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On-line high-performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea

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Teas represent a rich source of dietary antioxidants. This paper describes analysis of the antioxidant potential of individual tea phenolics using an on-line high-performance liquid chromatography (HPLC) method. Tea phenolics from Kenyan green and black teas were identified using liquid chromatography – mass spectrometry (LC-MS^n) in conjunction with the analysis of their 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radical scavenging ability. Antioxidant potential of flavan-3-ols, caffeoylquinic acids, flavonols, and theaflavins was assessed in comparison to the synthetic vitamin E analogue Trolox. (–)-Epigallocatechin gallate was identified as the most potent antioxidant with a Trolox equivalent antioxidant capacity (TEAC) value of 3.0, contributing approximately 30% of the total antioxidant capacity of green tea. Theaflavins retained antioxidant capacity similar to that of (–)-epicatechin monomers whilst conjugated flavonols did not contribute significantly to the antioxidant capacity of either green or black tea. After HPLC analysis of the antioxidant capacity of phenolics in black tea some 80% of antioxidant activity remained unaccounted for indicating the potential importance of thearubigens as antioxidants in black teas.

Keywords: Antioxidant capacity / *Camellia sinensis* / Flavan-3-ols / Flavonols / Hydroxycinnamates / Phenolics

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1 Introduction

Diets rich in fruits and vegetables are associated with a reduced risk of disease including coronary heart disease and some cancers [1]. Recent attention has focused on trying to identify those components within the diet which contribute to good health. Protection provided by fruits, vegetables, and beverages may, in part, be associated with phenolic compounds with antioxidant activity. Teas, prepared from the dried leaves of *Camellia sinensis*, are an especially rich source of phenolics. Unfermented green teas contain high levels of flavan-3-ols whereas oxidation followed by polymerisation occurring during the manufacture of black teas leads to a reduction in the concentration of flavan-3-ols and an increase in theaflavins and thearubigens. Additionally, green and black teas are known to contain lower levels of flavonols, flavones, and phenolic acids [2].

Many phenolic compounds have a structure that affords them antioxidant activity, and as such they may chelate transition metal ions thereby preventing the formation of iron-induced free radicals, act as hydrogen/electron donating radical scavengers, terminate propagatory chain reactions of lipid peroxidation or regenerate α-tocopherol [3]. Flavan-3-ols lack a 2–3 double bond and a carbonyl group at the 4-position, a combination known to contribute to strong antioxidant activity; instead they may possess B ring hydroxylation and/or a galloyl moiety at position 3 conferring antioxidant potential [4] (Fig. 1). Tea phenolics have been shown to elicit antioxidant activity in a range of assays. Terao *et al.* [5] studied their *in vitro* effects in a phospholipid bilayer. (–)-Epicatechin and (–)-epicatechin gallate were found to be powerful antioxidants preventing lipid peroxidation by conserving endogenous α-tocopherol and scavenging aqueous oxygen radicals. A study by Yokozawa *et al.* [6] showed that the flavan-3-ol fraction of a green tea extract produced a significant antiradical effect against the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^+$) radical, reduced lipid peroxidation and DNA fragmentation.

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Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; **PDA**, photodiode array; **TEAC**, Trolox equivalent antioxidant capacity; **Trolox**, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

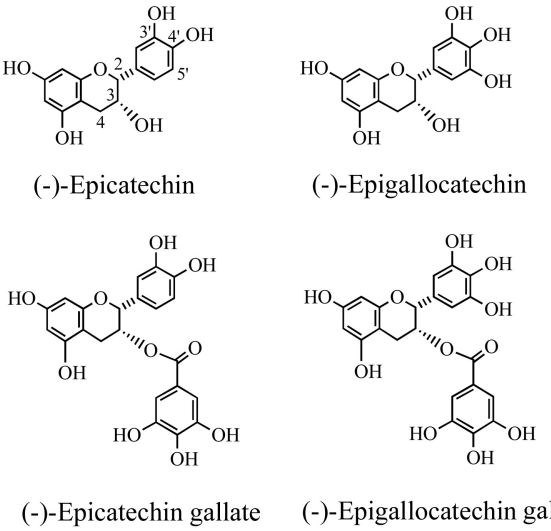

Figure 1. Examples of C-3 galloylation and B-ring hydroxylation of flavan-3-ols.

Recently, sensitive on-line HPLC methods for analysing radical scavenging activity have been developed [7, 8]. Such methods require a stable model free radical system, such as 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^+$), with radical scavenging activity assessed in comparison to the water-soluble synthetic vitamin E derivative 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). On-line assessment of antioxidant activity allows complex mixtures to be separated by HPLC and the antioxidant contribution of individual components can be evaluated.

The purpose of the current study was to assess the contribution of individual components of green and black tea to the overall antioxidant capacity of tea. Tea components were initially separated and identified using HPLC-MS^n, the analysis was then reproduced on an HPLC system linked to an $ABTS^+$-based on-line antioxidant detection system.

2 Materials and methods

2.1 Materials

James Finlay Ltd (Finlay Tea Solutions UK Ltd, London, UK) supplied fresh leaf and black tea from Kenya. (+)-Catechin, (−)-gallocatechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, gallic acid, caffeine, theobromine, quercetin-3-glucoside, quercetin-3-rutinoside, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$), and a black tea extract containing a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3-3′-digallate were obtained from Sigma (Poole, Dorset, UK). Kaempferol-3-rutinoside and 5-caffeoylquinic acid (chlorogenic acid) were supplied by AASC Chemicals (Southampton, UK). HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland).

2.2 Teas

Tea leaves were chemically dried using P_2O_5 under vacuum in a sealed dessicator and ground in a pestle and mortar prior to preparation of teas. Tea infusions were prepared by adding 300 mL boiling water to 3 g of tea leaves. After 3 min continuous stirring, the brew was filtered through a tea strainer to remove particulate matter. This approximates to the average weight of tea contained within one tea bag infused in a mug of water.

2.3 HPLC-diode array and MS^n analysis

Tea infusions were analysed using a Surveyor gradient HPLC system [2] comprising an HPLC pump, diode array absorbance detector scanning from 250 to 700 nm, and an autosampler cooled to 4°C (Thermo Finnegan, San José, CA, USA). Separations were carried out using a Phenomenex (Torrance, CA, USA) RP-MAX 4 μm 250 × 4.6 mm i. d. C_{12} reverse-phase column maintained at 40°C, eluted at 1 mL · min^{-1} with a 60 min gradient of either a 4–25% (analysis of catechins and hydroxycinnamates) or a 10–30% gradient (separation of flavonols and theaflavins) of acetonitrile in water containing 1% formic acid. Following separation catechins, hydroxycinnamates and theaflavins were detected by PDA analysis at 280 nm while flavonols were monitored at 365 nm. After the mixture passed through the flow cell of the absorbance monitor, the column eluate was split and 20% was directed to a Finnigan LCQ Decca mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from 150 to 2000 amu. Samples were analysed using both positive and negative ionisation modes. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400°C. Gallic acid, (−)-gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin gallate, (−)-epicatechin, 5-caffeoylquinic acid, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-glucoside, caffeine, and theobromine were all quantified by reference to standard calibration curves obtained with diode array detection at λ_{max} values. Standards were not available in all instances so 5-galloylquinic acid was quantified in gallic acid equivalents, 3-caffeoylquinic acid (neochlorogenic acid) in 5-caffeoylquinic acid equivalents and theaflavins in (−)-epicatechin equivalents. All quercetin-derived compounds, except rutin, were quantified in quercetin-3-gluco-

side equivalents and all kaempferol-based compounds were quantified by reference to kaempferol-3-glucoside.

2.4 ABTS$^+$ decolourisation assay

The antioxidant activity of green and black teas was determined using the ABTS$^+$ assay based on the methods used by Dapkevicius *et al.* [7] and Koleva *et al.* [8]. A 2 mM ABTS$^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated at room temperature in darkness overnight to allow for stabilisation of the radical. ABTS reagent was prepared by diluting the stock 8-fold in 0.1 M potassium phosphate buffer at pH 8. Samples, 5 μL aliquots of green tea or 10 μL of black tea, were injected into a Surveyor HPLC system. HPLC separations were carried out as described in the previous section. HPLC eluent from the PDA then arrived at a "T" piece, where the ABTS$^+$ was added. The ABTS$^+$ flow rate was 0.5 mL/min delivered by a Shimadzu LC-10 AD VP Liquid Chromatography pump. A Shimadzu GT-154 Vacuum Degasser was used to remove any oxygen in the reagent prior to mixing. After mixing through a 1.5 M × 0.4 mm loop maintained at 40°C, the absorbance was measured by a UV-detector at 720 nm (Nemphlar Bioscience, Lanark, UK). Data were analysed using ThermoFinnigan Chromquest™ chromatography software Version 4.0 (Fig. 2). Antioxidant potential was quantified by reference to a Trolox standard calibration curve. The antioxidant potential was calculated as the concentration of Trolox required to produce an equivalent antioxidant potential (μM) and as Trolox equivalent antioxidant capacity (TEAC), defined as the mM concentration of Trolox having equivalent activity to a 1 mM concentration of the compound under investigation.

3 Results

Infusions of green tea and black tea produced from the same batch of tea leaves were analysed by HPLC with photodiode array (PDA) and MS^n detection allowing the identification and quantification of flavan-3-ols, flavonols, caffeoylquinic acids, theaflavins and purine alkaloids

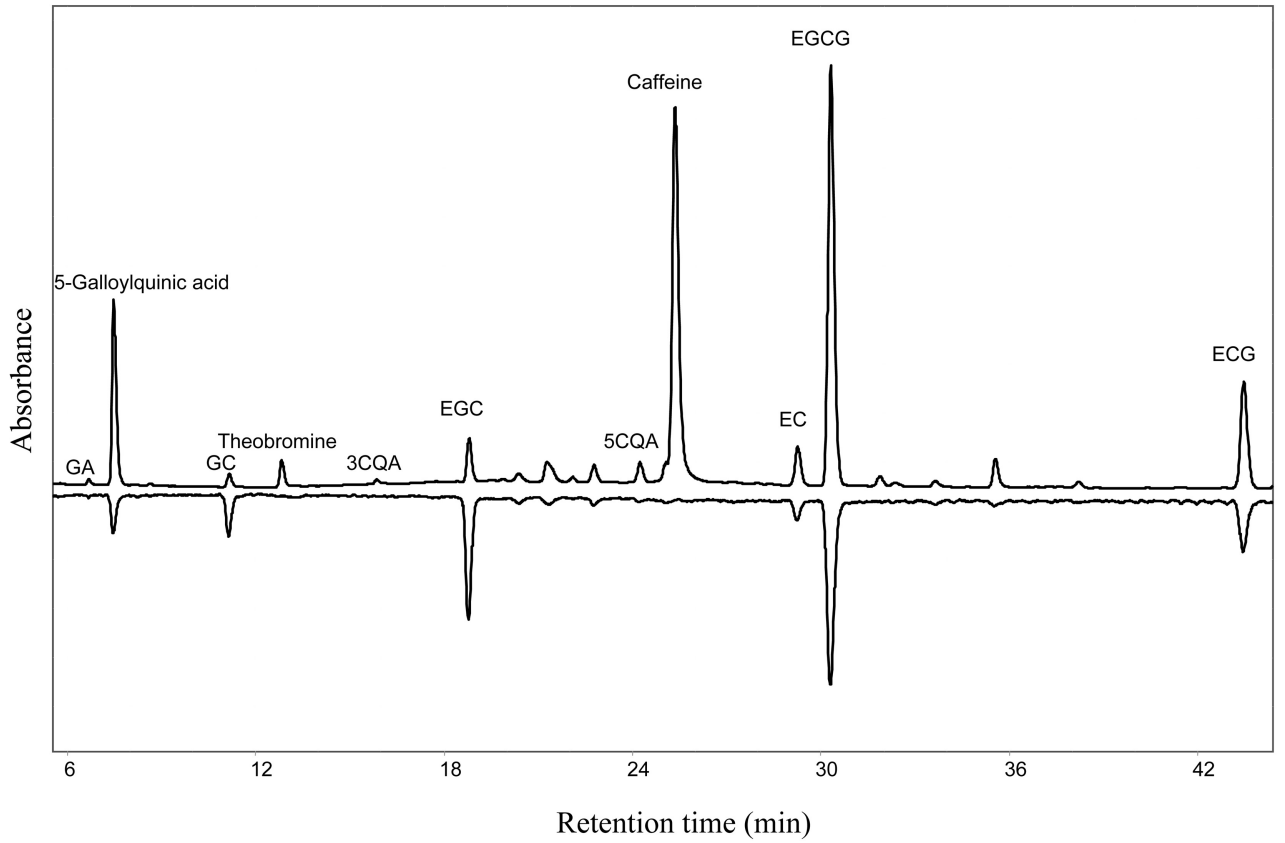

Figure 2. On-line HPLC ABTS$^+$ analysis of gallic acid derivatives, flavan-3-ols, hydroxycinnamates, and purine alkaloids in green tea. Teas were diluted 10-fold and 10 μL aliquots analysed by gradient reverse-phase HPLC with a PDA at 280 nm (positive trace) prior to reaction with ABTS$^+$ radical and analysis of antioxidant potential at 720 nm (negative trace). Abbreviations: GA, gallic acid; GC, gallocatechin; 3CQA, 3-caffeoylquinic acid; EGC, (−)-epigallocatechin; 5CQA, 5-caffeoylquinic acid; EC, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; ECG, (−)-epicatechin gallate.

Table 1. Mass spectral characteristics and identity of gallic acid derivatives, flavan-3-ols, hydroxycinnamates, and purine alkaloids present in tea

t_R (min)	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragment ions *(m/z)*
6.5	Gallic acid	169	125
7.5	5-Galloylquinic acid	343	191 (quinic acid; $[M-H]^-$-galloyl), 169
11.1	(−)-Gallocatechin	305	261, 221, 219, 179
12.9	Theobromine	181*	
15.4	3-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$-caffeoyl), 179
18.8	(−)-Epigallocatechin	305	261, 221, 219, 179
21.0	5-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$-caffeoyl)
25.4	Caffeine	195*	
29.0	(−)-Epicatechin	289	245, 205, 179
30.6	(−)-Eigallocatechin-3-gallate	457	331, 305, 169
43.4	(−)-Epicatechin-3-gallate	441	331, 289, 169

$[M-H]^-$, negatively charged molecular ion; * denotes positive ionisation.

Table 2. Mass spectral characteristics and identity of flavonols and theaflavins present in tea

t_R (min)	Compound	$[M-H]^-$ (*m/z*)	MS^n fragment ions (*m/z*)
26.9	Quercetin-rhamnosylgalactoside	609	301 (Q; $[M-H]^-$-Gal-Rham)
28.0	Quercetin-3-rutinoside	609	301 (Q; $[M-H]^-$-Glc-Rham)
28.8	Quercetin-3-galactoside	463	301 (Q; $[M-H]^-$-Gal)
29.8	Quercetin-hexose-rhamnose-rhamnose	755	609 ($[M-H]^-$-Rham), 301 (Q; $[M-H]^-$-Rham-Rham-Hex)
31.9	Kaempferol-rhamnose-hexose-rhamnose	739	593 ($[M-H]^-$-Rham), 431 ($[M-H]^-$-Rham-Hex), 285 (K; $[M-H]^-$-Rham-Hex-Rham)
33.9	Kaempferol-galactoside	447	285 (K; $[M-H]^-$-Gal)
34.3	Kaempferol-3-rutinoside	593	285 (K; $[M-H]^-$-Glc-Rham)
36.2	Kaempferol-3-glucoside	447	285 (K; $[M-H]^-$-Glc)
51.0	Theaflavin	563	
53.6	Unknown quercetin conjugate	901	755 ($[M-H]^-$-146), 609 ($[M-H]^-$-146-146), 301 (Q; $[M-H]^-$-146-146-308)
54.8	Theaflavin-3-gallate	715	
56.5	Unknown quercetin conjugate	901	755 ($[M-H]^-$-146), 609 ($[M-H]^-$-146-146), 463 ($[M-H]^-$-146-146-146), 301 (Q; $[M-H]^-$-146-146-308)
57.0	Theaflavin-3′-gallate	715	
57.9	Theaflavin-3,3′-digallate	867	
58.0	Unknown kaempferol conjugate	885	739 ($[M-H]^-$-146), 593 ($[M-H]^-$-146-146), 285 (K; $[M-H]^-$-146-146-308)

$[M-H]^-$, negatively charged molecular ion. Q, quercetin; K, kaempferol; Gal, galactosyl; Glc, glucosyl; Hex, hexosyl; Rham, rhamnosyl

(Tables 1 and 2). Compounds were identified by their retention time, UV spectra, parent mass and fragmentation pattern as described for other green and black teas by Del Rio *et al.* [2] and, where possible, by comparison with an authentic standard. Quantitative estimates of the various phenolics in the green and black tea infusions are presented in Tables 3 and 4.

The antioxidant potential of individual tea phenolics was assessed by HPLC using an on-line $ABTS^+$ method. Following HPLC separation, HPLC eluate was mixed with a stabilised solution of the $ABTS^+$ radical, and the solution directed to a UV-vis detector monitoring absorbance at 720 nm. The radical solution has a deep blue colour, any quenching of the $ABTS^+$ radical results in a loss of colour indicated by a negative peak on the absorption profile monitored at 720 nm. Figures 2 and 3 show the HPLC profiles of green and black tea analysed for flavan-3-ols (280 nm). Figures 4 and 5 show profiles of teas analysed for flavonols and theaflavins (365 nm). The antioxidant contribution associated with each identified compound is indicated by a negative peak recorded at 720 nm.

3.1 Analysis of green tea

Flavan-3-ols were the most abundant phenolics in the green tea, particularly (−)-epigallocatechin gallate and (−)-epigallocatechin (1202 and 1594 μM, respectively) and the individual flavan-3-ols demonstrated varying degrees of radical scavenging ability (Table 3). The concentration of each phenolic present within the tea samples was quantified by reference to an appropriate standard while the antioxidant potential was calculated as the concentration of Trolox

Table 3. Content and antioxidant potential of individual phenolic compounds from green tea

	Conc. (μM)	Trolox equiv. (μM)	TEAC
Phenolic acids			
Gallic acid	11 ± 8.4	22 ± 3.0	2.0
5-Galloylquinic acid	258 ± 8.7	529 ± 21	2.0
Total	269	551 (6.6%)	
Flavan-3-ols			
(−)-Gallocatechin	513 ± 7.2	552 ± 29	1.1
(−)-Epigallocatechin	1594 ± 114	2032 ± 146	1.3
(−)-Epicatechin	374 ± 25	398 ± 3.5	1.1
(−)-Epigallocatechin gallate	1202 ± 74	3606 ± 378	3.0
(−)-Epicatechin gallate	389 ± 1.7	1092 ± 32	2.8
Total	4072	7680 (92.1%)	
Caffeoylquinic acids			
3-Caffeoylquinic acid	9.4 ± 1.2	20 ± 1.4	2.1
5-Caffeoylquinic acid	65 ± 0.4	53 ± 1.5	0.8
Total	74	73 (0.9%)	
Flavonols			
Quercetin rhamnosyl galactoside	3.3 ± 0.0	0	0
Quercetin-3-rutinoside	24 ± 0.4	4.2 ± 1.7	0.2
Quercetin-3-galactoside	21 ± 0.7	7.1 ± 0.4	0.5
Quercetin-hexose-rhamnose-rhamnose	22 ± 0.3	25 ± 2.9	1.1
Kaempferol-rhamnose-hexose-rhamnose	4.0 ± 0.1	0	0
Kaempferol-galactoside	9.4 ± 0.9	0	0
Kaempferol-3-rutinoside	16 ± 1.2	0	0
Kaempferol-3-glucoside	29 ± 0.4	0	0
Total	129	36 (0.4 %)	
Theaflavins			
Theaflavin	n.d.	–	–
Theaflavin-3-gallate	n.d.	–	–
Theaflavin -3′-gallate	n.d.	–	–
Theaflavin-3-3′-digallate	n.d.	–	–
Total	0	–	–
Purine alkaloids			
Caffeine	1194 ± 11	0	0
Theobromine	54 ± 0.2	0	0
Total	1248	0	

Results represent concentration of phenolics and phenolic antioxidant potential in a water extract of Finlays green tea (3 g/300 mL).

Table 4. Content and antioxidant potential of individual phenolic compounds from black tea

	Conc. (μM)	Trolox equiv. (μM)	TEAC
Phenolic acids			
Gallic acid	183 ± 3.1	309 ± 6.7	1.7
5-Galloylquinic acid	146 ± 2.7	282 ± 1.9	1.9
Total	329	591 (39.6%)	
Flavan-3-ols			
(−)-Gallocatechin	n.d.	n.d.	–
(−)-Epigallocatechin	48 ± 0.3	36 ± 3.2	0.8
(−)-Epicatechin	34 ± 0.3	25 ± 0.7	0.7
(−)-Epigallocatechin gallate	52 ± 2.9	175 ± 3.2	3.4
(−)-Epicatechin gallate	58 ± 2.9	195 ± 5.9	3.4
Total	192	431 (28.8%)	
Caffeoylquinic acids			
3-Caffeoylquinic acid	n.d.	–	–
5-Caffeoylquinic acid	n.d.	–	–
Flavonols			
Quercetin rhamnosyl galactoside	4.5 ± 0.1	0	0
Quercetin-3-rutinoside	29 ± 0.9	17 ± 0.6	0.6
Quercetin-3-galactoside	27 ± 0.8	22 ± 3.3	0.8
Quercetin-hexose-rhamnose-rhamnose	25 ± 0.8	30 ± 2.8	1.2
Kaempferol-rhamnose-hexose-rhamnose	5.4 ± 0.1	0	0
Kaempferol-galactoside	11 ± 0.4	0	0
Kaempferol-3-rutinoside	21 ± 0.6	0	0
Kaempferol-3-glucoside	29 ± 0.9	0	0
Total	152	69 (4.6%)	
Theaflavins			
Theaflavin	117 ± 4.2	54 ± 2.5	0.5
Theaflavin-3-gallate	168 ± 7.3	151 ± 1.5	0.9
Theaflavin-3′-gallate	87 ± 3.8	66 ± 1.6	0.8
Theaflavin-3,3′-digallate	194 ± 6.0	132 ± 6.8	0.7
Total	566	403 (27.0%)	
Purine alkaloids			
Caffeine	1295 ± 20	0	0
Theobromine	49 ± 0.7	0	0
Total	1344	0	

Results represent concentration of phenolics and phenolic antioxidant potential in a water extract of Finlays black tea (3g/100mL water).

required to produce an equivalent antioxidant potential (μM) and also as TEAC, defined as the mM concentration of Trolox having equivalent activity to a 1 mM concentration of the compound under investigation.

The greatest antioxidant contribution to the green tea comes from the flavan-3-ols, which account for *ca.* 92% of the HPLC-derived antioxidant potential and 68% of the total antioxidant potential. Approximately 30% of the total antioxidant activity of green tea comes from (−)-epigallocatechin gallate. This significant contribution to antioxidant activity is due in part to the relatively high concentration of (−)-epigallocatechin gallate present in green tea. In addition (−)-epigallocatechin gallate was found to have the highest TEAC value of all the tea phenolics tested. In contrast, (−)-epigallocatechin, present in the highest concentration, 1594 μM, contributes approximately 18% of the total antioxidant potential. The TEAC value of (−)-epigallocatechin was found to be 1.3, which is less than half of the antioxidant potential of (−)-epigallocatechin gallate (Table 3).

The green tea also contained low levels of 3- and 5-caffeoylquinic acid. Both caffeoylquinic acids possessed antioxidative potential. A range of flavonol glycosides were also identified in green tea. Although the flavonol aglycone quercetin is widely reported to be a powerful antioxidant, once conjugated to sugars, as occurs in plant tissues, this antioxidant potential appears to be reduced markedly. Kaempferol conjugated to rhamnose and hexose sugars showed no detectable antioxidant potential in this system (Table 3).

3.2 Analysis of black tea

Flavan-3-ols are significantly reduced during the fermentation process and are, therefore, either absent or present in low levels in black tea. Those flavan-3-ols remaining in the black tea tended to have slightly lower TEAC than had been calculated with green tea (Tables 3 and 4). This is due to the flavan-3-ols being present in much lower concentrations in black tea which results in the antioxidant response being

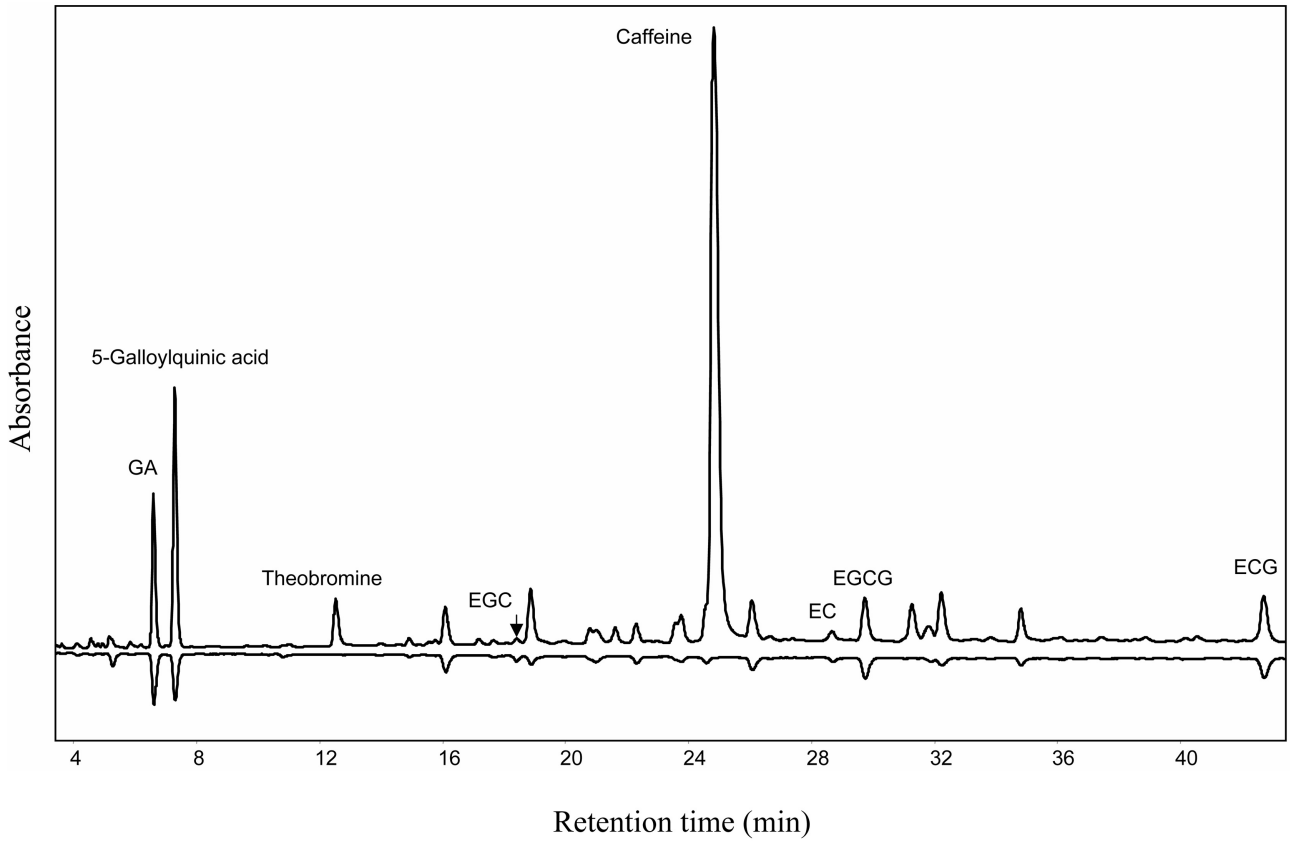

Figure 3. On-line HPLC ABTS$^+$ analysis of gallic acid derivatives, flavan-3-ols, and purine alkaloids in black tea. Aliquots of black tea (10 μL) were analysed by gradient reverse-phase HPLC with a PDA detector at 280 nm (positive trace) prior to reaction with ABTS$^+$ radical and analysis of antioxidant potential at 720 nm (negative trace). Abbreviations as in Fig. 2.

just above quantifiable limits. Standards of the flavan-3-ols were tested with the on-line HPLC system and the TEAC values obtained were the same as those obtained with with the green tea infusions.

Flavonol levels altered little during tea fermentation. The TEAC value for quercetin-3-rutinoside in black tea was higher than expected probably due to its elution in close proximity to (–)-epicatechin gallate (Figs. 4 and 5). Comparison with the antioxidant potential of an authentic standard showed an antioxidant potential of quercetin-3-rutinoside in line with that measured in green tea. The estimated antioxidant potentials for the individual flavonols were broadly similar for the green and black teas and the variation that did occur was, once again, due to the quantities injected being near to the limit of detection of the antioxidant assay (see Tables 3 and 4).

Black teas contain the flavan-3-ol dimers, theaflavins, formed during the fermentation process. Theaflavins appear to retain a level of free radical scavenging activity comparable with that of the (–)-epicatechin monomers. The antioxidant activity of theaflavins appeared to increase with the addition of a gallate moiety (Fig. 5, Table 4). In addition to the phenolics, green and black teas contained the purine alkaloids caffeine and theobromine. The concentration of both caffeine and theobromine was unchanged by tea fermentation. No antioxidant activity was associated with these compounds.

The total antioxidant content of the teas were measured using the reaction of an aliquot of green or black tea with the ABTS$^+$ radical solution using the on-line HPLC system but with the HPLC column removed. There was, therefore, no separation of tea components nor was any material retained on the column. The data obtained are presented in Table 5. Addition of the antioxidant potential of all the individual tea components separated by HPLC accounts for 74% of the antioxidant potential of the green tea but only 20% of the antioxidant potential of the black tea. The difference between the HPLC-derived and total antioxidant activity of the black tea probably represents the antioxidant activity of the thearubigens, which appear not to elute from the reverse-phase HPLC column [2].

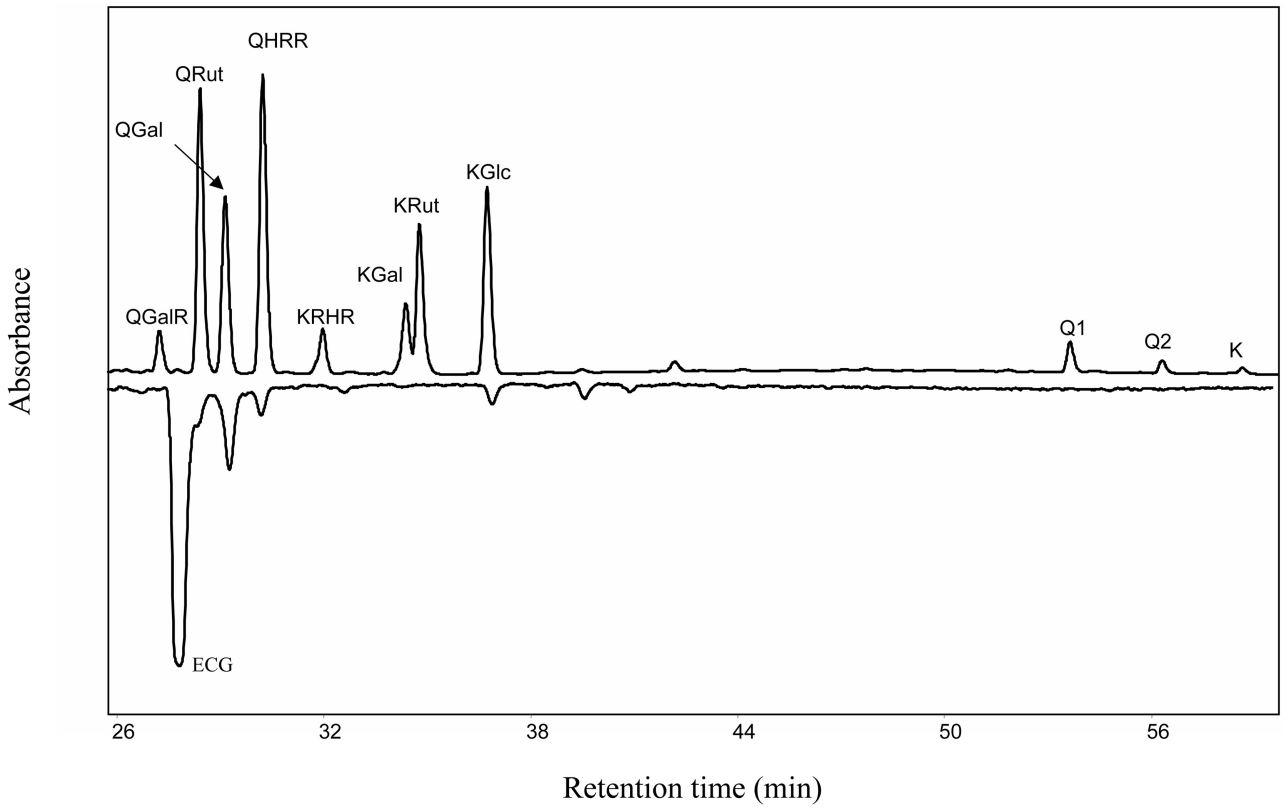

Figure 4. On-line HPLC ABTS$^+$ analysis of flavonols in green tea. Aliquots of green tea (10 μL) were analysed by gradient reverse phase HPLC with a PDA detector at 365 nm (positive trace) prior to reaction with ABTS$^+$ radical and analysis of antioxidant potential at 720 nm (negative trace). Abbreviations: QGalR, quercetin rhamnosyl galactoside; QRut, quercetin-3-rutinoside; QGal, quercetin-3-galactoside; QHRR, quercetin hexose rhamnose rhamnoside; KRHR, kaempferol rhamnose hexose rhamnoside, KGal, kaempferol galactoside; KRut, kaempferol-3-rutinoside, KGlc, kaempferol-3-glucoside; ECG, (−)-epicatechin gallate. Q1 and Q2 represent unidentified quercetin conjugates. K is an unidentified kaempferol conjugate.

Table 5. Total antioxidant capacity of green and black tea

	Trolox Equivalent (μM)	
	Green tea	Black tea
Total antioxidant capacity[a)]	11,302 ± 226 (100%)	7,382 ± 184 (100%)
HPLC antioxidant capacity[b)]	8,340 ± 60 (73.8%)	1,494 ± 4.0 (20.5%)
Difference	2,682 (26.2%)	5,888 (79.5%)

a) Trolox equivalent concentration of green or black tea
b) Addition of the Trolox equivalent concentrations calculated for each tea phenolic peak separated by HPLC

4 Discussion

Separation of the complex matrix of tea polyphenols by HPLC prior to 'on-line' reaction with the ABTS$^+$ stable radical solution allowed identification of the antioxidant potential of individual tea components and assessment of the relative contribution of each compound to the antioxidant capacity of the beverage. Flavan-3-ol monomers were the most abundant phenolics in green tea. The flavan-3-ols demonstrated varying degrees of radical scavenging activity dependant upon their structure. (−)-Epigallocatechin gallate contributed approximately 30% of the antioxidant activity of the green tea, due in part to the relatively high concentration of (−)-epigallocatechin gallate present in green tea. Additionally, (−)-epigallocatechin gallate was identified as a very potent radical scavenger as indicated by a high TEAC value (3.0). Previous studies have identified the structural characteristics of (−)-epigallocatechin gallate that contribute to antioxidant power. Important structural elements include the tri-hydroxyl structure on the B-ring and the presence of a galloyl moiety at the 3-position (see Fig. 1) [9]. (−)-Epicatechin gallate retains the galloyl moiety and has a TEAC value slightly lower (2.8) than that of (−)-epigallocatechin gallate. In contrast, (−)-epigallocatechin, which retains the trihydroxyl structure on the B-ring but lacks the galloyl group (Fig. 1), has approximately half the antioxidant capacity of (−)-epigallocatechin gallate (Table 3).

Determination of the antioxidant potential of flavan-3-ol extracts from tea leaf, and tea flowers show similar radical scavenging properties to those identified in the present

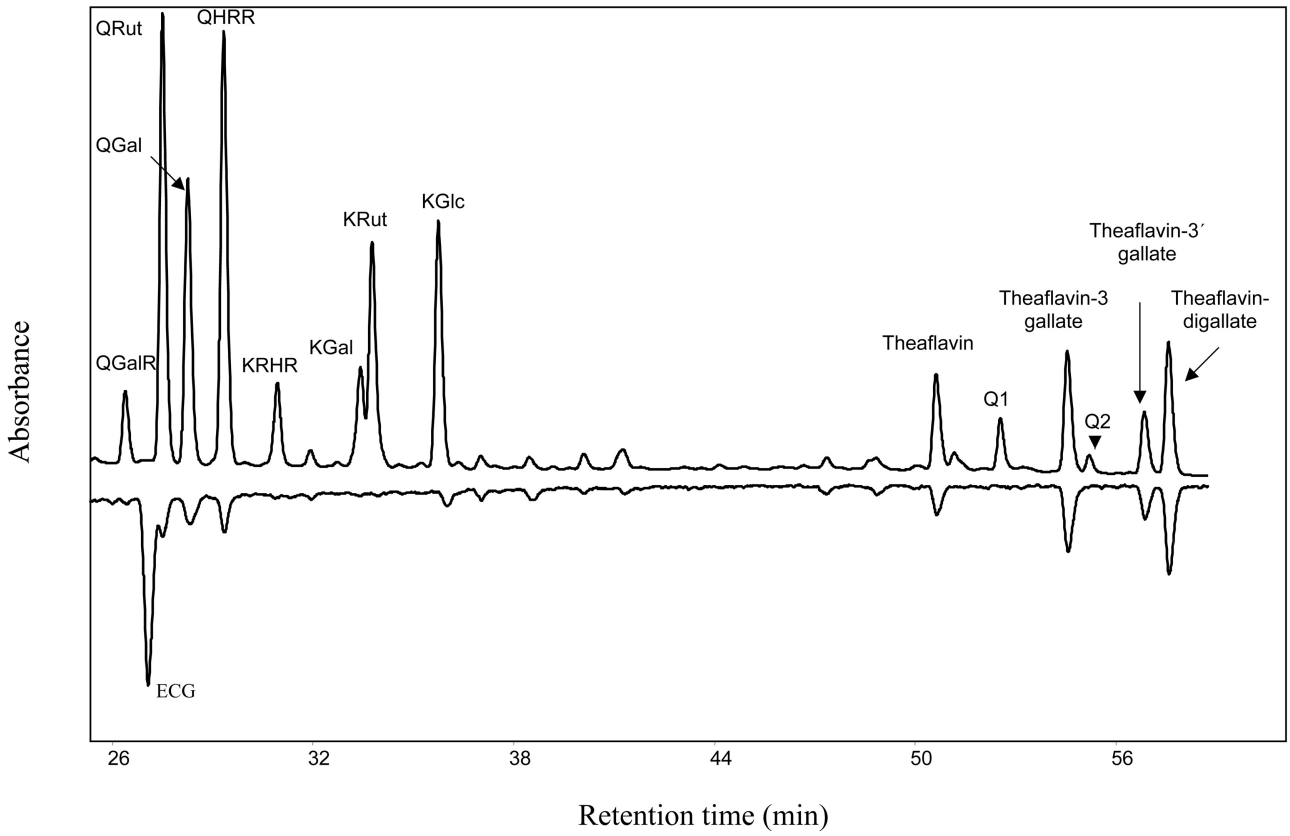

Figure 5. On-line ABTS^{+} analysis of flavonols and theaflavins in black tea. Aliquots of black tea (10 μL) were analysed by gradient reverse-phase HPLC with a PDA detector at 365 nm (positive trace) prior to reaction with ABTS^{+} radical and analysis of antioxidant potential at 720 nm (negative trace). Abbreviations as in Fig. 4.

study. Flavan-3-ols from tea flowers showed anti-radical activity greater than that of vitamin E [10]. Additionally the major anti-radical component of tea leaf extracts was identified as (−)-epigallocatechin gallate [11]. TEAC values for flavan-3-ols defined in the present study were lower than those previously defined by Salah [3] where values ranged from 4.93 for (−)-epicatechin gallate to 2.5 for (−)-epicatechin. This discrepancy may be due to the lack of radical stabilisation and/or the extended reaction time, which would not occur in a continuous flow on-line antioxidant detection system used in the present study.

Green but not black tea contained low levels of 3- and 5-caffeoylquinic acids. The caffeoylquinic acids have antioxidant potential, most notably the 3-caffeoylquinic acid, but they contributed less than 1% of the total antioxidant capacity of the green tea. Flavonols were present in similar levels in green and black teas indicating that the range of flavonol glycosides present in teas are relatively stable during the fermentation process. The three quercetin conjugates present in highest amounts had antioxidant activity whereas no antioxidant activity was associated with kaempferol conjugates. Quercetin aglycone is a potent antioxidant due to the trihydroxyl structure in the B-ring and the 3-hydroxyl group on the C-ring adjacent to a carbonyl group. Any conjugation, which disrupts either of these structural determinants, reduces antioxidative potential [12].

Black teas contain a further group of phenolics, which as yet cannot be separated and identified by HPLC, the thearubigens. Thearubigens are complex polymers of flavan-3-ols the antioxidant potential of which could not be measured directly using an on-line antioxidant detection system. The antioxidant contribution of thearubigens was, therefore, assessed indirectly by subtraction of the antioxidant potential of all the HPLC derived compounds from the total antioxidant potential of the black tea determined with the on-line system without the HPLC column. Results indicated that the source of ~80 % of the antioxidant capacity of black tea was not associated with the compounds which could be separated and identified by HPLC and indicates the possible importance of the thearubigens as antioxidants in black tea.

Recent studies indicate that flavan-3-ols are readily absorbed in humans and reach the circulatory system in the free form or as sulphated, methylated, or glucuronidated derivatives [13, 14]. Results indicate that catechins in their

free form may act as potent dietary antioxidants. In the future, on-line analysis systems such as the method described in this paper may be applied to the separation and analysis of complex biological samples such as urine and plasma allowing an insight into the antioxidant activity of catechin metabolites.

In conclusion, we demonstrate the use of HPLC with an on-line $ABTS^+$ system to separate the phenolic compounds in teas and simultaneously analyse and quantify the antioxidant potential of the individual tea components. We conclude that both green and black teas are a rich source of antioxidants. Gallated catechins, and those with di/trihydroxylation on the B ring demonstrated the highest level of radical scavenging activity. Total antioxidant potentials indicate that although the flavan-3-ols and theaflavins contribute to the antioxidant potential of the black tea, a more significant source of antioxidants may be the thearubigens.

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