode-array detection and LC–MS.

MATERIALS AND METHODS

Sep-Pak C_{18} cartridges (100 mg/1 ml) were obtained from Millipore Waters Chromatography (Watford, UK) and Bond-Elut SCX and CBA cartridges (100 mg/1 ml) from Jones Chromatography (Hengoed, UK). Chromatographic grade Amberlite 120 ion-exchange resin (CG-120) was obtained from BDH (Poole, UK) and Sephadex LH-20 from Pharmacia (Milton Keynes, UK). Whatman CF1 and microcrystalline chromatographic grade cellulose and 3MM chromatography paper were obtained from Whatman (Maidstone, UK).

The HPLC methods and the preparation of the tea liquor were as described previously [1,2]. Aqueous acetic acid was used with an untreated, 25 × 0.49 cm, 5 μm Hypersil ODS and a 15 × 0.49 cm, 5 μm Hamilton PRP-1 column, and an aqueous citrate buffer with an EDTA–citrate treated, 25 × 0.49 cm, 5 μm Hypersil ODS column. Chromatograms were monitored at 280 and 460 nm, the former wavelength being used to detect all phenolics and the latter to detect coloured compounds. In this paper, a chromatogram monitored at either of these wavelengths is referred to as a "280 nm chromatogram" or a "460 nm chromatogram", respectively. UV–Vis spectra were run on a Perkin-Elmer Lambda 5 spectrophotometer.

Mass spectra of group I pigments, isolated using a Sep-Pak C_{18} cartridge (designated as Sep-Pak eluent 2), were obtained using a VG Thermospray interface on a VG TS250, double-focusing air-cored magnet mass spectrometer, at the VG Tritech Applications Laboratory (Manchester, UK). An untreated Hypersil ODS column was used with isocratic elution, the solvent being 2% (v/v) acetonitrile in 0.05% (v/v) aqueous trifluoroacetic acid, with a flow-rate of 0.8 ml min^{-1} and injection of 50 μl. The chromatogram was monitored with a UV detector at 280 nm. The mass spectrometer scanned the mass range m/z 2000–200. All mass spectra described in this paper were acquired in the positive-ion mode.

Mass spectra of group I pigments, isolated using a column of Amberlite CG-120 cation-exchange resin (designated the CG-120 fraction), were obtained using a Finnigan Thermospray interface on the Finnigan TSQ70, triple quadrupole mass spectrometer at the Finnigan Applications Labs. (Hemel Hempstead, UK). Loop injections were carried out with 2% aqueous (v/v) acetic acid, at a flow-rate of 0.8 ml min^{-1}. The flow into the ion source was made up to 2 ml min^{-1} by post-column addition of 0.1 *M* aqueous ammonium acetate at a flow-rate of 1.2 ml min^{-1}. Volumes of 20–50 μl were injected. The mass spectrometer scanned the mass range m/z 1000–100.

Mass spectra of group I pigments, isolated using a Bond-Elut SCX cation-exchange cartridge (designated anthocyanidin pigments), were obtained using a Kratos Thermospray inter-

face on a Kratos MS80RFA double focusing mass spectrometer, in the Department of Food Science and Technology, University of Reading. An untreated Hypersil ODS column, eluted isocratically with 30% (v/v) methanol in 0.1 *M* ammonium acetate at a flow-rate of 1.0 ml min^{-1}, was used, 20–50 μl being injected. The mass spectrometer scanned the mass range *m/z* 800–100.

Isolation of Roberts' SI and SII thearubigin fractions

A tea liquor was prepared (tea, 10 g; water, 100 ml) and allowed to cool. The liquor was extracted with ethyl acetate (50 ml, ×4), in a separating funnel. The combined extracts were evaporated to dryness, under vacuum on a rotary evaporator, to give a red oil. The red oil was dissolved in acetone (10 ml) and chloroform (90 ml) added to precipitate a buff solid, which was filtered off, washed with chloroform, washed with light petroleum (b.p. 60–80°C), and dried in a vacuum desiccator over phosphorus pentoxide, to give a crude, solid SI fraction (0.56 g). This was dissolved in acetone (5 ml) and diethyl ether (300 ml) added to precipitate a buff solid, which was filtered off, washed with diethyl ether, washed with light petroleum (b.p. 60–80°C), and dried in a vacuum desiccator over phosphorus pentoxide, to give the SI fraction (0.13 g).

The aqueous layer from the ethyl acetate extraction was extracted with isobutanol (50 ml, ×2) and the combined extracts evaporated to dryness, under vacuum on a rotary evaporator, to give a pale yellow solid, which contained traces of solvent. The pale yellow solid was dissolved in methanol (8–10 ml) and diethyl ether (300 ml) added to precipitate a buff solid, which was filtered off, washed with diethyl ether, washed with light petroleum (b.p. 60–80°C), and dried in a vacuum desiccator over phosphorus pentoxide. This filtrate was evaporated to dryness, the precipitation repeated, and the solids combined, to give a solid SII fraction (0.24 g).

The isolation of "polar" and "non-polar" fractions from a neutral Sep-Pak C_{18} cartridge

A Sep-Pak C_{18} cartridge was conditioned first with methanol and then with water. A tea liquor was prepared (tea, 4 g; water, 100 ml) and an aliquot (2–5 ml) applied to the cartridge. The cartridge was washed with 35% (v/v) aqueous methanol (2–5 ml) and then with 80% (v/v) aqueous methanol (2–5 ml), the eluents being collected as separate fractions.

Isolation of group I pigments using Sep-Pak C_{18} cartridges

Sep-Pak 1 was conditioned first with methanol and then with water, and Sep-Pak 2 was conditioned first with methanol and then with 0.01 *M* HCl. A tea liquor was prepared (tea, 4 g; water, 100 ml) and an aliquot (2 ml) applied to Sep-Pak 1, the eluent (eluent 1) being collected and the Sep-Pak set aside. Eluent 1 was then applied to Sep-Pak 2 and the eluent (eluent 2) collected. The material retained on Sep-Pak 2 was eluted from it with pure methanol (eluent 3).

Isolation of group I pigments using Sephadex LH-20

Sephadex LH-20 (25 g) was washed in 35% (v/v) aqueous acetone (150 ml, ×3), the suspension allowed to settle, the liquid containing fines decanted off, and the LH-20 allowed to stand overnight in the solvent. A column (30 × 1 cm) was packed and equilibrated with the same solvent (100 ml). The void volume of the column was measured with Dextran Blue and found to be 10.5 ml. A tea liquor was prepared (tea, 4 g; water 100 ml), made 35% (v/v) in acetone and an aliquot (2 ml) applied to the column. The chromatogram was developed with 35% aqueous acetone at a flow-rate of about 0.33 ml min^{-1}. Coloured bands eluting from the column were collected separately and the volume of the fractions measured.

Isolation of group I pigments using Whatman CF1 and microcrystalline cellulose

Columns of Whatman CF1 Cellulose and Whatman Microcrystalline Cellulose (35 × 3 cm) were packed in water and washed with methanol (500 ml) and 2% (v/v) aqueous acetic acid (500 ml). Tea liquor was prepared (tea, 4 g; water, 100 ml), an aliquot (10 ml) applied to each of the above columns, and each chromatogram developed stepwise with 1% (v/v) aqueous acetic acid (200 ml), methanol (200 ml) and 5% (v/v)

aqueous acetic acid (200 ml). Fractions were collected and monitored for an absorption maximum at 510 nm. The fractions showing such absorption were combined and concentrated under vacuum on a rotary evaporator. When the liquid had been reduced to about 2 ml, ethanol (10 ml) was added and the solution evaporated to dryness under vacuum on a rotary evaporator.

Isolation of group I pigments using a Bond-Elut SCX (sulfonic acid) cation-exchange cartridge

A Bond-Elut SCX cartridge was conditioned first with methanol and then with 1% (v/v) aqueous acetic acid. A tea liquor was prepared (tea, 4 g; water, 100 ml) and an aliquot (5 ml) applied to the cartridge. The cartridge was washed with water until the washings were colourless, and then with 2% (v/v) concentrated hydrochloric acid in methanol (5 ml), the latter eluent being collected.

Isolation of group I pigments using a Bond-Elut CBA (carboxylic acid) cation-exchange cartridge

A Bond-Elut CBA cartridge was conditioned first with methanol and then with 1% (v/v) aqueous acetic acid. A tea liquor was prepared (tea, 4 g; water, 100 ml) and an aliquot (5 ml) applied to the cartridge. The cartridge was washed with water until the washings were colourless, and then with 2% (v/v) concentrated hydrochloric acid in methanol (5 ml), the latter eluent being collected.

Isolation of group I pigments using Amberlite CG-120 (sulfonic acid) cation-exchange resin

Amberlite CG-120 resin was washed with 0.1 *M* hydrochloric acid (200 ml, ×3), the suspension allowed to settle and the liquid containing fines decanted off. A column (30 × 1 cm) was packed in 0.1 *M* HCl and washed with distilled water until the washings were the same pH as the water. A tea liquor was prepared (tea, 4 g; water, 100 ml) and an aliquot (10 ml) applied to the column. The chromatogram was developed, stepwise, with distilled water (100 ml), 20% (v/v) aqueous methanol (100 ml), 80% (v/v) aqueous methanol (100 ml) and 2% (v/v) conc. HCl in methanol (100 ml). Fractions (5 ml) were collected and their composition was monitored by UV–Vis spectroscopy and HPLC.

Paper chromatography of tea liquors and tea fractions

Squares (25.5 × 25.5 cm) of Whatman 3MM paper were used. The paper was held in plastic racks, separated by plastic spacers, and developed by upward displacement. The chromatograms were developed first in isobutanol–acetic acid–water (4:1:2.2, v/v/v), removed from the tank, dried, and developed in the second direction in 2% (v/v) aqueous acetic acid. The paper was removed from the tank, dried and sprayed with 2% (w/v) ferric chloride–2% (w/v) potassium ferricyanide reagent, to which a few drops of 1% (w/v) potassium permanganate solution had been added.

RESULTS AND DISCUSSION

The isolation and analysis of Roberts' SI and SII fractions and "polar" and "non-polar" fractions

Roberts' SI and SII thearubigin fractions were isolated by the method of Roberts *et al.* [8] (Table I). The 280 nm chromatograms of the SI and SII fractions on an EDTA–citrate-treated Hypersil ODS column showed peaks close to the void volume of the column, a convex broad band, and resolved and partially resolved peaks with the broad band as baseline. They showed that both fractions contained gallic acid, cinnamic acid derivatives, flavonol glycosides, flavanol gallates, caffeine and some theaflavins [1]. The corresponding 460 nm chromatograms (SI: Fig. 1; SII: Fig. 2) showed pigments from all three groups I–III [2] and clear differences between the two fractions. The Hamilton PRP-1 column gave better 460 nm chromatograms of the group III pigments (SI: Fig. 3; SII: Fig. 4), showing clearly that the broad band from the SI and SII fractions differed, the maximum of the former being at a longer retention time than that of the latter.

Photodiode-array UV–Vis spectra taken from a 460 nm chromatogram of the SI fraction on an EDTA–citrate-treated Hypersil ODS column showed the presence of the four main theaflavins

TABLE I

SUMMARY OF THE ISOLATION AND ANALYSIS OF THE FRACTIONS

Fraction	Ref.	HPLC column	Comments
SI	7, 8	Treated[a] Hypersil ODS and Hamilton PRP-1	Pigments from all three groups, distinguishable from SII
SII	7, 8	Treated Hypersil ODS and Hamilton PRP-1	Pigments from all three groups, distinguishable from SI
"Polar"	9	Treated Hypersil ODS and Hamilton PRP-1	SII-like
"Non-polar"	9	Treated Hypersil ODS and Hamilton PRP-1	SI-like
Sep-Pak C_{18}, eluent 2	2	Untreated Hypersil ODS	Group I pigments: first peak an anthocyanidin pigment, second and third peaks brown pigments
Sephadex LH-20, first brown band	2	Untreated Hypersil ODS	Group I pigments: first peak an anthocyanidin pigment, second, third and fourth peaks brown pigments
Bond-Elut, SCX fraction	2	Untreated Hypersil ODS	Anthocyanidin pigments: first peak a group I pigment and second peak tricetinidin
Bond-Elut CBA fraction	2	Treated Hypersil ODS and Hamilton PRP-1	Anthocyanidin pigments (as above) and SI-like thearubigins
Amberlite CG-120 fraction	2	Untreated Hypersil ODS	Anthocyanidin pigments (as above)

[a] Treatment of the Hypersil ODS column was with EDTA and citrate as described in ref. 2.

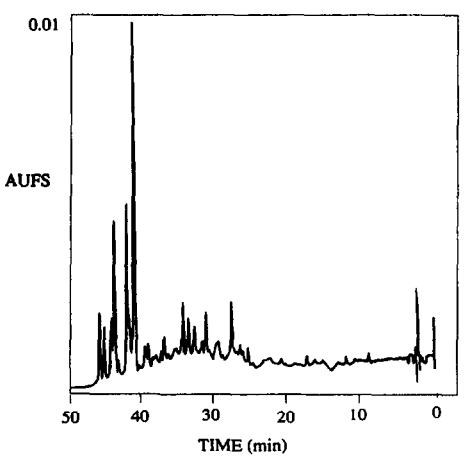

Fig. 1. The chromatogram (460 nm) of the SI fraction on an EDTA–citrate-treated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous citrate buffer. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate = 1.5 ml min^{-1}.

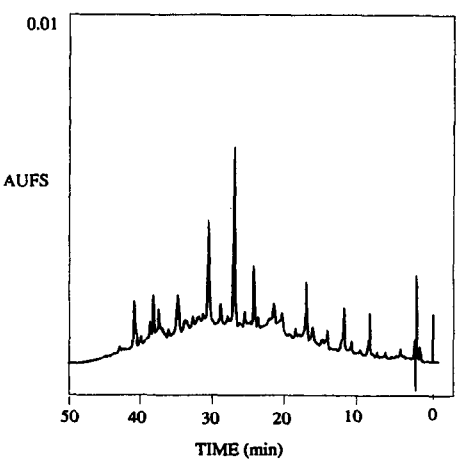

Fig. 2. The chromatogram (460 nm) of the SII fraction on an EDTA–citrate-treated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous citrate buffer. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate = 1.5 ml min^{-1}.

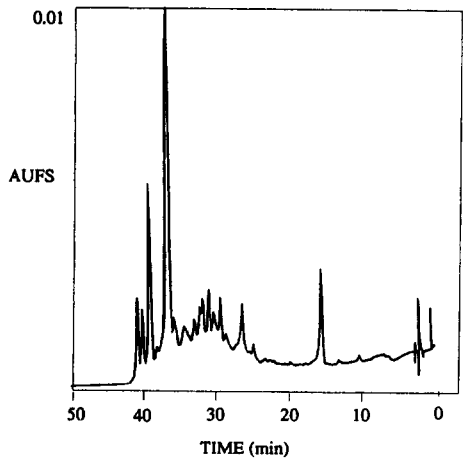

Fig. 3. The chromatogram (460 nm) of the SI fraction on a 5-μm Hamilton PRP-1 column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 5 to 33% solvent B over 50 min. Flow-rate = 1.0 ml min^{-1}.

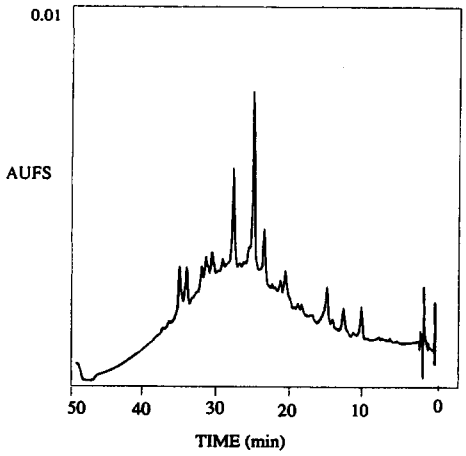

Fig. 4. The chromatogram (460 nm) of the SII fraction on a 5-μm Hamilton PRP-1 column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 5 to 33% solvent B over 50 min. Flow-rate = 1.0 ml min^{-1}.

found in all black tea liquors [1,2], plus one other theaflavin [2], three theaflavic acids [2], three type I resolved pigments [2] and five type II resolved pigments [2]. It was not possible to obtain good quality spectra from all of the peaks due to the low solubility of the SI fraction in the solvents used. The collection of photodiode-array spectra from the SII fraction was more difficult, as the fraction was less soluble in the HPLC solvent than SI, and many of the spectra were noisy and difficult to classify. However, a few photodiode-array UV–Vis spectra from the chromatogram could be used for classification. The theaflavin levels were lower than in the SI fraction, and there were only two theaflavic acids. Four peaks were assigned to type II resolved pigments, and one to a type I resolved pigment. The remaining peaks were very small and did not give spectra good enough for classification.

Wellum and Kirby [9] obtained a "polar" fraction from a Sep-Pak C_{18} cartridge with 35% (v/v) aqueous methanol, and a "non-polar" fraction with 80% (v/v) aqueous methanol. Since Wellum and Kirby [9] did not publish details of the first-eluted "polar" fraction, the procedure was repeated and both fractions were analysed by HPLC (Table I). A black tea liquor was applied to a neutral Sep-Pak C_{18} cartridge and a polar fraction eluted from the cartridge with 35% (v/v) aqueous methanol, before a "non-polar" fraction consisting of flavonol glycosides and theaflavins and other pigments was eluted with 80% (v/v) aqueous methanol. The 280 nm chromatograms of the "polar" fraction on an EDTA–citrate-treated Hypersil ODS column were complex, with many polyphenols, caffeine, and a convex broad band [1]. The 460 nm chromatograms of the "polar" fraction on the EDTA–citrate-treated Hypersil ODS (Fig. 5)

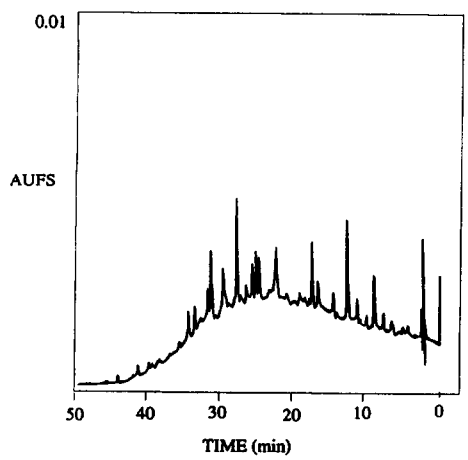

Fig. 5. The chromatogram (460 nm) of the "polar" Sep-Pak fraction on an EDTA–citrate-treated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous citrate buffer. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate = 1.5 ml min^{-1}.

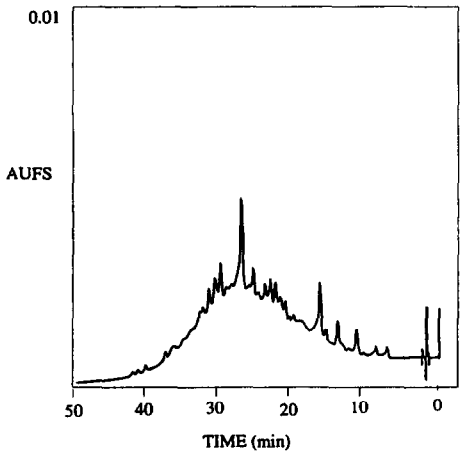

Fig. 6. The chromatogram (460 nm) of the "polar" Sep-Pak fraction on a 5-μm Hamilton PRP-1 column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 5 to 33% solvent B over 50 min. Flow-rate = 1.0 ml min^{-1}.

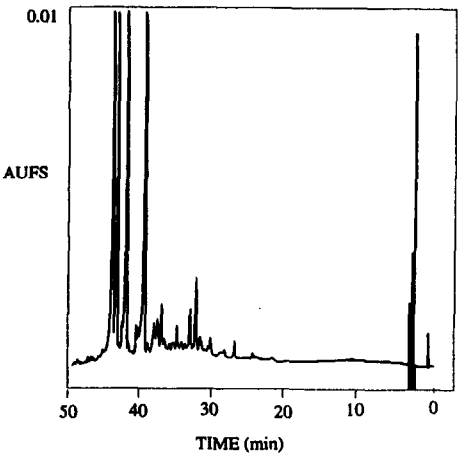

Fig. 7. The chromatogram (460 nm) of the "non-polar" Sep-Pak fraction on an EDTA–citrate-treated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous citrate buffer. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate = 1.5 ml min^{-1}.

and a Hamilton PRP-1 columns (Fig. 6) were SII-like (*cf.* Figs. 1–4). The "non-polar" fraction was eluted from the cartridge with 80% (v/v) aqueous methanol. The 280 nm chromatograms of this fraction on an EDTA–citrate-treated Hypersil ODS column showed mainly flavonol glycosides and theaflavins [1], the broad band not being prominent. The 460 nm chromatograms of the "non-polar" fraction on an EDTA–citrate-treated Hypersil ODS and a Hamilton PRP-1 columns (Figs. 7 and 8) were SI-like.

Thus HPLC showed the Roberts' SI and SII thearubigin fractions to be mixtures of pigments from all three groups, similar to those found in black tea liquor, and showed them to be distinguishable. The "polar" and "non-polar" fractions isolated previously by Wellum and Kirby [9] were shown to be pigment fractions chromatographically similar to Roberts' SII and SI fractions, respectively.

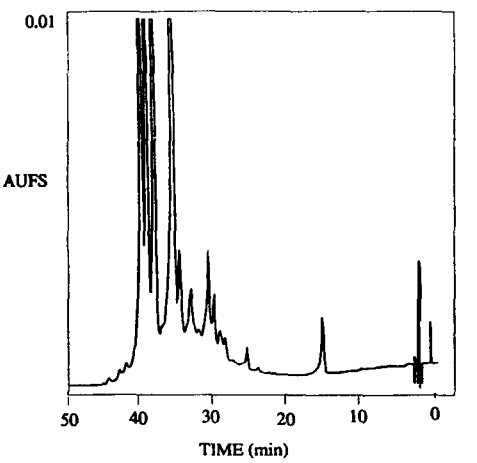

Fig. 8. The chromatogram (460 nm) of the "non-polar" Sep-Pak fraction on a 5-μm Hamilton PRP-1 column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 5 to 33% solvent B over 50 min. Flow-rate = 1.0 ml min^{-1}.

The isolation and analysis of group I pigments

Since group I pigments were excluded from all the HPLC columns [2], it was thought that the fraction not retained by a Sep-Pak C_{18} cartridge would be enriched in these pigments (Table I). Jaworski and Lee [10] fractionated grape phenolics using Sep-Pak C_{18} cartridges, so a further fractionation of the group I pigments by this method was attempted. Using this method, a fraction, not retained by a neutral cartridge, was collected for further fractionation on a second cartridge conditioned with 0.01 *M* HCl. The following designations are used below (*cf.* Materials and Methods): eluent 1 was not retained by a neutral Sep-Pak, eluent 2 was not retained by a Sep-Pak conditioned with 0.01 *M* HCl and eluent 3 was retained by the Sep-Pak conditioned with 0.01 *M* HCl.

The 280 nm chromatogram of eluent 1, on an untreated Hypersil ODS column, showed group I pigments, as well as gallic acid, theogallin, chlorogenic acid, *p*-coumaroylquinic acid, flavanol gallates, caffeine and some group III pigments [1,2]. Most, but not all, of the theaflavins and flavonol glycosides were retained on the cartridge. Thus eluent 1 was enriched in group I pigments, as expected, but was still a complex fraction. The 280 nm chromatogram of eluent 2, on an untreated Hypersil ODS column (Fig. 9), showed group I pigments, gallic acid and theogallin. Few group III pigments were present and the remaining peaks were very small. Thus eluent 2 was less complex than eluent 1. The 280 nm chromatograms of eluent 3, on an untreated Hypersil ODS column, showed group I pigments, polyphenols, caffeine and group III pigments.

Thermospray mass spectra of eluent 2 showed interesting high-mass ions, to which plausible ion compositions were assigned. The purity of the chromatographic peaks was not known, however, so more complete spectral interpretation was not attempted. Three peaks were observed on the UV trace in the first 3 min, but only the first peak (*ca.* 1 min) appeared on the ion chromatogram. Spectra extracted from the first peak, showed an ion at *m/z* 1822, for which two plausible compositions can be written, namely,

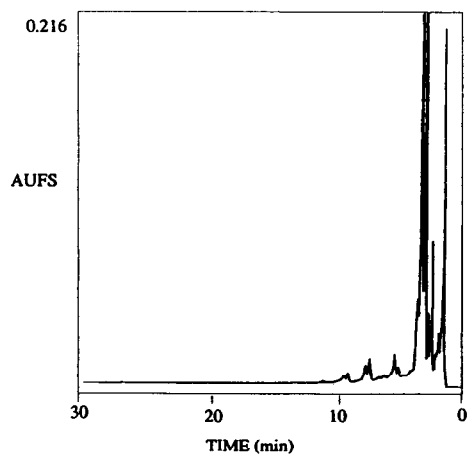

Fig. 9. The chromatogram (280 nm) of eluent 2 on an untreated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate 1.5 ml min^{-1}.

[4GCG − 6H − 4] and [3GC + 2GCG − 8H − 4], where GCG and GC are the masses of gallocatechin gallate and gallocatechin monomer units, respectively. The formation of a tetramer would require the loss of six H atoms from the sum of the monomer masses, leaving a difference of four H atoms to explain. Oxidation of one monomer unit to the anthocyanidin level, would account for a further three H atoms, leaving only one H atom outstanding. Similar reasoning was applied to the pentameric ion. In a similar way, two compositions for an ion at *m/z* 1529 are [1C + 2GC + 1GCG − 6H + 23] and [1C + 1GC + 2GCG − 6H + 23], where C is the mass of a catechin monomer unit. The mass difference of +23 suggests that the fragment was cationised by sodium. Spectra taken from a small peak eluting after the first group showed an ion at *m/z* 1973, which could have been a pentamer [2GC + 3GCG − 8H − 5] or hexamer [5GC + 1GCG − 10H − 5], again with one unit oxidised to the anthocyanidin level. However, assignments for these two ions were treated with even more caution, as the peak on the ion-chromatogram was very small. Thus MS suggests that some of the group I pigments were charged flavanol oligomers with end-groups oxidised to the anthocyanidin level.

That only the first of the main group of peaks, visible on the UV trace, was observed on the ion chromatogram suggested that only the first of the main early running peaks (group I pigments) possessed such an anthocyanidin group, the charged species being detected efficiently by the thermospray interface. The first peak of the photodiode-array three-dimensional plot of tea liquor, on EDTA–citrate-treated Hypersil ODS and Hamilton PRP-1 columns, showed an absorption at *ca.* 510 nm, suggestive of anthocyanidin chromophore [1,2] and of pigments similar to the anthocyanidin pigments found in wine [11]. The present oligomers, detected by MS, could be pigments of this type. Immobile, pink pigments are also observed at the origin of paper chromatograms of black tea liquor run using the method of Roberts *et al.* [8]. Experiments arising from this observation are discussed later in this paper.

Pursuing this approach, it was decided to

collect fractions that were excluded from a column of Sephadex LH-20, so a black tea liquor was applied to a column of Sephadex LH-20, packed and equilibrated in 35% (v/v) aqueous acetone, and the chromatogram developed, isocratically, with the same solvent (Table I). Three discrete brown bands ran ahead of coloured material that was strongly retained at the top of the column. These bands were collected and analysed by HPLC. The photodiode-array three-dimensional plot of the chromatogram of the first brown band (Fig. 10) on an untreated Hypersil ODS column showed that the fraction contained group I pigments. Four peaks were present in the first 5 min of the chromatogram, each with a spectrum that tailed off into the visible region, suggestive of a brown pigment [2]. However, the first peak in the plot (*ca.* 1.5 min) also showed an absorption at *ca.* 510 nm, that could have been due to an anthocyanidin chromophore. Once again, this peak was similar to the peak on photodiode-array three-dimensional plots of black tea liquor tentatively assigned to an anthocyanidin pigment [1,2]. This material could also have been a flavanol oligomer or polymer with endgroups oxidised to the anthocyanidin level, analogous to the anthocyanidin pigments in wine [11], although further work is required to substantiate this suggestion. The other peaks appeared to be brown pigments but remain not otherwise characterised.

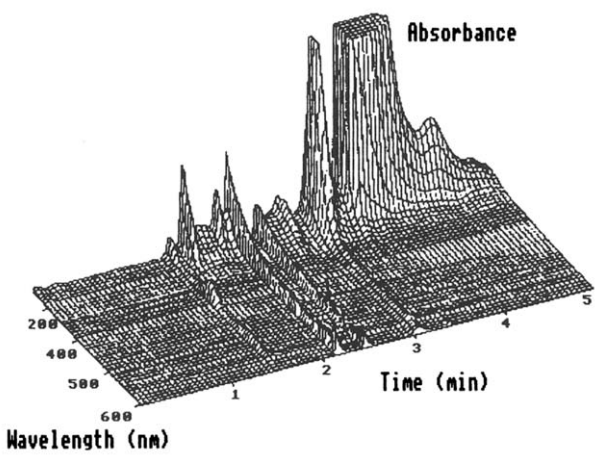

Fig. 10. The photodiode-array three-dimensional plot of the first brown band on an untreated 5-μm Hypersil ODS column. Solvent A = 2% (v/v) aqueous acetic acid. Solvent B = acetonitrile. Gradient 8 to 31% solvent B over 50 min. Flow-rate = 1.5 ml min^{-1}.

The photodiode-array three-dimensional plot of the second brown band on an untreated ODS Hypersil column showed group I pigments, chlorogenic acid, *p*-coumaroylquinic acid and caffeine. The observation of simple polyphenols co-eluting with the group I pigments does not rule out the possibility that group I pigments are excluded from the HPLC columns by size, as, with the solvents used, polyphenols show adsorption and/or partition as well as size exclusion, on Sephadex LH-20 [12]. The photodiode-array three-dimensional plot of the chromatogram of the third brown band on an untreated Hypersil ODS column showed group I pigments, polyphenols, caffeine and the broad band.

Since anthocyanidin pigments adsorbed strongly on paper, an attempt was made to isolate them from black tea liquor by selective adsorption on a column of cellulose. Columns of Whatman CF1 and microcrystalline cellulose were packed and equilibrated in 1% (v/v) aqueous acetic acid. Black tea liquor was applied to both columns, and the chromatograms were developed stepwise with 1% (v/v) aqueous acetic acid and methanol. Fractions were collected and analysed by UV–Vis spectroscopy and HPLC (on an untreated Hypersil ODS column). All the fractions were complex mixtures and none showed a band at 510 nm, suggestive of an anthocyanidin chromophore. Much coloured material remained on the columns after development with methanol, so the columns were washed with 5% (v/v) aqueous acetic acid, and this resulted in the elution of pink fractions with an absorption maximum at 510 nm. Fractions showing this band were combined and concentrated under vacuum on the rotary evaporator, ethanol being added to assist in the removal of the last traces of water. On addition of ethanol, the colour of the solution changed from light pink to purple and the absorption maximum shifted from 510 and 570 nm, the pink colour and the absorption maximum at 510 nm returning on addition of glacial acetic acid. This behaviour is typical of an anthocyanidin [13] and supported the suggestion that anthocyanidin pigments were present in the pink fraction. How-

ever, this method for the isolation of these pigments lacked reproducibility and much coloured material remained on the column.

To pursue the idea that some of the group I pigments possess anthocyanidin chromophores, their isolation using cation exchange was explored. Cation-exchange chromatography has been used to isolate polyphenolics and anthocyanidins from plant extracts [13]. Therefore, methods for the isolation of anthocyanidin pigments, using Bond-Elut cation-exchange cartridges, were explored (Table I). Black tea liquor was applied to a Bond-Elut SCX (sulfonic acid) cartridge, conditioned in turn with methanol and 1% (v/v) aqueous acetic acid. After washing with water until the washings were colourless, anthocyanidin pigments were eluted from the cartridge with 2% (v/v) concentrated hydrochloric acid in methanol. The UV–Vis spectrum of the anthocyanidin pigments showed a band with absorption maximum at about 505 nm.

The 280 nm chromatograms of the anthocyanidin pigments on an untreated Hypersil ODS column showed a peak at 1.8 min and a very small peak at 11.2 min. The 460 nm chromatogram on the Hamilton PRP-1 column showed two peaks, at 1.4 and 18.6 min. A photodiode-array three-dimensional plot of the chromatogram suggested that the peak at 18.6 min was an anthocyanidin, and the spectrum extracted for the peak confirmed this suggestion. This spectrum was the same as that of the first retained peak of the 460 nm chromatogram of black tea on the Hamilton PRP-1 column [2]. The photodiode-array UV–Vis spectrum of the peak at 1.4 min was difficult to interpret, as it was very close to the "refractive index peak" and showed much noise. The pigments were applied to paper and run using the method of Roberts *et al.* [8]; much of the material was completely immobile, remaining at the origin, supporting the suggestion that some of the pigments were polymeric anthocyanidin pigments. A faint coloured spot, mobile in butanol–acetic acid–water, but immobile in 2% (v/v) aqueous acetic acid, suggested the presence of Roberts' tricetinidin [7,8]. No standard was available to confirm this suggestion, but, in support of it, an ion was observed in a thermospray spectrum of the fraction at m/z 287, the correct mass for tricetinidin. On applying the fraction to an untreated Hypersil ODS column and eluting isocratically with 2% (v/v) aqueous acetic acid 0.1 *M* in ammonium acetate, ions at m/z 287 (M_r of tricetinidin = 287) and 403 [287 + 2NaCl] were observed. Fast atom bombardment spectra of anthocyanins show a peak at the mass of the flavylium ion [14,15], so the assignment of the ion at m/z 287 to the anthocyanidin tricetinidin is reasonable. Intense peaks due to clusters of sodium chloride were observed in this scan, showing the presence of sodium chloride in the sample.

Tea liquor was applied to a Bond-Elut CBA (carboxylic acid) cartridge, conditioned in turn with methanol and 1% (v/v) aqueous acetic acid (Table I). After washing with water until the washings were colourless, brown pigments were eluted from the cartridge with 2% (v/v) concentrated hydrochloric acid in methanol. The brown solution showed an absorption maximum at 510 nm. This suggested that the fraction was a mixture of anthocyanidin and thearubigin pigments. The 460 nm chromatograms of the fraction, on EDTA–citrate-treated Hypersil ODS and Hamilton PRP-1 columns, were SI-like. Thus the CBA cartridge gave a mixture of SI-like pigments and anthocyanidin pigments, and was less useful than the SCX cartridge. It is relevant to note the consistency of the presence of the anthocyanidin peak at *ca.* 18.5 min on the 460 nm chromatograms, on the Hamilton PRP-1 column, in the SI, Bond-Elut SCX and Bond-Elut CBA fractions, and in black tea liquor.

The Bond-Elut SCX cartridge isolation method was scaled up using a column of Amberlite CG-120 chromatographic grade sulfonic acid resin (Table I). The column was packed in distilled water, the tea liquor applied, and the column eluted, stepwise, with water, 20% (v/v) aqueous methanol, 80% (v/v) aqueous methanol and 2% (v/v) conc. HCl in methanol. The first three solvents were from a method of Ribéreau-Gayon [13], and the last solvent was chosen as one likely to elute anthocyanidin pigments from the column. The UV–Vis spectra of the pink fractions were similar to those of the pink fractions from the Bond-Elut SCX cartridges.

Similar pigments were obtained from the CG-120 column with 0.5% conc. HCl in methanol, suggesting that the pigments were unlikely to be merely degradation products. Paper chromatograms of these pigments, by the method of Roberts *et al.* [8], were similar to those isolated with Bond-Elut SCX cartridges. The fraction was heavily contaminated with sodium chloride, and ions consisting of clusters of tricetinidin, sodium chloride and water were observed in its thermospray spectrum.

CONCLUSIONS

This paper describes the isolation and HPLC analysis of fractions consisting of various black tea pigments, the results being summarised in Table I. HPLC showed the Roberts' SI and SII thearubigin fractions to be complex mixtures of pigments from all the groups I–III [2], including theaflavins, theaflavic acids, type I and type II resolved pigments (resolved thearubigins), and unresolved thearubigins. That both fractions are composed of pigments previously observed on black tea chromatograms gives increased confidence that the pigments observed on black tea chromatograms are, in fact, pigments previously designated as thearubigins. The SI fraction showed more theaflavins and a different distribution of group II and III pigments than SII. The convex broad bands from SI and SII were distinguishable by HPLC, their shapes being different, and their maxima at different retention times (Figs. 1 to 4).

"Polar" and "non-polar" fractions were isolated from black tea liquor using Sep-Pak C_{18} cartridges, following a method due to Wellum and Kirby [9]. HPLC showed the "polar" fraction to be SII-like, and the "non-polar" fraction (the fraction discussed by Wellum and Kirby [9]) SI-like. This provides a rapid, simple and robust method for the isolation SI-like and SII-like fractions for routine assessments, *e.g.*, as part of tea quality surveys. However, the relation of the SI-like and SII-like fractions to tea quality remains to be established.

Methods have been developed for the isolation of group I pigments, pigments that elute at the void volume of all the HPLC columns [2]. A similar group of pigments was also not retained by a Sep-Pak C_{18} cartridge or a Sephadex LH-20 column, and this enabled these pigments to be partially purified. A fraction of such pigments, designated eluent 1, was obtained from a Sep-Pak C_{18} cartridge. Further fractionation of these pigments gave eluent 2, a relatively simple fraction with an HPLC peak that gave thermospray ions at masses up to m/z 1973. These ions appeared to be from linear flavanol oligomers with one unit oxidised to the anthocyanidin level, and could be related to the anthocyanidin pigments discussed previously [1,2]. The chromatogram was monitored by UV and mass spectrometry, the 280 nm chromatogram showing a group of three peaks in the first 5 min, but the ion–chromatogram only the first peak of the group. These chromatograms were consistent with the chromatogram of the first brown band, excluded from a column of Sephadex LH-20, that showed only one peak with a UV–Vis absorption maximum at *ca.* 510 nm, the other three peaks having the spectra of brown pigments. The anthocyanidin pigments could be similar to the pink pigments found in wine [11].

Pink group I pigments were isolated from black tea using selective adsorption on a column of cellulose. However, the procedure lacked reproducibility, and coloured material was irreversibly adsorbed onto the cellulose. Anthocyanidin pigments were isolated using a Bond-Elut SCX (sulfonic acid) cation-exchange cartridge, the method being scaled up using a column of Amberlite CG-120 cation-exchange resin. HPLC, on the Hamilton PRP-1 column, showed these fractions to consist of an anthocyanidin, probably Roberts' tricetinidin [7,8], and a peak running close to the void volume of the column. The latter was similar to the pink pigments discussed above. The anthocyanidin was also prominent on black tea chromatograms run on the Hamilton PRP-1 column, but was not prominent on chromatograms run using an EDTA–citrate-treated Hypersil ODS column. An ion at m/z 287 in the thermospray mass spectra of the fraction provided evidence for the presence of tricetinidin. The Bond-Elut CBA cartridge was not as useful, as it gave a mixture of SI-like pigments and anthocyanidin pigments (460 nm

chromatograms on EDTA–citrate-treated Hypersil ODS and Hamilton PRP-1 columns). Thus the evidence is that the group I pigments are a mixture of pink anthocyanidin pigments and brown pigments.

The experiments described in this paper show the usefulness of our HPLC methods [1,2] for the qualitative analysis of black tea pigments. Used in conjunction with a range of separation methods, LC–MS, and photodiode-array detection, the methods have increased our knowledge of these pigments. However, much work remains to be done, particularly in the field of LC–MS. The long-term aim is to provide routine methods for the evaluation of tea quality for use in tea-producing countries. The Sep-Pak isolation methods could form the basis of a simple, rapid and robust method for the determination of thearubigin levels in black tea. However, this must await a better appreciation of the role of SI and SII thearubigins in tea quality.

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