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HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia

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Abstract

As part of a 4-year project to study phenolic compounds in tea shoots over the growing seasons and during black tea processing in Australia, an HPLC method was developed and optimised for the identification and quantification of phenolic compounds, mainly flavanols and phenolic acids, in fresh tea shoots. Methanol proved to be the most suitable solvent for extracting the phenolic compounds, compared with chloroform, ethyl acetate and water. Immediate analysis, by HPLC, of the methanol extract showed higher separation efficiency than analyses after being dried and redissolved. This method exhibited good repeatability (CV 3–9%) and recovery rate (88–116%). Epigallocatechin gallate alone constituted up to 115 mg/g, on a dry basis, in the single sample of Australian fresh tea shoots examined. Four catechins (catechin, gallocatechin, epicatechin and epigallocatechin) and six catechin gallates (epigallocatechin gallate, catechin gallate, epicatechin gallate, gallocatechin gallate, epicatechin digallate and epigallocatechin digallate) have been identified and quantified by this HPLC method. In addition, two major tea alkaloids, caffeine and theobromine, have been quantified, while five flavonol glycosides and six phenolic acids, including quinic acids and esters, were identified and quantified.

Keywords: Alkaloids; Flavanols; Fresh shoots; HPLC; Photodiode array detection; Phenolic acids; Tea

1. Introduction

Phenolic compounds are major tea constituents and are closely associated with the sensory properties and hence the quality of a brew (Graham, 1992; Hara, Luo, Wickremasinghe, & Yamanishi, 1995; Harbowy & Balentine, 1997). The HPLC technique was developed to analyse black and green tea constituents in the 1970s and showed good separations of the tea phenolic compounds (Hoefler & Coggon, 1976). However, the quantitative reproducibility of HPLC analysis was still very poor until the late 1980s before photodiode array (PDA) detection was incorporated into the HPLC system for the study of thearubigins in a model in vitro fermentation system (Opie, Clifford, & Robertson, 1993; 1995; Opie, Robertson, & Clifford, 1990; Powell, Clifford, Opie, Ford, Robertson, & Gibson, 1993; Powell, Clifford, Opie, & Gibson, 1995). The latter improved HPLC analysis has since contributed effective separation and identification of the black tea pigments from the chemical oxidation of green tea polyphenols, epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) (Bailey, Nursten, & McDowell, 1990, 1991, 1992, 1993; Wan, Nursten, Cai, Davis, Wilkins, & Davies, 1997; Yao & Nursten, 1997, 1998). Recent studies (Wang & Helliwell, 2001; Khokhar & Magnusdottir,

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2002; Zuo, Chen, & Deng, 2002) showed that a similar system of HPLC analysis can be used to determine (simultaneously), catechins (including their gallates), caffeine and gallic acids in green, oolong, black and puerh teas. However, no available method is suitable for the analysis of these tea phenolics in fresh young tea shoots (leaves), because the solvent extraction of tea components from these fresh tea shoots (leaves) may stimulate the action of polyphenol oxidase that causes the oxidation of tea polyphenols resulting from crushing of cells, which brings the polyphenols into contact with the enzymes (Hara et al., 1995). Moreover, the polyphenols are prone to form complexes with other components after being extracted. Thus, the type of solvent, the extraction time, and the method of extraction are considered important in the development of the extraction protocol prior to HPLC analysis.

The total area in which tea is grown in Australia is about 900 ha, which produces about 1300 tonnes of black tea and supplies 10–15% of the domestic consumption annually (Chudleigh, 1999). This present work is part of a project to study the phenolic compounds in tea shoots over the growing seasons and during black tea processing, so that a suitable database can be established for monitoring the quality of Australian grown and made tea.

2. Materials and methods

2.1. Plant materials

Tea [*Camellia sinensis* var. *assamica* (L.) O. Kuntze] fresh leaves (shoots), consisting of one apical bud and two adjoining leaves, were hand-plucked from the fields of a tea farm at Malanda in north Queensland, Australia, during harvest seasons from April 2000 to May 2001 (tea is harvested all year in Australia). After collection, the samples were wrapped in washed calico and packed in dry ice in polystyrene foam boxes. All samples were then delivered with dry ice by overnight cargo flight from Cairns to Brisbane, and to the laboratory at Gatton by road transportation. After arrival, all the samples were stored in a freezer at −80 °C prior to analysis. This protocol was developed in an endeavour to prevent oxidation of tea polyphenols by the enzymes present in the fresh shoots.

2.2. Equipment

The Shimadzu Class-VP HPLC system used consists of a computer-controlled system with the upgraded Class-VP 5.03 software and an SCL-10A VP System controller. Other accessories were a Shimadzu GT-104 Degasser, an FCV-10AL Mixer, two LC-10AD Shimadzu Liquid Chromatography Pumps, an SIL-10a XL Auto Injector, a CTO-10A Column Oven, and an SPD-M10A VP Diode Array Detector.

2.3. Chemicals

The solvents used for the extraction of tea samples were analytical grade methanol and Milli-Q (Millipore Australia Pty. Ltd., North Ryde, New South Wales, Australia) distilled water, while those used for the HPLC analysis were HPLC grade acetonitrile and acetic acid, and Milli-Q distilled water.

Caffeine, caffeic acid, coumarin, *p*-coumaric acid, epicatechin gallate (ECG), epigallocatechin gallate (EGCG), gallic acid (GA), 3-(*p*-hydroxyphenyl)-propionic acid (3PA), kaempferol and theobromine were obtained from the Chinese Tea Research Institute, Zhejiang, China and the College of Food Science, Southwest Agricultural University, Chongqing, China. Chlorogenic acid (CA) and epicatechin (EC) were purchased from Sigma Chemical Co., St Louis, MO, USA. Catechin (C), catechin gallate (CG), ECG, epigallocatechin (EGC), gallocatechin (GC) and gallocatechin gallate (GCG) were kindly provided by the Food Research Laboratories, Tokyo Food Techno. Co. Ltd., Miyabara, Fujieda, Shizuoka, Japan.

2.4. Solvent extraction

The method of extraction is based on an international standard (ISO 3103, 1980) and previous work (Bailey et al., 1990, 1991; Opie et al., 1990, 1995; Robertson & Bendall, 1983). Approximately 15 g of hand-plucked fresh tea shoots are blended with a solvent. The ratio of solvent to tea shoots on a dry weight basis is about 60:1 (v/w) for all the extractions. In this study, both boiling water and methanol were used in method development and its optimisation.

2.4.1. Method 1: extraction of phenolic compounds using boiling water

Fresh tea shoots (ca. 15 g) were blended with boiling water (180 ml, 100 °C) for 4 min (from 95 to 86 °C) in a blender (MX-T30GP Panasonic Super Blender, Matsushita Electric, Taiwan, 2 L). The mixture was filtered through cotton wool and the residue was washed with distilled water (3×10 ml). The combined extract was then concentrated to dryness in a rotary evaporator under reduced pressure at 40 °C. The dry extract was stored at −24 °C prior to HPLC analysis. Re-extraction of the dry extract was required for HPLC analysis. Various solvents were selected for trials involving the re-extraction of tea phenolic compounds, including chloroform, ethyl acetate, methanol and water. In each trial, solvent was added to completely dissolve the tea components in the dried water extract, and diluted to 25 ml with a ratio of 1:100 (w/v). Each solution was then

filtered through a 0.45-μm membrane filter for HPLC analysis. Each experiment was duplicated.

2.4.2. Method 2: extraction of phenolic compounds using methanol with a drying step

Fresh tea shoots (ca. 15 g) were blended with methanol (180 ml) for 4 min at 20 °C. The volume of the methanol extract was reduced due to evaporation occurring during the blending. The mixture was filtered through cotton wool and the residue was washed with methanol (3×10 ml). The rest of the trial was as for the boiling-water extract, except that methanol was added to completely dissolve the tea components in the dried extract, and the solution was then diluted to 25 ml with methanol. The whole trial was duplicated.

2.4.3. Method 3: extraction of phenolic compounds using methanol without a drying step

Design of this method was based on the results of Method 2. Fresh tea shoots (ca. 15 g) were blended with methanol (180 ml) for 4 min at 20 °C. The volume of the methanol extract was reduced due to evaporation occurring during the blending. The resultant mixture was filtered through cotton wool and the residue was washed with methanol (3×10 ml). The combined extract was then diluted to 200 ml with methanol. The whole experiment was duplicated.

2.5. Trials on blending times

Method 3 was carried out, but with blending for 3, 4, 5, 6 or 7 min. Each blending time was repeated four times according to a completely randomised design.

2.6. Test of enzymic activity

The enzymic activity was examined by using guaiacol (*o*-methoxyphenol) as an indicator, as described by Miller (1998). The fresh tea shoots (ca. 15 g) were blended with methanol (180 ml) for 0.5, 1, 2, 3, 4, 5, 6 and 7 min, respectively. Extract (2 ml) was added to distilled water (20 ml). Guaiacol solution (0.5% in 50% ethanol) (1 ml) and hydrogen peroxide solution (0.8%; 1 ml) were added. A control was run without addition of guaiacol and hydrogen peroxide solution. Peroxidase activity was indicated by the development of a reddish colour within 3.5 min. The trial for each blending time was duplicated.

2.7. Repeatability trials on the methanol extraction method

Extraction Method 3, using a 5 min blending time, was repeated seven times using different sub-samples of fresh tea shoots from the same gross sample. The blending time was adjusted to 5 min according to the recovery of main tea polyphenols extracted in the trials. Each extract was filtered through a 0.45-μm membrane filter and directly injected into the HPLC. Means, standard deviations and coefficients of variation for the levels (mg/g) of the compounds EC, ECG, EGC, EGCG and caffeine and for the total levels of these compounds in the extract were compared.

2.8. Recovery trials for the main components in the methanol extraction method

Fresh tea shoots (ca. 15 g) and five added standards were blended with methanol (180 ml) for 5 min. The extraction was carried out according to Method 3. The standards added were the four principal tea compounds, ECG, EGCG, GA and caffeine, and one minor tea component, coumarin. The amounts of each standard added for the trial were 50 mg of ECG, 50 mg of EGCG, 80 mg of GA, 80 mg of caffeine and 50 mg of coumarin. The whole experiment was repeated on three successive days.

2.9. Final optimised method for the extraction of polyphenols

This was as Method 3, but the fresh tea shoots (ca. 15 g) were blended for 5 min.

2.10. The HPLC analysis

The tea solution was analysed by HPLC, using a 5 μm Hypersil ODS S5 250×4.6 mm reversed phase column (TheroQuest Hypersil, Runcorn, Cheshire, UK). A 10×4 mm guard column, packed with Exsil ODS 5 μm packing material and held in a 10GCH-guard cartridge holder (SGE Exsil, Ringwood, Victoria, Australia), was used. The spectra were recorded from 220 to 600 nm and the chromatograms were monitored at 280, 310, 340, 380, 450 and 510 nm. The temperature of the column oven was set at 35 °C. The elution consisted of a linear gradient program from 8 to 31% acetonitrile in 2% aqueous acetic acid over 50 min (Bailey et al., 1991), then ramped to 100% acetonitrile over 2 min, maintained at 100% acetonitrile for 3 min, and then returned to 8% acetonitrile in 2% aqueous acetic acid for 10 min. The flow rate was 1.2 ml min^{-1}. An auto injector was used to inject 20 μl of the test solution into the HPLC system.

2.11. Identification of the tea phenolic compounds

The multi-wavelength detector can monitor phenolic compounds continuously between 190 and 800 nm, which enables the UV and visible (vis) regions to be monitored simultaneously. The spectrum of each standard was recorded and stored in the HPLC spectrum

library. The criteria for the identification of tea compounds were established based on comparisons of the retention time and spectrum of an unknown compound with HPLC library data of standards. The purity of the peaks was determined to ensure the identification. For peaks too close in the chromatogram, an integration program in the HPLC software was used to split the peaks and produce data for the calculation. Namely, for well resolved or symmetric peaks, such as Peaks 1, 6, 10, 13, 18 in Fig. 1, the spectrum match of an unknown compound was at a level of 99% or more against the spectrum of a standard compound, while for less resolved or asymmetric peaks, such as the pairs of Peaks 7 and 8, 14 and 15, enlargement of these peaks and then manual splitting with the peak purity control programme were used. Following this process, re-integration per peak was applied by performing the chromatographic programme. All of these peaks were identified by their spectrum matches at a level of 90% or above with the spectra of their standards. For those less than 90% of spectrum matches, the peaks were not included in the identification for further analysis.

2.12. Quantification of the tea phenolic compounds

The quantification was carried out using the external standard method. Solutions of each standard, at various concentration levels, were injected into the HPLC system and the peak areas and thus the calibration curves and response factors were recorded under the same conditions as for the samples. The concentration of a compound was calculated as peak area of the compound × response factor. For compounds where no standards were available, selection of the reference standard was based on the chromatographic and spectroscopic behaviour under the same conditions. For example, the response factor of kaempferol at 280 nm was used for the quantification of flavonoid glycosides (QG, Q3G, Q3RG, KG and K3RG, Table 1). ECDG and EGCDG were quantified using the response factors of ECG and EGCG, respectively. Isochlorogenic acid, theogallin and *p*-coumarylquinic acid were quantified against the chlorogenic acid, GCG and caffeic acid at 280 nm, respectively. All the results in this study are reported on a dry weight basis.

2.13. Statistical analysis

Quantitative data from HPLC analysis were compared using either the coefficients of variation (CV%) or analysis of variance (ANOVA). For variables where significant F-values ($P<0.05$) were found, Fisher's Least Significant Difference (LSD) has been used to compare means (Walpole, 1990).

3. Results and discussion

3.1. Identification of tea components in Australian-grown fresh tea shoots

The chromatogram of an extract of Australian-grown fresh tea shoots is shown in Fig. 1, while Table 1 shows the retention times, spectral data and identities for the peaks numbered in the chromatogram.

The PDA spectrum (Fig. 2) of Peak 1 in Fig. 1 is very similar to the spectrum of gallic acid. The spectrum of Peak 1 was at first mistaken as that of a digallic acid (Cartwright & Roberts, 1954a) before being confirmed as that of theogallin (Cartwright & Roberts, 1954a,

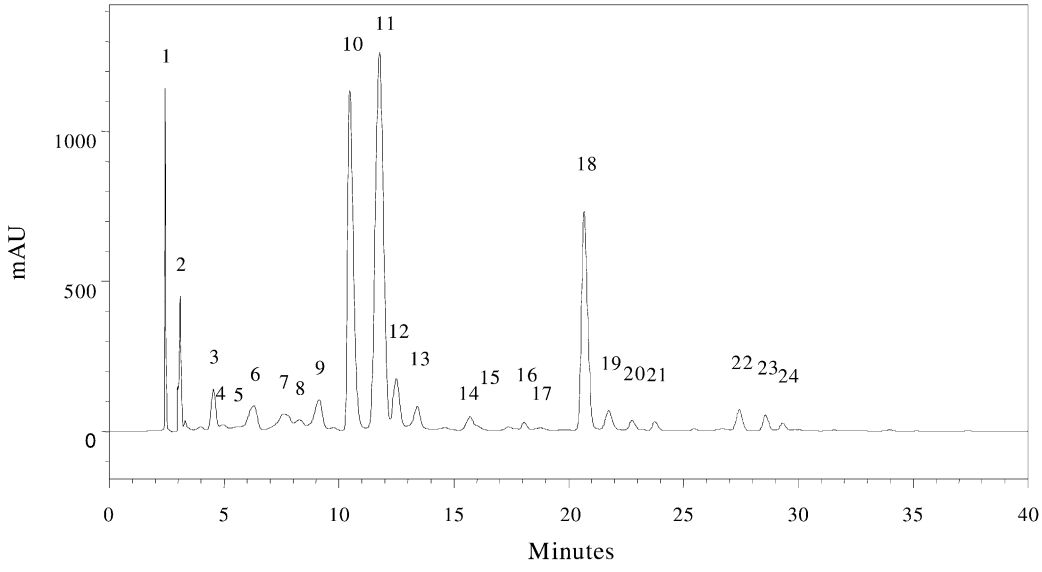

Fig. 1. HPLC trace of an extract of Australian-grown fresh tea shoots monitored at 280 nm: linear gradient 8–31% acetonitrile in aqueous 2% acetic acid over 50 min.

Table 1
UV absorption and identity of peaks in the chromatogram

Peak	Time (min)	Peak maxima (nm)	Identity	Peak maxima (nm) in literature
1	2.43	236, 271	Theogallin	276[b], 226.5[c], 274.5[c], 275[f]
2	3.09	232, 273	Gallic acid	272[a], 273[b], 226.5[c], 270.5[c]
3	4.53	233, 272	Theobromine	226.5[c], 272.5[c]
4	4.97	235, 298sh, 323	Isochlorogenic acid	296.5[c], 324.5[c]
5	5.60	234, 275	Gallocatechin (GC)	271[a]
6	6.30	235.5, 269	Epigallocatechin (EGC)	271,[a] 272[b]
7	7.58	233, 263	Catechin (C)	280[a]
8	8.28	234, 283, 325	*p*-Coumarylquinic acid	313[b], 228.5[c], 310.5[c], 314[c]
9	9.14	235, 301sh, 325	Chlorogenic acid	292.5[c], 320.5[c]
10	10.48	234, 270	Caffeine	226.5[c], 272.5[c]
11	11.78	236, 272	Epigallocatechin gallate (EGCG)	275[a], 279.5[a], 277[b], 226.5[c], 274.5[c]
12	12.50	234, 277	Epicatechin (EC)	280[a]
13	13.42	234, 310	*p*-Coumaric acid	
14	15.71	232.5, 276	3-(*p*-Hydroxyphenyl)propionic acid (3PA)	
15	16.00	235, 273	Gallocatechin gallate (GCG)	
16	18.06	257, 306sh, 354	Quercetin 3-rhamnosylglucoside (Q3RG)	256.5[c], 264[sh,c], 352.5[c], 259[e], 266[sh,e], 359[e]
17	18.77	237, 275	Epigallocatechin 3,5-digallate (EGCDG)	283[d]
18	20.67	234, 275.5	Epicatechin gallate (ECG)	280[a], 226.5[c], 276.5[c], 279[d]
19	21.75	233, 277	Catechin gallate (CG)	
20	22.75	255, 265sh, 353	Quercetin 3-glucoside (Q3G)	256.5[c], 264[sh,c], 354.5[c], 257[e], 269[x,e], 362[e]
21	23.76	254, 262sh, 352.5	Quercetin glycoside (QG)	256.5[c], 264[sh,c], 352.5[c]
22	27.44	266, 346	Kaempferol 3-rhamnosylglucoside (K3RG)	264.5[c], 344.5[c]
23	28.57	264, 346	Kaempferol glycoside (KG)	264.5[c], 344.5[c]
24	29.31	233, 278	Epicatechin 3,5-digallate (ECDG)	282[d]

[x] = shoulder.
[a] Bradfield and Penny (1948).
[b] Roberts and Williams (1958).
[c] Bailey et al. (1990).
[d] Coxon et al. (1972).
[e] Mabry et al. (1970).
[f] Cartwright and Roberts (1954a, 1954b).

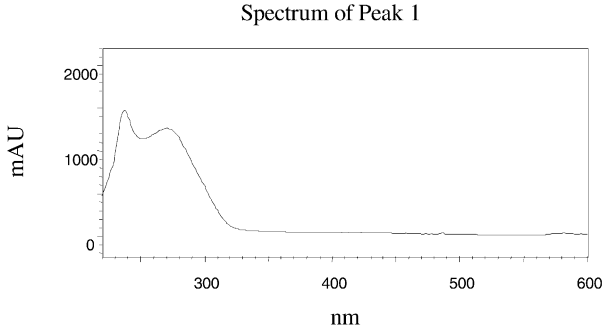

Fig. 2. Photodiode array UV spectrum of theogallin.

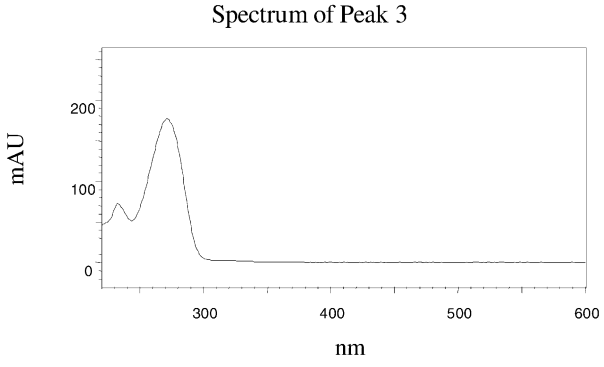

Fig. 3. Photodiode array UV spectrum of theobromine.

1954b; Roberts & Myers, 1958). In addition, the HPLC retention time of Peak 1 was found to be close to that of theogallin (Bailey et al., 1990) (Table 1). Therefore, Peak 1 was assigned to the compound, theogallin.

In contrast to Peak 1, the PDA spectra and retention times of Peaks 2 and 3 compare satisfactorily with the standard samples of gallic acid and theobromine. The spectrum of Peak 3 (Fig. 3) contains details that were also similar to the UV spectrum described by Bailey et al. (1990). Thus, the Peaks 2 and 3 were assigned to gallic acid and theobromine, respectively.

The PDA spectra of Peaks 4 and 9 (Fig. 4) are characteristic chlorogenic acid spectra. The similarity UV spectra of Peaks 4 and 9 suggests that isomers were present. Roberts, Cartwright and Wood (1956) isolated and identified three compounds in tea as chlorogenic acids (Roberts, 1962), which were later identified as positional *cis*- and *trans*-isomers (Bailey et al., 1990, 1991). The spectrum and retention time of Peak 9 compared satisfactorily with those of standard chlorogenic acid (3-*o*-caffeoylquinic acid), whereas Peak 4 was assigned to its isomer, isochlorogenic acid (5-*o*-caffeoyl-

quinic acid). This assignment is supported by the HPLC analysis of black tea liquor (Bailey et al., 1990), which showed similar chromatographic and spectroscopic behaviour for these two compounds. The PDA spectrum of Peak 8 has a similar spectrum, to chlorogenic acids with two partially resolved bands at 234 and 325 nm (Fig. 4), suggesting a quinic acid derivative. Moreover, it also has a UV spectrum similar to authentic *p*-coumaric acid. Based on the observation of Roberts (1962) and the HPLC analysis of Bailey et al. (1990), Peak 8 was assigned to *p*-coumarylquinic acid.

The PDA UV spectra of Peaks 5 to 7, 11, 12, 15, 18 and 19 are spectra characteristic of green tea catechins and their gallates. All of them have two specific resolved bands (Fig. 5). Band I ranges from 232 to 235 nm and is little affected by galloylation. Band II spans 260–278 (285) nm, galloylation tending to shift the absorption to longer wavelengths. The retention times and spectra of a number of standard catechins and their gallates compared satisfactorily with those of these compounds (Table 1). The four principal polyphenolic compounds present in green tea leaves are EC, ECG, EGC and EGCG (Fig. 5), which together make up 70% of the polyphenols in fresh tea shoots (Roberts, 1962; Sanderson, 1972; Millin, 1987).

With regard to Peaks 13 and 14, their PDA spectra and retention times compared satisfactorily with those of the authentic compounds, *p*-coumaric acid and 3-(*p*-hydroxyphenyl)-propionic acid (3PA), respectively. Further study may be necessary for the identification of 3PA since this is the first discovery of this compound in tea shoots with minor concentration.

The PDA spectra of Peaks 17 and 24 in Table 1 are very similar to the spectra of EGCG and ECG, respectively. The former compound, eluted after EGCG, has a UV absorption maximum at 283 nm in ethanol, whereas the UV absorption maximum of EGCG is 275 nm (Coxon, Holmes, Ollis, Vora, Grant, & Tee, 1972). Thus, Peak 17 was assigned to EGCDG (Table 1). In similar manner, Peak 24, which eluted after ECG, was assigned to ECDG (Table 1).

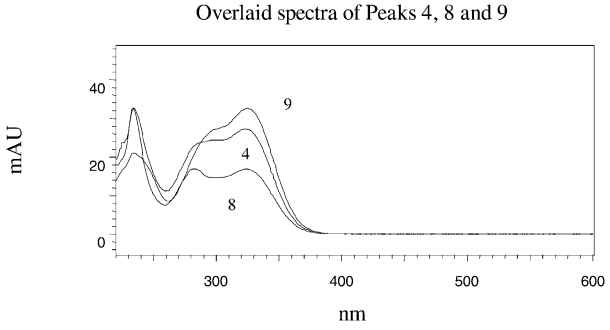

Fig. 4. Photodiode array UV spectra of quinic acid derivatives: isochlorogenic acid (4), *p*-coumarylquinic acid (8) and chlorogenic acid (9).

The chromatogram in Fig. 1 also shows five peaks (16, 20, 21, 22 and 23) between 18 and 30 min of retention time with PDA UV/vis spectra (Fig. 6) characteristic of flavonol glycosides: i.e., Band I 240–285 nm and Band II 300–550 nm. Bailey et al. (1990) compared the PDA spectra of these five peaks using HPLC and authentic compounds. Based on the HPLC retention times and UV/vis spectra (Table 1), Peaks 16 and 20–23 were assigned as in Fig. 6 and Table 1.

As there are no obvious peaks in the chromatogram of Fig. 1 after 30 min of elution, the oxidation products of green tea polyphenols, which would be the slowest eluting compounds during HPLC analysis, are absent, suggesting that the extraction of polyphenols did not result in oxidation.

3.2. Extraction of fresh tea shoots with water

Researchers usually use boiling water for the extraction of polyphenols from dried green and black teas (Khokhar & Magnusdottir, 2002; Roberts et al., 1956; Wood et al., 1964). Aqueous ethanol (Wang & Helliwell, 2001) and methanol (Zuo et al., 2002) were recently applied to the extraction of flavonoids from dried black, green, oolong and/or pu-erh teas. However, there is no systematic and detailed method available for the extraction of polyphenols from fresh young tea shoots, so boiling water was tested as a method to extract phenolic compounds from the fresh tea shoots. In the current study, hot distilled water extracted significantly higher ($P<0.05$) amounts of all compounds examined, than chloroform and, except for EC, than ethyl acetate. The levels of the main components found for this trial are shown in Table 2. Further trials proved that methanol was the most efficient solvent for the examined tea compounds since it extracted these compounds at significantly higher ($P<0.05$) levels from the dry tea solids than chloroform, ethyl acetate, or water (Table 2). Therefore, methanol is selected for subsequent trials for the extraction of polyphenols from fresh tea shoots.

3.3. Comparison of the extraction of water and methanol

The mean contents of four main tea catechins (EC, EGC) and catechin gallates (ECG, EGCG) showed higher levels for the methanol extraction than those for the water extraction (Table 3), but there were no significant differences ($P>0.05$) in the levels of the tea polyphenols EC, EGC, ECG and EGCG in the final extracts, regardless of whether water or methanol was used for the initial extract. Similarly, for the principal tea alkaloid caffeine there were no significant differences ($P>0.05$) in the levels extracted by either of these two solvents. The four principal catechins and catechin gallates were chosen for the trials since they usually make

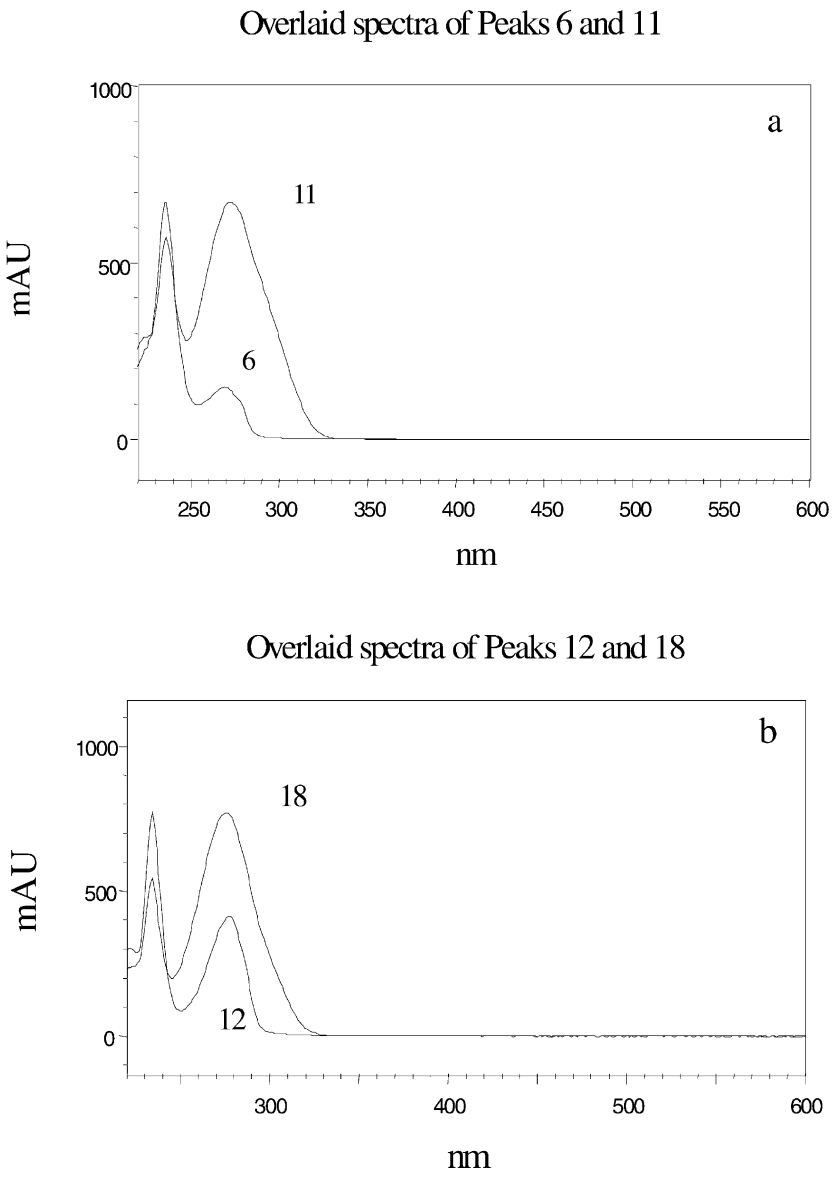

Fig. 5. Photodiode array UV spectra of the principal green tea polyphenols: a. EGC (6) and its gallate EGCG (11); b. EC (12) and its gallate ECG (18).

Table 2
Main tea components re-extracted with different solvents from a water extract

Solvent	Main compound (mg/g, dry basis)[a]				
	EGC	EC	EGCG	Caffeine	ECG
Chloroform	0.35 a	0.04 a	0.40 a	0.45 a	0.14 a
Ethyl acetate	2.46 b	1.19 b	9.49 b	0.26 a	2.59 b
Water	9.58 c	0.99 b	28.7 c	9.35 b	11.8 c
Methanol	25.2 d	2.89 c	38.7 d	14.3 c	17.9 d
LSD	1.36	0.21	2.39	0.72	1.00

[a] Means in columns followed by a common letter are not significantly different ($P > 0.05$).

Table 3
Main components from fresh tea shoots extracted with boiling water and methanol

Solvent	Main compound (mg/g, dry basis)[a]				
	EGC	EC	EGCG	Caffeine	ECG
Boiling water	24.2 a	15.1 a	55.1 a	29.2 a	16.5 a
Methanol	31.0 a	15.8 a	70.8 a	25.4 a	29.0 a
LSD	14.43	5.21	46.4	7.11	14.9

[a] Means in columns followed by a common letter are not significantly different ($P > 0.05$).

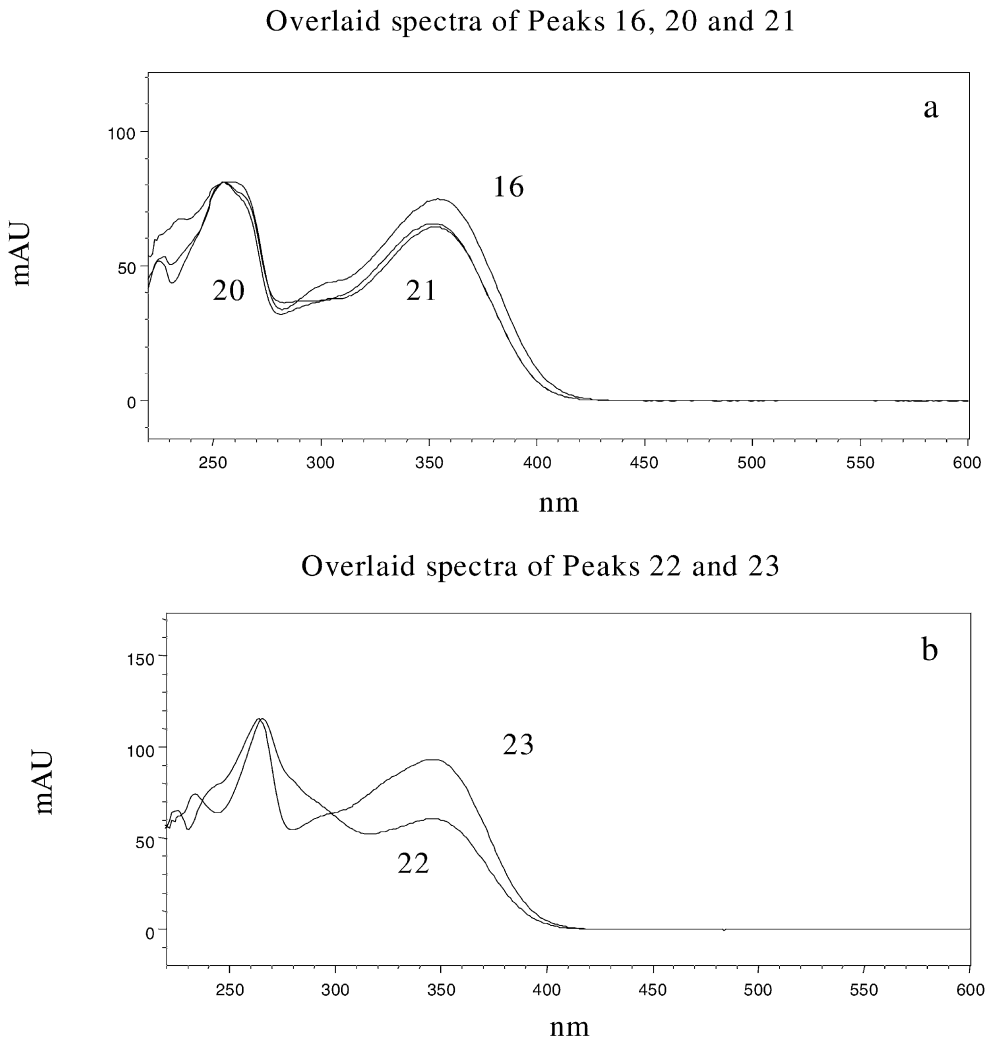

Fig. 6. Photodiode array UV spectra of the flavonol glycosides: a. Q3RG (16), Q3G (20) and QG (21); b. K3RG (22) and KG (23).

up 70% of polyphenols in tea shoots (Roberts, 1962; Sanderson, 1972; Millin, 1987; Harbowy & Balentine, 1997).

Methanol is supposed to be a solvent suitable for the extraction of phenolic compounds, including flavonoids, from fresh plant tissues (Harborne, 1988; Harborne, Mabry, & Mabry, 1975; Mabry, Markham, & Thomas, 1970; Waterman & Mole, 1994). Ethanol has also been used for the extraction of polyphenols from fresh tea shoots for paper chromatographic analysis, which included a heat treatment of fresh leaves immersed in ethanol and a homogenising process and the subsequent tea liquor was evaporated to dryness and re-extracted with methanol (Roberts & Fernando, 1981). In another study, Taylor and McDowell (1991) showed that methanol was efficient in extracting pigments from fresh tea shoots. Therefore, methanol was chosen for subsequent trials based on its use in the literature, as well as its ease of evaporation compared to water.

3.4. Comparison of methanol extraction with and without a drying step

Regarding the drying of the polyphenolic extracts, Degenhardt, Engelhardt, Lakenbrink, and Winterhalter, (2000); Degenhardt, Engelhardt, Wendt, & Winterhalter (2000) used aqueous methanol to extract catechins from green tea and the solvent was evaporated under vacuum. Zuo et al. (2002) reported a method of extracting catechins and catechin gallates from made teas using methanol and then used the extract directly for HPLC analysis. However, no methods are available for the extraction of polyphenols from fresh tea shoots for HPLC analysis. Therefore, it became necessary to compare the behaviour of methanol extracts from fresh tea shoots to see whether a drying process affects the composition of the extract and, if so, how.

The levels for two of the four principal catechins in fresh tea shoots, EGC and ECG, were not significantly

Table 4
Main components in tea methanol extracts with and without dryness

Treatment	Main compound (mg/g, dry basis)[a]				
	EGC	EC	EGCG	Caffeine	ECG
Dried	31.0 a	15.8 a	70.8 a	25.4 a	29.0 a
No drying	32.7 a	18.5 b	106.0 b	31.5 b	33.4 a
LSD	10.8	2.70	10.1	1.50	7.15

[a] Means in columns followed by a common letter are not significantly different ($P > 0.05$).

different ($P > 0.05$) between the treatments with and without a drying step (Table 4). However, levels for the other two principal compounds, EC and EGCG, and the tea alkaloid caffeine were significantly different ($P < 0.05$) between treatments, with the levels detected in methanol extracts without a drying step being significantly higher ($P < 0.05$) than those analysed with a drying step (Table 4). Thus, the HPLC analysis of tea polyphenols is better done directly on the methanol extract obtained initially, rather than that obtained after drying and re-dissolution.

Not only does EGCG comprise 30–40% of the polyphenols in fresh tea shoots (McDowell & Taylor, 1993; Millin, 1987; Roberts, 1962; Sanderson, 1972), but EGCG decreased proportionately the most during the drying process (Table 4). This indicates that EGCG is most vulnerable. Thus, the extraction of EGCG needs to be maximised through omission of the drying step.

3.5. Optimisation of the blending time for the extraction of tea polyphenols

Although 5 min extraction achieved the maximum quantity of the four main tea polyphenols, no significant ($P > 0.05$) differences were found for all of the main compounds extracted from fresh tea shoots by methanol with blending times of 3–7 min, except for EGC, where a significant ($P < 0.05$) increase in the level extracted occurred for a 5 min blending time compared with the 4 min one (Table 5). These results also indicate that, after blending for 3 min, most of the tea components had already been extracted by methanol. Considering the consistence of whole experiments and analytical practice, 5 min was selected as the blending time in this study, because of the maximum quantity of polyphenols being obtained with this blending time.

Table 5
Main tea phenolic components and caffeine extracted with different blending time

Time (min)	Main compound (mg/g, dry basis)[a]				
	EGC	EC	EGCG	Caffeine	ECG
3	47.1 ab	16.8 a	103.1 a	38.3 a	36.3 a
4	45.0 a	16.6 a	104.0 a	39.6 a	35.8 a
5	47.9 b	17.5 a	115.0 a	41.6 a	40.7 a
6	46.8 ab	16.8 a	113.1 a	43.6 a	39.8 a
7	46.8 ab	16.8 a	113.15 a	40.4 a	38.9 a
LSD	2.7	2.15	18.2	5.55	7.12

[a] Means in columns followed by a common letter are not significantly different ($P > 0.05$).

3.6. Test of enzymic activity in the methanol extracts

Using the peroxidase test, no enzymic activity was detected in the solution of fresh tea shoots after blending from 0.5 to 7 min with methanol. In fact, there was no enzymic browning in a methanol extract 1 h after the blending. This shows that blending the fresh tea shoots with methanol effectively inactivated the enzymic oxidation.

3.7. Repeatability of the final extraction method

Methanol extracts of seven sub-samples from one gross sample of fresh tea shoots were obtained. The coefficients of variation (CV) of the levels of the main catechins, the alkaloid caffeine, and the total amount of compounds (the 24 identified compounds in Table 1) in the extract from fresh tea shoots were determined (Table 6). The CV of EGCG, ECG, and the total extract were 4.12, 4.15 and 4.11%, respectively, all below 5.00%, which indicates excellent repeatability for the extraction of these compounds. The CV of EC and EGC were 5.09 and 8.55%, respectively, suggesting a good repeatability for the extraction of these two compounds.

3.8. Recovery trial for the final extraction method

Five authentic compounds, ECG, EGCG, GA, caffeine and coumarin, were added to the fresh tea shoots before blending as part of a recovery study. The results from this recovery trial showed that the mean recovery of the standards tested varied from 88.9 to 116% (Table 7). These are very high recovery rates bearing in mind the complexity of the analyses. The CVs of gallic acid, caffeine and coumarin were

Table 6
Coefficients of variation for the extraction method with fresh tea shoots

Statistical analysis	Main compound (mg/g, dry basis)[a]					
	EGC	EC	EGCG	Caffeine	ECG	Total[a]
Mean	32.5	15.0	101.0	32.1	31.0	252.0
CV%	8.55	5.09	4.12	3.74	4.15	4.11

[a] Mean of content of all twenty-four compounds extracted (Table 1).

Table 7
Coefficients of variation for the recovery test of some tea standard chemical compounds

Statistical analysis	Recovery of standard compound				
	GA	Caffeine	Coumarin	ECG	EGCG
Mean recovery (%)	110.0	88.9	90.4	116.0	105.0
CV%	3.45	4.25	1.47	5.82	8.66

3.45, 4.25 and 1.47%, respectively, all below 5.00%, exhibiting very good precision; the CV of ECG and EGCG were 5.82 and 8.66%, respectively, showing good precision.

4. Conclusion

Four catechins (catechin, gallocatechin, EC and EGC) and six catechin gallates (EGCG, CG, ECG, GCG, ECDG and EGCDG), two major tea alkaloids, caffeine and theobromine, as well as five flavonol glycosides and six phenolic acids, including four quinic acids and esters have been identified and quantified using the HPLC method developed and optimised in this study. EGCG is the main flavanol in Australian fresh tea shoots, constituting up to 115 mg/g of tea shoots on a dry basis. Methanol proved to be the most suitable solvent for extracting the phenolic compounds in fresh tea shoots compared with chloroform, ethyl acetate and water. For fresh tea shoots, blending for 5 min with methanol at a ratio of 1:18 (w/v) maximised the extraction of phenolic compounds. Immediate analysis by HPLC of the methanol extract showed higher separation efficiency, and (good repeatability and recovery rate) than those analysed after being dried and redissolved. This method showed a good repeatability (CV 3–9%) and recovery rate (88–116%).

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